Cerium Oxide Nanoparticles as a Novel Nanodrug

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by

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In the loving memory of my grandmother, Hanife Özel. Thank you for being one of the biggest supports in my life grandma!
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ABSTRACT

Cerium oxide nanoparticles (nanoceria) have been shown to effectively modulate reactive oxygen species (ROS) levels within cells due to its unique physiochemical structure. In ceria oxides, both oxidation states (III) and (IV) can coexist, producing a redox couple that is responsible for ceria’s catalytic activity. The central focus of this thesis is to engineer nanoceria to induce ROS generation in vitro to fight severe diseases such as cancer, inflammation, and infection.

ROS are considered oncogenic, as they are present at elevated amounts in cancer cells and they contribute to the initiation, progression and metastasis of cancer. However, in non-surgical cancer treatment approaches, such as radiotherapy, ROS generation within cancer cells is a common way to target cancer cells. For the first project, we engineered sub 5 nm nanoceria (+4) to effectively kill the cancer cells at different pH values, resembling the cancerous and non-cancerous environment. We found that dextran-coated nanoceria were much more efficient at killing bone cancer cells (osteosarcoma cells) in a slightly acidic environment (pH 6), compared to physiological and basic pH values (pH 7 and pH 9). In contrast to the cancer cells, approximately 2–3 fold higher healthy bone cell viabilities were observed compared to osteosarcoma cell viabilities when cultured with nanoceria at pH 6. Moreover, osteosarcoma cells showed elevated ROS generation under these conditions, whereas healthy osteoblast cells did not have show significant increases after 1 day treatment.
Secondly, the antibacterial activity of nanoceria (+4) was examined against *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* by varying the nanoparticle concentration and the pH of the solution. Findings suggested that nanoceria particles were much more effective at killing *P. aeruginosa* and *S. epidermidis* at basic pH values (pH = 9) compared to acidic pH values (pH = 6). For both bacteria, a 2 log reduction and elevated amounts of ROS generation per colony were observed and *P. aeruginosa* showed drastic morphological changes as a result of cellular stress after 6 hours of treatment (with nanoceria at pH 9) compared to untreated controls.

Overall, ROS are detrimental by-products of cellular systems, and it has been shown that oxidative stress is linked with cancer and infection. In this study, we engineered cerium oxide nanoparticles to generate ROS to selectively kill the cancer cells and inhibit bacterial infection by controlling the pH of the solution.
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CHAPTER 1

Introduction

1.1 Cancer Biology

Cancer is one of the most serious health challenges in the 21st century that people have been fighting, and in fact arises from healthy cells with genetic alterations affecting tightly controlled systems for growth control.\textsuperscript{1, 2} In other words, healthy cells turn into heterogeneous multicellular entities containing cells of multiple lineages.\textsuperscript{3} Figure 1 represents the initiation and progression of a tumor starting from a healthy tissue (Figure 1a). Basically when one cell acquires enough mutations, it becomes cancerous (Figure 1b) and divides at an accelerated rate (Figure 1c) until it reaches steady state, where tumor cells grow and die at a steady state (Figure 1d).

![Figure 1 Schematic of tumor progression. (a) Healthy layer of tissues (demonstrated in white), (b) One cell becomes cancerous after acquiring enough mutations (demonstrated in black), (c) Cancer cells divide at an accelerated rate and displace healthy tissues, and (d) Tumor cells will continue to grow and die at a steady state size.](image)
Cancer cells interact with each other, the extra cellular matrix (ECM), and other soluble molecules in their vicinity (Figure 2). Tumor formation is a dynamic process and favors cell proliferation, movement, differentiation, and ECM metabolism. Thus, it is almost impossible to find one general cure for all types of cancer. The type of the cancer is the most important parameter when trying to cure cancer effectively.

Figure 2 Representation of the tumor microenvironment, showing tumor cell interactions with cellular and non-cellular components. Adapted from Reference 4
1.2 Current Treatment Options in Cancer

Cancer, being the second leading cause of death in the United States, still suffers from proper treatment methods. Current treatment options in cancer treatment are surgery, radiotherapy and chemotherapy, where the basic approach is to remove or kill the diseased cells faster than the healthy cells. Surgical resection, the most decisive way of treatment, is only an option if cancer is diagnosed at early stages. Limited tumor accessibility for resection and the risk of damaging the healthy tissue surrounding the cancerous tissue has been an obstacle for surgery.

In chemotherapy, a major issue to avoid treatment failure is to accumulate and retain therapeutically relevant drug concentrations at the site of the tumor. For an effective treatment at the tumor site, prolonged exposure of the tumor to sufficiently high drug concentrations is a prerequisite. However, physiochemical properties like molecular weight, stability, and lipophilicity of the compound often result in sub-therapeutic drug levels at the tumor site. Additionally, most chemotherapeutic agents (such as cisplatin, doxorubicin, and methotrexate) suffer from non-selective toxicity and harm healthy cells as well as cancer cells. Therefore, a cancer treatment that is generally less invasive and more selective is badly needed.

Radiation is efficient in terms of the elimination of cancer cells, but healthy cells within close proximity of cancer cells may be exposed to this radiation and could be harmed. During radiation therapy, there are several ionizing reactions, which result in the
formation of free radicals. These free radicals are capable of killing cancer cells as well as healthy cells, and they react with cellular DNA and RNA to alter molecular interactions.\textsuperscript{13,14}

Since the 1950’s, alternative ways to protect healthy cells from radiation damage has been an important area of research. In an effort to protect healthy cells, natural free radical scavengers (such as glutathione, vitamin E, vitamin C, and B- carotene) as well as synthetic antioxidants (as amifostine, NAC, and captopril) have been tested for their capability to scavenge reactive oxygen species.\textsuperscript{15-17}

To date, amifostine has been found to be the most effective free radical scavenger.\textsuperscript{13} It prevents radiation-induced cell death and mutagenesis while facilitating the repair of normal cells.\textsuperscript{18,19} However, its clinical use is a major problem for amifostine due to its short half-life in serum.\textsuperscript{13} It has been reported that more than 90% of the drug disappears from the plasma compartment 6 minutes after intravenous (i.v.) administration.\textsuperscript{20} Clearly, new materials are needed to fight cancer.

1.3 Bacteria and Bacterial Infection

Bacteria are single celled microorganisms that can thrive in many different types of environments. There are thousands of different kinds of bacteria, many different types of bacteria can live in the human body, and some of the bacteria can live in extreme conditions like very cold and very hot temperatures. The vast majority of bacteria are harmless, and some are even beneficial to human the health, but some strains, called
pathogens, can be very harmful to public health. Bacterial infection is the growth and proliferation of these harmful bacteria strains. When a pathogenic bacteria strain enters the human body, it begins to reproduce and grow in tissues, and bacterial diseases occur. Some common pathogenic bacteria and diseases that they cause are listed in Table 1.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli and Salmonella</em></td>
<td>Food poisoning</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Gastritis and ulcers</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>Gonorrhea</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>Meningitis</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Boils, cellulitis, abscesses, wound infections, toxic shock syndrome, pneumonia, and food poisoning.</td>
</tr>
</tbody>
</table>

Variations between the different strains of bacteria are discussed in a later section.

1.4 Cancer Related Infections

Cancer patients not only suffer from tumors blocking organ function, but also an increased susceptibility to infection. The link between tumors and infection was formed as early as the 19th century when scientists realized tumors often occur at the site of chronic inflammation and biopsied tumor samples contained inflammatory cells.21, 22 The
The link between infection and cancer is not just related to the formation of tumors but it is also a problem encountered during disease progression. During disease or treatment of cancer, patients are at increased risk of developing infections. An elevated risk of infectious complications among cancer patients is due to a patient’s weakened immune system or complications during surgical resection. Moreover, some strains of bacteria (such as *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*) are known to be opportunistic bacteria, meaning that they mostly affect the host, whose immune system has already been compromised. Often, infectious diseases are an important cause of morbidity and mortality in patients with cancer. 15-20% of all deaths from cancer worldwide have been shown to be related to infections and inflammatory responses. Thus, effective strategies to anticipate, prevent and manage infectious complications should be developed; even better are strategies which can simultaneously use the same approach to both kill cancer and infection.

### 1.5 Current Treatment Options in Infection Treatment

High rates of mortality and morbidity were a fact of life for the world’s population before the development and administration of antibiotics in the early 1940’s. Arguably, antibiotics have been proven to be one of the most important medical innovations in human history, as they have reduced infectious disease-related mortality rates and healing time greatly. In the United States alone, infection-related mortality rates dropped drastically between 1900 and 1996, from 797 to 59 deaths per 100,000 people.
Unfortunately, the application of antibiotics may not be a viable long-term solution for infectious diseases. Over the last several decades, the excessive and improper use of antibiotics has driven the ruthless emergence of drug-resistant bacteria. Many routinely-used antibiotics are already ineffective in the clinic; some even speculate that the 21st century will come to be known as the ‘post-antibiotic’ era. Based on Centers for Disease Control projections, by 2050, more deaths will result from infection than all cancers combined. Thus, the need for novel antibacterial agents has never been greater.

1.6 Nanoparticle Based Therapies

Due to their unique optical, electronic and magnetic properties at the nanoscale, nanosized materials are attractive for a wide range of applications including biological applications. The motivation behind introducing nanoscale materials for enhanced interactions for biological applications stems from the similarity between the nanoscale size range of proteins and other macromolecules with nanoscale materials (Figure 3). Cells and their constituent organelles lie on the sub-micron to micron size, whereas proteins and macromolecules found in cells are at the nanometer scale. Thus, materials at the nanoscale have become ideal for probing, imaging, diagnostics, drug and gene delivery and tissues engineering applications.
Figure 3 Schematic of the interaction of nanorough surfaces with proteins and other macromolecules which may be controlled to enhance adsorption and/or minimize such interactions due to altered surface energy.  

1.6.1 Nanoparticle based therapies in cancer treatment

Nanoparticle-based therapies may be the answer to cancer treatment as they have been utilized to deliver therapeutic agents to a diseased site and promote the accumulation of therapeutic levels of drugs at the target site. So far, liposomes (nanoparticles based on solid lipids), polymeric nanoparticles, and magnetic nanoparticles have all demonstrated great promise for targeted cancer drug delivery. Among them, liposomes have been approved for clinical use due to their colloidal stability and selectivity of drug accumulation at the tumor site.  

Such attractive properties of nanocarriers stem from their ability to extravasate through the leaky and highly permeable tumor vasculature and accumulate in the tumor interstitium as demonstrated in Figure 4. The ability of nanoparticles to extravasate through the
inherently leaky and loosely compacted vasculature to reach the tumor space and stay there due to the inability of lymphatic drainage in tumors was shown by Matsuma et al. for the first time in 1986. Later, a phenomenon called the enhanced permeability and retention (EPR) effect was introduced to explain nanoparticle accumulation in tumors. Various properties of nanoparticles (such as size, shape, surface charge and their dynamic and continuous interactions with components of the vasculature) govern their ability to exhibit the EPR effect. For example, Cabral et al. showed that polymeric nanoparticles that are 30-100 nm in diameter could penetrate into the highly permeable tumor. However, for poorly permeable adenocarcinoma cells which have low vascularity and dense fibrosis, particles larger than 50 nm were not able to accumulate in the tumor.

![Figure 4 Schematic of the extravasation of nanoparticles in the tumor interstitium. Adapted from reference 37](image)

With their small size and macromolecule functionalized surface, nanosized therapeutic agents tend to stay longer in the blood circulation. The longer they stay in the
circulation, the more likely it is for them to extravasate into the tumor. However, longer circulation also means that drugs can extravasate into the healthy tissue just at a slower rate.\textsuperscript{38} Thus, even though nanoparticles access tumor tissue and their presence can be increased temporarily due to the local EPR effect, there is room for improvement for the specific uptake of these nanoparticles by tumor cells. Many nanoparticles still suffer from cytotoxicity to healthy cells.\textsuperscript{39, 40} For these reasons, new carriers, which do not possess such healthy cell cytotoxicity properties, still need to be identified.

### 1.6.2 Nanoparticle based therapies in bacterial infection

With the emerging need for novel antimicrobial agents, nanoparticles have been proposed to treat infections as they utilize different mechanisms for killing bacteria than conventional antibiotics, making them promising candidates to overcome current issues faced with antibiotic drug resistant bacteria.\textsuperscript{41} To date, nanoparticles of many different elements (such as zinc,\textsuperscript{42} copper,\textsuperscript{43} titanium,\textsuperscript{44} selenium,\textsuperscript{45} magnesium,\textsuperscript{46} iron oxide\textsuperscript{47} and silver\textsuperscript{48}) have been studied for their antimicrobial properties. It is important to note that while some of these metals, such as silver and copper, are inherently antibacterial even in their bulk form, other materials such as iron oxide\textsuperscript{47}, only exhibit antimicrobial properties on the nanoscale.\textsuperscript{47, 49} So far, more than 10 different nanoparticle-based products have been commercialized for applications in bacterial diagnosis, antibiotic delivery, and medical device development.\textsuperscript{25} However, the long-term effects associated with the use of
these nanosized products are still being questioned; especially, the use of chemistries like silver, where silver has been shown to be a very effective antibacterial agent but demonstrates high toxicity to mammalian cells, or iron oxide, where iron oxide has been very efficient in killing bacteria only at high concentrations. Thus, the demand for developing effective antibacterial agents at low enough doses with minimal toxicity to mammalian cells is still badly needed.

1.7 Cerium Oxide: Background and Motivation

Cerium (Ce) is the most abundant element in the earth’s crust (at 66.5 ppm) belonging to the lanthanide group in the rare earth family.$^{50,51}$ The electron configuration of cerium is [Xe] 4f$^2$ 6s$^2$, adding distinction among the lanthanides for these partially-filled 4f and 5d orbital electron subshells allowing for potential excited states. Unlike most of the rare earth elements, which are usually in a trivalent oxidation state, there are two stable oxidation states for cerium: + 3 and + 4.$^{51,52}$ Due to the relative ease of switching between these two oxidation states according to its chemical environment, significant changes may occur in cerium’s valence structure, which makes it attractive for a number of applications.$^{53,54}$

In ceria oxides, both oxidation states (III) and (IV) can coexist, producing a redox couple that is responsible for ceria’s catalytic activity (Figure 5). This rapid change in oxidation state is a result of reversible storage and release of oxygen from the lattice.$^{54}$ The corresponding number of vacancies, enriched at the surface, compensates for the
reduction in positive charge of Ce$^{3+}$. Previous research has shown that decreases in particle size directly results in an increase in the lattice constant of crystalline cerium oxide nanoparticles (nanoceria) due to the increase in oxygen vacancies. At the nanoscale, not only the surface area to volume ratio increase (which thus increases oxygen exchange and redox reactions), but also migration enthalpy of the oxygen vacancy in nanoceria decreases. Thus, the presence of oxygen vacancies, which makes nanoceria an efficient oxygen buffer, is more likely at the surface of smaller particles. Particles around 5 nm in diameter with a high surface to volume ratio, enables nanoceria to regenerate its catalytic activity, thus, it acts in an autocatalytic capacity. The oxidation state and the defect structure of cerium oxide is dynamic and may change spontaneously or as a response to changing physical parameters such as temperature, presence of other ions and oxygen partial pressure.

![Figure 5 Crystal structures of Ce$_2$O$_4$ and Ce$_2$O$_3$. Adapted from reference](image-url)
Due to its abundance and unique chemical properties, cerium oxide (CeO$_2$) is a technologically important material. So far, it has been used in a variety of applications including sensors, membrane systems, fuel cells, mechanical polishing, ultraviolet absorbent, catalysis and more recently in biotechnology, medicine and environmental chemistry.$^{51,52,62}$ In nanocrystalline solids, a decrease in particle size leads to an increase in density of interfaces, and leads to a reduction in energy for defect formation. Thus, increased levels of non-stoichiometry and electronic carrier formation have been observed as particle size is reduced.$^{51}$

In spite of the wide range of applications in which nanoceria is being used, the scope of this thesis is restricted to biological applications of nanoceria, for which it has seen little attention. The concepts and principles relevant to investigating the effectiveness of nanoceria for tissue engineering applications will be reviewed in the critical literature review section of this thesis.

1.8 Organization of the Dissertation

The central theme underlying this dissertation is analyzing the biological activity of uniformly distributed cerium oxide nanoparticles in three different areas, namely, its anti-cancer properties, antibacterial properties, and its reactive oxygen scavenging capability resulting in such properties. At different pH values cerium oxide nanoparticle physiochemical properties (such as it hydrodynamic radius, surface charge, valance state, etc.) can be modified, as a new ion balance is introduced to the system. Subsequently, the
particles interact with their environment differently. Thus, the primary emphasis of this thesis is focused on engineering certain biological properties that cerium oxide nanoparticles can achieve by altering the pH of the environment.

This dissertation is divided into five chapters, including an introduction section on the background of cancer biology, current treatment options of cancer, the need for better alternatives, why infection is a problem especially for cancer patients, what are cerium oxide nanoparticles, and why they might be an alternative to cancer treatment and inhibition of bacteria growth when they are engineered correctly.

Chapter 2 provides a critical literature review on what has been done so far using cerium oxide nanoparticles in biological applications. A detailed discussion is included on the efficacy of cerium oxide nanoparticles in cancer treatment; inhibition of bacteria growth and mechanism of action as analyzed by examining reactive oxygen species generating properties of the nanoparticles. Chapter 2 concludes with an introduction of the biological organisms used in this dissertation.

Chapter 3 gives the details of the experimental methods that were used in this thesis. In chapter 4, results obtained from all three different projects are listed and discussed in detail. Results given in this chapter reflect different manuscripts written by the author that are either already published in peer-reviewed journals or have been submitted for publication.
In the final chapter, the results from all the experiments with nanoceria are summarized and further suggestions presented to advance our understanding of possible medical applications of cerium oxide nanoparticles.
CHAPTER 2

Critical Literature Review

2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive, oxygen containing molecules which either contain one or more unpaired electrons such as hydroxyl ions (OH\(^{-}\)), and superoxide ions (O\(_{2}^{-}\)) or species which can easily be converted into a radical like hydrogen peroxide (H\(_{2}\)O\(_{2}\)).\(^{63-65}\) Endogenously, ROS are derived from the excitation and partial reduction of molecular oxygen and exist in all aerobic species at low levels in mitochondria.\(^{52,65}\) Tightly regulated ROS are not always harmful byproducts of oxygen metabolism. There is now growing evidence on how they are involved in enzymatic reactions and regulate cell proliferation, cell differentiation, signal transduction, and ion transport.\(^{66-68}\) ROS can be eliminated by elaborate mechanisms, known as antioxidant defense mechanisms.\(^{16}\)

Oxidative stress is generated when there is an imbalance between the generation and elimination of ROS.\(^{69,70}\) Oxidative stress within the cell has damaging effects on DNA, proteins, lipids and other cellular components.\(^{66,71,72}\)

Accumulation of reactive oxygen species or oxidative stress is important to biomedical society as it is associated with numerous diseases like neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS), insulin resistance, aging and cancer which cause over 500,000 deaths per
ROS accumulation can drive both the initial development and progression of cancer since it affects enzymes which normally would combat such oxygen radical production. Or ROS may interfere with cytoplasmic and nuclear signal transduction pathways, causing structural alterations in DNA that can modulate genes related to cell proliferation, apoptosis and differentiation.

Antioxidants, as defined by Halliwell and Gutteridge in 1989, are ‘any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate’. This definition is collective of enzymatic and non-enzymatic antioxidant compounds.

Antioxidants function in three different mechanisms: prevention of the formation of ROS scavengers, by donating an electron to free radicals to neutralize them such that these radicals cannot damage the cells anymore, interception into damaging species, and repair of the damage. Within the cell, redox balance is maintained by antioxidant defense systems that consists of common antioxidant molecules like, vitamin C, vitamin E, glutathione, and antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase. The duty of this complex system is to minimize the ROS accumulation while allowing ROS to perform cell signaling and redox regulation. Synthetic molecules like amifostine, captopril and NAD are also studied for their ability to eliminate ROS. However, there are two main challenges about them. The first one is the uptake of the molecule by tumor and the healthy cells. In a tumor microenvironment, passive diffusion
dominates the uptake, which is slow for hydrophilic molecules like amifostine. Recent studies by Brown et al. showed that hydrophilic compounds might be useful when designing radioprotectants. They showed an increase in the therapeutic gain for 6 hydrophilic thiols compared to lipophilic thiols. The second issue in the use of the synthetic antioxidants is the limit that cells can uptake before they become antioxidant. WR-1065 was tested against several different cancer cell lines. Concentrations above 25-30 nmol/10⁶ induced toxicity when cells were not irradiated. Thus, there are still limitations with the use of synthetic antioxidants.

2.2 Biological Applications of Cerium Oxide Nanoparticles

CeO₂ has been shown to be effective in fighting inflammation and cancer to protecting cells from radiation, and oxidative stress associated diseases. The oxygen defect structure on its surface, alternating oxidation states between +3 to +4, and low reduction potential between the oxidation states give it a unique property of modulating reactive oxygen species (ROS) levels. The ability of cerium oxide switching oxidation states was found to be comparable to that of biological antioxidants (such as superoxide dismutase and glutathione). The ratio of Ce³⁺ to Ce⁴⁺ has been shown to determine the biological property that nanoceria will attain. With a higher +3/+4 ratio, the number of oxygen vacancies at the particle surface and superoxide dismutase (SOD) mimetic activity will increase. Thus, it is more effective against diseases associated with oxidative
stress. With a lower +3/+4 ratio, particles exhibit more catalase-like activity and possess anticancer and antibacterial properties. Thus, cerium oxide nanoparticles have extensive potential as therapeutic agents in ROS mediated diseases, anti-cancer, and anti-inflammatory applications.

2.2.1 Nanoceria as ROS modulator

Among various nanoparticles, nanoceria can effectively regulate/scavenge reactive oxygen / nitrogen species (i.e., act like an antioxidant) including superoxide radicals, hydrogen peroxide, hydroxyl radical, peroxynitrite, and nitric oxide radicals. As opposed to their larger counterparts, nanoceria nanoparticles have a large number of surface defects which are primarily oxygen vacancies. The catalytically active sites of nanoceria are known to be oxygen defects at the surface of the particles and/or delocalized electron density at the surface of the nanoparticles. The presence of these vacancies is responsible from the change in the local electronic stability and the valance state of the particles. Nanoceria’s unique ability to switch its oxidation states between +3 (reduced) to +4 (oxidized) based on environmental conditions makes it a desirable material in biomedical science, especially for antioxidant applications. Studies have shown that the crystal structure of cerium oxide nanoparticles consists of cerium atoms in the +4 oxidation state, but as the particle size is reduced, O2 vacancies are formed on the surface of the particles which then results in higher levels of cerium atoms in the +3 oxidation state (CeO2-x).
In recent studies, nanoceria has been reported to mimic naturally existing enzymes such as superoxide dismutase, converting superoxide to hydrogen peroxide ($\text{H}_2\text{O}_2$) and catalase (Figure 6), converting $\text{H}_2\text{O}_2$ to water and antioxidants such as glutathione, and uric acid.\textsuperscript{50, 87-89} Thus, it is a good candidate as a potent artificial antioxidant. The essence of the antioxidant behavior of these nanoparticles and the underlying mechanism behind the antioxidant behavior have been attributed to the ability of the nanoparticles to modulate its valance states, and the coexistence of valence states of Ce$^{+3}$ and Ce$^{+4}$ on the surface under the right redox environment.\textsuperscript{13, 72, 87, 88, 90} The proposed mechanisms for nanoceria’s SOD and catalase mimetic activity is given in Figure 7 and Figure 8.\textsuperscript{57, 91} In a recent study performed by Pirmohamed et al., nanoceria’s activity was shown to be changing in a redox-state dependent manner.\textsuperscript{86} Cerium atoms in the +4 state exhibit significant catalase mimetic activity, and less SOD mimetic activity.\textsuperscript{62} These findings were supported by Li et al. when they observed that particles greater than 5 nm in diameter had a negligible ratio of Ce$^{+3}$/Ce$^{+4}$, thus, they didn’t exhibit minimal superoxide dismutase mimetic activity.\textsuperscript{92} In another study performed by Asati et al., the antioxidant property of nanoceria was shown to be dependent on pH and suggested that nanoceria acts like an anti-oxidant at physiological pH values, and it acts like a pro-oxidant at acidic pH values.\textsuperscript{90} There is also growing evidence on how acidic pH promotes SOD mimetic activity of nanoceria while inhibiting its catalase mimetic activity (resulting in the increased accumulation of $\text{H}_2\text{O}_2$).\textsuperscript{74, 89} Due to the array of different ideas,
whether nanoceria is an anti- or pro-oxidant, some groups suggest them as free radical modulators instead of free radical scavengers. \(^{89}\)

\[
a) \quad 2 \text{O}_2^* + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2 \\
b) \quad \text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2 \text{HO}_2 + \text{O}_2
\]

**Figure 6** The reaction catalyzed by SOD (a) and catalase (b)

![Figure 6](image)

**Figure 7** Proposed mechanism of nanoceria’s SOD mimetic activity. Adapted from reference \(^5\)
Figure 8 Proposed mechanism of nanoceria’s catalase mimetic activity. Adapted from reference 57

Ceria nanoparticles potentially have superior properties compared to conventionally used antioxidants (such as amifostine). One major problem with the use of current antioxidants is their short circulation time (in the order of minutes) in which macromolecule coated nanoceria may be able to overcome. It has been shown by a few groups that surface coatings (such as dextran and polyacrylic acid) do not alter nanoceria’s antioxidant properties. The defective surface of nanoceria (i.e., oxygen vacancies) may act as chemical spin-traps, or compounds that have the ability to stabilize or trap free radicals, in a similar manner to conventionally used antioxidant molecules. The significance of nanoceria is dependent on its size as it can offer many spin-trap sites per particle, whereas conventionally used antioxidant molecules can only offer one active
site per molecule. Additionally, these defects on the surface of the particles have the potential for regeneration in which an autoregenerative reaction cycle (\(\text{Ce}^{3+} \rightarrow \text{Ce}^{4+} \rightarrow \text{Ce}^{3+}\)) continues on the surface of the particles. Thus, unlike currently used antioxidants, the use of nanoceria as an antioxidant does not require repetitive dosage. This regenerative property of nanoceria makes it a great candidate as a potent antioxidant.\textsuperscript{13,52,94}

The following set of reactions was proposed by Chen et al. to elucidate the radical scavenging mechanism of nanoceria.\textsuperscript{52}

\[
\begin{align*}
\text{Ce}^{3+} & \leftrightarrow \text{Ce}^{4+} + e^- \\
\text{Ce}^{3+} + \text{OH}^\cdot & \rightarrow \text{Ce}^{4+} + \text{OH}^- \\
\text{Ce}^{4+} + \text{O}_2^- & \rightarrow \text{Ce}^{3+} + \text{O}_2
\end{align*}
\]

A detailed study was performed by Chen et al. to evaluate the ability of nanoceria in scavenging reactive oxygen intermediates (ROI) within retinal neurons both during \textit{in vitro} cell culture systems and \textit{in vivo} albino rat light-damage models.\textsuperscript{52} Photoreceptor cells have the highest rate of oxygen metabolism and are continuously exposed to the detrimental effects of oxidative stress (i.e., damage to cells and cellular components exerted by exposure to highly unstable small molecules).\textsuperscript{95,96} In every kind of blindness, the concentration of ROIs rises chronically or acutely and initiates cell death pathways.\textsuperscript{97} The ability of nanoceria particles to protect retinal neurons in culture was tested initially. A 5 nM nanoceria treatment was found to be enough to have fewer apoptotic retinal cells compared to non-treated retinal cells after 4 weeks. For a further demonstration of the
protective effects of nanoceria, ROI-induced apoptosis that occurs in retinal neurons when exposed to exogenously added H₂O₂ was also prevented when cells were treated with nanoceria up to 20 nM concentrations. Under the same conditions, nanoceria was also shown to prevent the intracellular accumulation of ROIs.

2.2.2 Nanoceria treatment against growth of tumor cells

Nanoparticles of many different kinds have been extensively studied as cancer treatment agents or drug carrying molecules due to their unique physiological, optical, and magnetic properties to increase the efficacy of treatment and to reduce the side effects of the treatment. There is now growing evidence on how reactive oxygen species are associated with the initial development and progression of cancer. Since nanoceria’s efficacy on modulating ROS levels have been shown by many different groups, subsequently, its efficacy has also been tested against cancer cells. So far, cerium oxide nanoparticles have been tested against many different cancer cell lines, including bone, ovarian, pancreas, bone, and lung cancer cells.

The cytotoxic effect of 20 nm cerium oxide nanoparticles was evaluated using a human broncoalveolar carcinoma derived cell line (A549) by Lin et al. A549 cells were treated with 3.5 µg/mL, 10.5 µg/mL, and 23.3 µg/mL cerium oxide nanoparticles up to 3 days in culture. At the end of the 3rd day, the cell viability was reduced to 88 %, 67.7 %, 53.9% when treated with 3.5 µg/mL, 10.5 µg/mL, and 23.3 µg/mL cerium oxide nanoparticles, respectively. It was reported that the ceria nanoparticles induced
significant oxidative stress (up to 181% increase when treated with 23.3 µg/mL for 24 hours), revealed by increased ROS and reduced glutathione levels. In fact, after the first day of the treatment, cell viability was found to be strongly correlated with oxidative stress levels. Results showed that cell viability was dependent to the dose of the particles and the incubation time.

Cytotoxicity of an agent against a cancer cell is a very common way to analyze its anti-cancer efficacy, but it is not the only way to determine it efficacy, and definitely not enough to make a solid conclusion. Abnormal angiogenesis (that is, formation of new blood vessels), is required for the growth and metastasis of solid tumors. Before a tumor grows beyond 1-2 mm in size, it requires blood vessels for the transportation of nutrients and oxygen. Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors, which acts as a mitogen for vascular endothelial cells in vitro, and as an angiogenic factor in vivo. Levels of VEGF within the cell is indicative of tumor progression.

A very detailed study on nanoceria’s anti-cancer properties was performed by Giri et al. 3-5 nm individual crystallites, which were loosely aggregated to 15-25 nm cerium oxide nanoparticles, were used to test their anti-cancer efficacy in vitro and in vivo against ovarian cancer cells. Three different ovarian cancer cell lines were used in this study (A2780, SKOV3, and C200) to assess the proliferation of ovarian cancer cells when treated with cerium oxide nanoparticles between 25 µM to 200 µM doses. 3 days
after the treatment, viability of the cells were assessed. Results showed that the particles did not inhibit the growth of ovarian cancer cell lines \textit{in vitro} after 3 days of treatment. Even though the particles were found to be ineffective \textit{in vitro} against ovarian cancer cells, it was found that they inhibited all growth factor-mediated cell migration (such as SDF1, HB-EGF, VEGF\textsubscript{165}, and HGF).

Even though there wasn’t any significant reduction in cell viability \textit{in vitro}, \textit{in vivo} studies, performed in a A 2780 ovarian carcinoma cell line bearing mouse model, demonstrated that there was a significant reduction in tumor growth (nanoceria treated tumor growth in mice was 33\% less in weight compared to controls), and attenuation of metastatic nodule size and numbers in the lung.

\textbf{2.2.3 Nanoceria as antibacterial agent}

While its effects on mammalian cells have been explored, relatively few studies have investigated nanoceria’s interaction with bacteria. Among these studies, some have concluded that nanoceria particles display no apparent antibacterial activity\textsuperscript{110, 111}. However, results from other research groups have not been as clear. For example, Thill \textit{et al.} investigated the impact of 7 nm nanoceria particles dispersed in water on Gram-negative bacterium \textit{Escherichia coli} (\textit{E. coli})\textsuperscript{112} and suggested that cerium oxide nanoparticles, which are positively-charged at physiological pH values, showed an electrostatic affinity towards the negatively-charged outer membrane of bacteria, thereby increasing the rate of particle attachment onto the cell surface. The main conclusion that
can be drawn from their study is that direct spatial contact has to be made in order to provoke a cytotoxic effect of cerium oxide nanoparticles against *E. coli*.

Differences in the reported antibacterial activity of nanoceria can be ascribed to many factors such as materials used during synthesis, size, media in which the ceria was contained, and surface chemistry of the particles. In a study performed by Pelletier *et al.*, a broad range of parameters, such as concentration (50–150µg/mL), size (6–45nm), exposure time, growth medium and pH were varied to test the growth and viability of *E. coli, Bacillus subtilis* (*B. subtilis*) and *Shewanella oneidensis* (*S. oneidensis*).\(^{113}\) Results showed a size dependent inhibition of *E. coli* and *B. subtilis*, whereas *S. oneidensis* appeared to be unaffected by the presence of the particles at all concentrations tested. The growth inhibition of *E. coli* decreased as the sample size increased, where 6 nm particles were the most effective. An adverse effect was observed for *B. subtilis*, growth inhibition increased with a decrease in size of the particle, and 45 nm particles were the most effective. Interactions of *E. coli* and 15 nm cerium oxide nanoparticles were observed under TEM and a general stress response was observed in Gram- negative *E. coli* cells.

Most of the studies assessing the antibacterial activity of nanoceria particles have used uncoated particles.\(^{113-115}\) Even though some reports claimed enhanced antibacterial activity with increasing nanoparticle concentrations, some reports suggested that this toxicity was due to the aggregation of unstabilized particles and some suggested that uncoated particles interacted more with the rich culture medium, thus, exhibited less
antibacterial activity due to their coated counterparts. In a study by Cuahtecontzi-Delint et al., the antibacterial activity of 100 nm ceria nanoparticles was enhanced by the addition of non-ionic surfactants. Wang et al. performed a study that compared the antibacterial activity of dextran and polyacrylate coated nanoceria against P. aeruginosa. Ceria particles (3-4 nm) coated with dextran had a higher ability to inhibit bacteria growth, mainly due to the smaller size of the particles. Similarly, in Shah et al.’s study, 2–4 nm dextran coated cerium oxide nanoparticles at pH 6 showed enhanced antibacterial activity (65 %) against E. coli.

Contrasting antibacterial responses may not only be caused by differences in treatment conditions, but also by differences in bacteria strain. Importantly, variations in thickness and constituents of the cell wall in Gram-positive and Gram-negative bacteria leads to different membrane structures, surface charge density, and metabolic processes. These structural variations may also cause differences in responses to the same treatment. For instance, Pelletier et al. showed that nanoceria was able to inhibit the growth of E. coli, and B. subtilis, but not S. oneidensis, due to the fact that S. oneidensis is a metal-reducing bacterium. Thus, the authors postulated that S. oneidensis may be inherently more resistant to metal oxide nanoparticles than bacteria without this ability.

Overall, while reports on nanoceria’s antibacterial activity have been mixed, those studies which have shown antibacterial activity emphasize the importance of the culturing conditions. Further, the mechanism for its bacteria toxicity was shown to be
dependent on whether ceria can be internalized by cells or not. For non-internalized ceria, where direct contact of the particle and cell membrane occurs, toxicity was found to be associated with reactive oxygen species (ROS) generation,\textsuperscript{121} membrane disruption\textsuperscript{122} or interference with nutrient transport functions. Membrane disruption by nanoceria is shown in Figure 9. In contrast, when ceria was internalized with lysosomal injury,\textsuperscript{123} oxidative stress\textsuperscript{104} was proposed to be the only mechanism of action.\textsuperscript{124}

\begin{center}
\includegraphics[width=0.8\textwidth]{figure1.png}
\end{center}

\textbf{Figure 1} Transmission electron micrograph of \textit{P. subcapiatana} untreated (a,e), treated with 5 nm nanoceria (b,f), and 7 nm nanoceria (c,g). Adapted from reference\textsuperscript{124}
2.2.4 Radiation protection by nanoceria

Nanoceria has been also utilized to scavenge the ROS generated during a widely
used treatment method of cancer, radiation therapy (RT).\textsuperscript{13, 89, 94} Ionizing radiation is
being used in RT to induce the radiolysis of water molecules, and then generates ROS as
superoxide and hydroxyl radicals.\textsuperscript{125-127} Colon \textit{et al.} utilized 3-5 nm ceria particles to
provide protection against radiation damage \textit{in vitro} (normal lung fibroblasts) and \textit{in vivo}
(athymic nude mice). Their findings suggests that nanoceria may be an effective
radioprotectant for healthy cells which still are in close proximity of cancer cells.\textsuperscript{94}
Tarnuzzer \textit{et al.} also assessed nanoceria against radiation damage with a breast carcinoma
(MCF-7) and a normal breast epithelial (CRL8798) cell line. Both cell lines showed a 40-
50\% decrease in viability when irradiated with 10 Gy radiation. However, 10 nM
nanoceria with particle sizes around 3-5 nm and pretreatment for 24 hours protected
almost 100\% of CRL8798 cells whereas it did not show any significant protection to
MCF-7 cells.\textsuperscript{13} Even though nanoceria was taken up at the same rate by cancer and
healthy cells, the differences in pH and the physiological differences between cancer and
healthy cells may be a reason for the different response to nanoceria treatment. The
relaxed chromatin structure of tumor cells exposes more bases in comparison to healthy
cells.\textsuperscript{128} Thus, the same amount of particles can protect more sites for healthy cells and
fewer sites for cancer cells.
In Wason et al.’s study, nanoceria was utilized as a radiation sensitizer in order to reduce the side effects of radiation therapy (RT).\(^8^9\) The effect of nanoceria treatment prior to RT on the accumulation of ROS and its impact on pancreatic cancer cell survival both \textit{in vitro} and \textit{in vivo} was examined. Their findings suggested that the pretreatment of pancreatic cancer cells with nanoceria (10 \(\mu\)M) followed by RT sensitized the cancer cells, and selectively killed cancer cells (> 2 fold). Previous research that has been done in this field already suggested that the acidity promoted SOD mimetic activity of nanoceria while inhibiting catalase mimetic activity (leading to higher amounts of H\(_2\)O\(_2\)). To further enhance this knowledge, Wason et al. studied the role of oxidation state (Ce\(^{3+}\) versus Ce\(^{4+}\)) on the activity change of the particles. RT further enhanced the SOD mimetic activity in the acidic microenvironment by possibly inducing a switch between +4 to +3. The results were supported by \(\text{O}_2^{2-} + \text{CeO}_2\_x \text{ (Ce}^{3+}\text{)} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{CeO}_2\text{ (Ce}^{4+}\text{)}\). These promising results on sensitizing pancreas cancer cells to RT while protecting the surrounding healthy cells from the toxic side effects of RT can be further studied in other types of cancer.

In summary, ROS that has detrimental effects on living organisms can be generated in many different ways endogenously as oxygen metabolism or exogenously as ionizing radiation, UV-light etc. With its unique chemical properties and minimal toxicity to healthy cells, vacancy engineered nanoceria has proven to be an efficient ROS modulator. To date, nanoceria’s antioxidant properties have been shown to be dependent
on many factors, but mainly on its oxidation state, and pH of the environment. At acidic pH values, nanoceria favors the scavenging of superoxide radicals, whereas at neutral pH values, it can scavenge both superoxide radical and \( \text{H}_2\text{O}_2 \). Additionally, \( \text{Ce}^{+3} \) has higher activity on scavenging superoxide radicals, contradictorily \( \text{Ce}^{+4} \) is much more active in scavenging \( \text{H}_2\text{O}_2 \). Now that there is such a control over nanoceria’s ROS modulating capacity, its efficacy should be further analyzed for diseases generated by other causes which eventually leads to accumulation of ROS such as in Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, etc.

2.2.5 Size and surface coating effects on nanoceria’s biological functions

To date, nanoceria has been engineered at different size ranges and with different surface coatings to be utilized in several different applications, including high-performance catalysis. Previous studies showed that nanoceria with a sub 5 nm particle size has inherent superoxide scavenging ability due to its higher \( \text{Ce}^{+3}/\text{Ce}^{+4} \) ratio.\(^{84}\)

To prevent the agglomeration of any kind of nanoparticle, a surface coating is essential. In the case of nanoceria, a coating is essential to also stabilize the catalytically active sites on the surface which are oxygen vacancies (Figure 10).\(^{90}\) It has been reported that nanoceria particles greater than 3 nm can not maintain a high \( \text{Ce}^{+3}/\text{Ce}^{+4} \) ratio under ambient conditions in comparison to bulk ceria.\(^{92}\) Without a surface coating, even sub 5 nm ceria particles would lose its inherent SOD mimetic activity due to \( \text{Ce}^{+3} \) oxidation.
Several different macromolecules were used for surface functionalization of cerium oxide nanoparticles including polyethylene glycol, dextran, poly(acrylic acid).\textsuperscript{90, 93, 118, 129, 130} Among these, a dextran and poly(acrylic acid) coating was shown to not affect the redox properties of the particles. To date, particles with the smallest size (2-4 nm) could be synthesized with a dextran coating.\textsuperscript{93, 103}

![Figure 10 Defective nanoceria scavenging superoxide radical after electron transfer. Adapted from reference 92](image)

Based on the aforementioned previous reports on nanoceria’s biological activity, it is clear that nanoceria can be engineered to have certain biological properties. Here in this dissertation, we hypothesized that altering the pH of the solution, that nanoceria is dispersed in, would allow us to control certain biological properties.
2.3 Eukaryotic and Prokaryotic Cells Used in This Thesis

In the next chapter, the structural differences between eukaryotic and prokaryotic organisms are discussed followed by an introduction of the cells used in this thesis.

2.3.1 Eukaryotic cells

Eukaryotic cells or eukaryotes are organisms whose cells have a nucleus, which contains the genetic material and is enclosed by a nuclear envelope, and other organelles enclosed within membranes. All multicellular organisms (plant and animal cells) including human cells are eukaryotes. In the following sections, details about the human cells used in this study will be listed.

2.3.1.1 Osteoblast cells

Osteoblast cells are single nucleus cells that are responsible for bone formation. Working as a group, their main function is to make and deposit a protein mixture called an osteoid. This mixture mainly contains collagen. Later, osteoblast cells deposit minerals like calcium into the protein mixture, osteoid, to make bone. Various in vitro culture models from different origins have been used to advance our understanding of the role that osteoblasts play in bone formation, remodeling, healing etc. In the era of advanced research in bone disease and bone tissue engineering, there is a growing need for reliable cell culture models that would resemble in vivo conditions as much as possible. Thus,
the primary human osteoblasts used in this study were isolated from femoral trabecular bone tissue from the knee or hip joint region.

2.3.1.2 Osteosarcoma cells

Osteosarcoma, also known as osteogenic sarcoma, is a malignant bone tumor, and the most common type of bone cancer. Types of osteosarcoma can range from low grade (where it is usually treatable with surgery) to high grade. It usually starts in the arm, leg or pelvis bones. It occurs more frequently in children and adolescents, between the age of 10-30, and in males than females.\textsuperscript{134} Osteosarcoma is often treated with a combination therapy that includes surgery, chemotherapy, and radiotherapy. Patients with high-grade osteosarcoma, usually go through three months of chemotherapy and then a surgery to remove the tumor. The drugs used most often include, methotrexate, doxorubicin, cisplatin, carboplatin or epirubicin. Depending on the size and the location of the tumor, amputation is the best way to safely remove the tumor. Patients with high-grade osteosarcoma in one location have survival rates around 60\%.\textsuperscript{135}

There are several different osteosarcoma derived cell lines, such as MG-63, Saos-2, and U2-OS.\textsuperscript{136} Osteosarcoma cells share some osteoblastic features, but due to chromosomal changes, they have abnormal cellular and molecular functions. Every cell line produces a differently composed extracellular matrix due to the difference in their characteristic labeling profile.\textsuperscript{136} Just like osteoblast cells, osteosarcoma cells are responsible for making bones and forming a bone matrix. Cancer cells, in general, have
higher metabolism compared to healthy cells. A study performed by Shapovalov et al., studied the metabolic difference between healthy, benign (Saos2) and aggressive bone tumors (143B). Their results suggest that benign or aggressive osteosarcoma cells, have larger mitochondria and an increased number of mitochondria in comparison to healthy osteoblasts, possibly leading to excessive generation of ROS. Through gene expression studies, they deduced that the mitochondrial single strand DNA binding protein, a key catalyst of mitochondrial replication, was significantly up-regulated in 143B cells. Mitochondrial dysfunction in cancer cells correlated with abnormally increased mitochondrial replication.

Among all different osteosarcoma cell lines, MG 63, is considered to show a number of features that are typical to undifferentiated osteoblasts, like the synthesis of type I and type III collagen. In this thesis, MG-63 cells were used for the in vitro assays performed due to the need for more effective osteosarcoma treatment. This cell line was derived from a 14 year old white Caucasian male.

2.3.1.3 Human dermal fibroblast cells

Fibroblast cells are found in most tissues, and their main function is that they deposit collagen and elastic fibers of the extra cellular matrix in connective tissues. Fibroblasts in different body sites arise from different embryonic origins. Hence, there is a great heterogeneity between different fibroblast lineages. Human dermal fibroblast (HDF) cells are responsible for producing the extra cellular matrix of connective tissue of
the skin and play an important role in wound healing. HDF cells used in this thesis were
derived from the dermis of adult skin.

2.3.2 Prokaryotic cells

Prokaryotic cells or prokaryotes are single celled organisms that do not have any
organelles (i.e., lacking a nucleus and membrane bound organelles). Bacteria are one of
the main categories of prokaryotes and are a diverse group of organisms with probably a
million different species existing on Earth. Bacteria exist almost everywhere from soil
to the human body and can withstand extreme conditions like alkaline lakes, acid rivers,
and high pressures. Based on where they live and what they can withstand, different
strains of bacteria differ greatly from each other. Some bacteria are quite useful, joining
to nitrogen and sulfur cycles, but some have detrimental effects to humankind.

2.3.2.1 Gram- positive and Gram-negative bacteria

Bacteria can be classified into two categories based on their staining: Gram-
positive and Gram-negative. Gram staining is a special technique that was developed by
Hans Christian Joachim Gram in 1884 to distinguish different types of bacteria by their
stain-ability. When the crystal violet stain is applied to bacterial cells, they change their
color and become crystal violet. If a cell can retain this color then it is called Gram-
positive bacteria, if it loses the color in time due to its thin cell membrane then it is called
Gram-negative bacteria. This staining technique differentiates between the bacteria
based on their cell wall structure and constituents. Gram-positive bacteria do not possess an outer membrane, but have a thicker cell wall (20-30 nm). Gram-negative bacteria have an outer membrane, but its cell wall is thinner 8-12 nm. Figure 11 depicts the differences between the two types of bacteria.

The outer cell surface of the bacteria dictates its interaction with its surroundings. The molecular components of the surface consist of macromolecules with phosphate, carboxylate and amino functional groups which are ionized at physiological pH, and thus determine the overall charge of these prokaryotic cells.

The bacterial surface possesses a net negative charge for both Gram-positive and Gram-negative bacteria. For Gram-positive species, the presence of teichoic acids on peptidoglycan cell walls dictates a negative charge as teichoic acid has phosphoryl groups. On the other hand, for Gram-negative bacteria, the source of the negativity stems from the lipopolysaccharides on the outer membrane.\textsuperscript{144}

The next section will talk about the structural details of the different strains of bacteria used in this thesis.
2.3.2.1.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a rod-shaped, versatile Gram-negative bacterium. With the help of its single polar flagellum it can move, and in fact, it is known to be one of the fastest swimming bacteria\textsuperscript{146} (Figure 12). *P. aeruginosa* is a versatile bacteria. Thus, it is found in a variety of places, usually in moist environments, like soil, marine habitats, sewage, plants, animal tissues, and marshes, etc.\textsuperscript{147, 148} Due to its versatility, it is a typical plant and animal pathogen. It is an opportunistic human pathogen, meaning it mostly affects the individuals whose immune system has already been compromised, patients with cancer, AIDS, cystic fibrosis and patients with severe burns.\textsuperscript{149, 150} To date, it is known to be the most common isolated pathogens in people hospitalized for more than a week. Treatment of *Pseudomonas* infections may involve the
use of one or a combination of the following antibiotics: ceftazidime, ciprofloxacin, gentamicin, cefepime, and aztreonam.

2.3.2.1.2 *Staphylococcus epidermidis*

*Staphylococcus epidermidis* (*S. epidermidis*) is a Gram-positive bacterium that is characterized by its round shape. It is a skin colonizer, but it has a benign relationship with its host. It is also an opportunistic pathogen. Unless there is a major breach in the host’s immune system, it can rarely cause a problem. The most common source of infections caused by *S. epidermidis* is indwelling implants like intravascular catheters,

**Figure 12 Scanning electron micrograph of *P. aeruginosa***.\(^{146}\)
cardiac pacemakers, and orthopedic implants.\textsuperscript{151, 152} As \textit{S. epidermidis} is a permanent colonizer of human skin, it increases the probability of device contamination during the insertion.\textsuperscript{153} Implantation contaminations due to \textit{S. epidermidis} rarely turn into a life threatening diseases. However, considering the frequency and the fact that they are hard to treat, it causes a huge economic burden for public health. \textit{S. epidermidis} has developed resistance to many common antibiotics as methicillin and novobiocin. Currently, vancomycin and rifampin are used to treat \textit{S. epidermidis} infections.

\section*{2.4 Motivation of the Thesis}

Cerium oxide nanoparticles have been studied intensively for their biological applications. As mentioned, the interest in ceria’s unique chemistry stems from its ability to switch oxidation states by gaining and losing an electron from its crystal lattice. It has been shown that this electron transfer is easier when particles are smaller than 5 nm and its biological activity is dependent on its surface oxidation state. The focus of this thesis was to engineer sub 5 nm dextran coated nanoceria particles to:

i) Selectively kill osteosarcoma cells,

ii) Prevent Gram-positive and Gram-negative bacteria growth, and

iii) Determine the mechanisms behind its biological activity

in a pH, time and dose dependent manner. This thesis is the first to show cerium oxide nanoparticles (+4) can be engineered to increase the ROS levels within: i) cancer cells
and effectively kill them at acidic pH values and ii) Gram-positive and Gram-negative bacteria as well as prevent their growth at basic pH values.
CHAPTER 3

Experimental

3.1 Synthesis and Characterization of Dextran Coated Nanoceria

In this section, the experimental details of the three different projects are given along with the synthesis and characterization of 0.1 M dextran coated nanoparticles. The particles used in the three parts of the projects were the same, thus, details regarding the synthesis and chemical and physical characterization of the dextran coated ceria is given at the beginning of the chapter. Following that, biological assays performed with eukaryotic and prokaryotic cells, cell culture conditions and characterization of nanoparticles in biological fluids are given as a separate sub-chapter for each project.

3.1.1 Synthesis of dextran coated nanoceria

Dextran coated ceria nanoparticles were synthesized according to a modified protocol published from Perez et al. in 0.1 M dextran T-10 (Pharmacosmos, Holback, Denmark) concentrations. Briefly, 1 mL of an aqueous solution of 1 M cerium nitrate (Sigma Aldrich, St Louis, MO) and 2 mL of 0.1 M dextran were mixed and the prepared solutions were added dropwise to 6 mL of a 30% ammonium hydroxide (Sigma Aldrich, St. Louis, MO) solution while stirring for 24 hours at 25 °C. Upon the addition of the precursor into ammonium hydroxide, the solution turned light yellow. With the formation of stabilized dextran coated cerium oxide nanoparticles, the solution became dark brown.
After 24 hours, particles were centrifuged at 4000 rpm for 30 min to remove any debris and any large agglomerates as well as unattached dextran. The final nanoparticle solution was stored in a refrigerator at 4 °C (Figure 13).

![Diagram of nanoparticle synthesis](image.png)

**Figure 13 0.1 M Dextran coated cerium oxide nanoparticle synthesis**

Before starting any experiments, 1 mL of the nanoparticle solution, which was on weighing cups, was dried on a hotplate until all of the water evaporated at 60 °C. Following, all nanoparticle concentrations were calculated based on the dried nanoparticle concentrations (weight/mL).
3.1.2 Characterization of nanoceria

3.1.2.1 Particle size and size distribution

Following nanoparticle synthesis, first, transmission electron microscopy (TEM) (JEM 1010, JEOL) was used to observe particle size, distribution, and morphology at an 80 keV accelerated voltage. Particles were analyzed on a carbon coated copper grid. Dynamic light scattering (DLS) (Zetasizer, NanoZS, Malvern Instruments) experiments were performed to analyze the particle size and distribution of the nanoparticles.

3.1.2.2 Chemical characterization

X-ray photoelectron spectroscopy (XPS) was used to determine the valence state of ceria. XPS consists of a dual source, non-monochromated X-ray source (Phi model 04-548) and a hemispherical analyzer (Phi model 10-360). The aluminum anode used in this study produces Al Kα X-rays (1486.6 eV) at 100 W. The system was calibrated using Au 4f and Cu 2p, and had a minimum full width half maximum (FWHM) of 1.2 eV with an 80% Gaussian/Lorentzian distribution at a pass energy of 35.75 eV. Background subtraction was performed using the integrated Shirley method.

UV-visible absorption spectroscopy measurements were performed in order to observe the changes in the oxidation states of ceria in solution depending on the pH. Particles were dispersed in DMEM at 1000 µg/mL concentrations and the pH of the solution was adjusted to pH 6 and pH 7 with acetic acid (mentioned in detailed below).
Spectra for the cerium oxide nanoparticles were acquired using a SpectraMax M3(MT05412) at room temperature in 3 mL cuvettes.

3.2 pH Dependent Activity of Nanoceria on Prohibiting Osteosarcoma

Cell Growth

In this section, experimental details of dextran coated nanoceria’s pH dependent activity on prohibiting osteosarcoma growth while protecting healthy osteoblast cells is given. Particles were synthesized and characterized as explained in the previous section. Further characterization of the particles in the cell culture media is given in this section.

3.2.1 Nanoceria in mammalian cell culture media

The size and surface charge of the synthesized nanoceria particles varied after dispersing them in cell culture media (Dulbecco’s Modified Eagle Medium (DMEM) (ATCC® 30-2003™), supplemented with 10% Fetal Bovine Serum (FBS) (ATCC® SCRR-30-2020™), 1% Penicillin/Streptomycin (P/S) (ATCC® 30-2300™) or osteoblast basal medium (PromoCell, C-27010), supplemental mix (PromoCell, C-39615), and 1% P/S at different pH values (pH 6, 7 and 9). Particle concentration was fixed to 1000 µg/mL. The zeta potential of the particles was measured by DLS (90Plus Zeta, Brookhaven Instruments) and the software provided by the manufacturer. In addition, the size of the nanoceria particles (1000 µg/mL, pH 9.8) were examined in DMEM media supplemented with 0, 1, 2.5, 5, and 10 % FBS and 1% P/S.
3.2.2 \textit{pH adjustments}

To conduct experiments at different pH values, at each nanoparticle concentration (10 \(\mu\)g/mL, 50 \(\mu\)g/mL, 100 \(\mu\)g/mL, 250 \(\mu\)g/mL, 500 \(\mu\)g/mL and 1000 \(\mu\)g/mL), the pH of the solution was adjusted to be basic (pH 9), neutral (pH 7), and acidic (pH 6, pH 5 and pH 3) values. The pH ranges were decided depending on the characteristic features of nanoceria and the tumor microenvironment. pH 6 is the approximate pH value of the bone tumor microenvironment \cite{30}. pH 7 is the relative value for regular cell growth. pH 9 is also a closer pH value of the synthesized nanoceria. Although pH 5 and pH 3 are very low pH values for regular cell growth, these pH values were also tested due to possible nanoceria free radical scavenger properties that may affect the survival of cells. Thus, the effect of these particles at pH 6 was tested on healthy osteoblast cells as well.

To adjust the pH values, nanoparticles were dispersed in the corresponding cell culture media at each concentration; an aqueous solution of 1 M to 10 M acetic acid (Sigma Aldrich, USA) was added to fix the pH values at the above mentioned acidic pH values. For pH 9, an aqueous solution of 1 M and 10 M sodium hydroxide (Sigma Aldrich, USA) was added dropwise. The pH of the solutions was measured with a pH meter (S230 Seven Compact, Mettler Toledo).
3.2.3 Osteoblast and osteosarcoma cell culture

In this study, two types of cell lines were used. One was an osteosarcoma cell line MG-63 (ATCC CRL-1427) and the other one was an osteoblast non-cancerous cell line (PromoCell, C-12720). Cells at passage numbers 4 to 7 were used in these experiments. Osteosarcoma cells were cultured in DMEM supplemented with 10% FBS and a 1% P/S solution. Healthy osteoblast cells were cultured with osteoblast basal medium with a supplement mix and a 1% P/S solution. Both of the cell lines were incubated at 37°C in air supplemented with 5% CO₂ until 80% confluency was achieved. Cells were enzymatically detached from the surface of the T-75 using trypsin (0.5%, Gibco Life Technologies) and collected by centrifugation at 1100 rpm for 5 minutes.

When not in use, both cell lines were stored in 90% FBS and a 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) solution and kept in a cyrofreezer. Prior to experiments, cells were thawed and dispersed in their own cell culture media. To remove the excess DMSO, cells were centrifuged at 1100 rpm for 5 mins.

3.2.4 Cytotoxicity (MTS) assays

CellTiter 96® AQucous One Solution Cell Proliferation Assays (MTS) were carried out for up to 5 days in culture using DMEM, 10% FBS and a 1% penicillin-streptomycin solution for osteosarcoma cells. For healthy osteoblast cells, MTS assays were also carried out for up to 5 days in culture using osteoblast basal medium with a supplement mix and a 1% penicillin-streptomycin solution. Both of the cell lines were seeded into a
96 well plate at 5,000 cells/well (15,000 cells/cm²) and were allowed to adhere at 37 °C in 5% CO₂ for 24 hours. The next day, the cells were treated with dextran coated ceria nanoparticles at 10 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL for each pH value. As a control group, cells were cultured in the pH-adjusted media only (no nanoceria). Following 24 hours of incubation with nanoparticles, 200 µL of media were removed from each well and 200 µL of an MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3581 Promega) reagent (1:5 ratio with cell culture media) was added to each well and incubated for 3 hours. At the end of incubation, a color change from pink to dark brown was observed, and absorbance of each well was measured by a SpectraMax M3 (MT05412) at 490 nm (Figure 14).

Figure 14 Schematic of nanoceria treatment against cancer and healthy cells.
To create a standard curve, serial dilutions of osteosarcoma cells and healthy osteoblast cells were seeded into a 96 well plate (50,000 cells/cm² to 0 cells/cm²) and were left to equilibrate for 2.5 hours. After the cells adhered, 200 µL of an MTS reagent (1:5 ratio with appropriate cell culture media) was added to each well and incubated for 3 hours. Again, the absorbance of the solution was measured at 490 nm.

Viability graphs were plotted by normalizing the data with respect to control groups, pH adjusted media with no ceria, from the first day of each experiment. Data of cell viability experiments performed at pH 3 and pH 5 were not reported due to the cell death that occurred in all control groups and nanoceria treated cells due to the very low pH.

To create a standard curve, serial dilutions of osteosarcoma cells and osteoblast cells were seeded into a 96 well plate (50,000 cells/cm² to 0 cells/cm²) and were left to equilibrate for 2.5 hours. After the cells adhered, 200 µL of an MTS reagent (1:5 ratio with appropriate cell culture media) was added to each well and incubated for 3 hours. Again the absorbance of the solution was measured at 490 nm (See Appendix).

3.2.5 IC-50 value calculation

IC₅₀ represents the concentration of a drug that is required for 50% inhibition of cell growth/metabolic and/or biochemical function. Here, IC-50 values were calculated and indicated in Table 3 for each pH condition for osteosarcoma cells and pH 6 and 7 for healthy osteoblast cells.
3.2.6 Reactive oxygen species measurements

Reactive oxygen species (ROS) measurements were performed using the Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, I36007) for osteosarcoma and osteoblast cells for one day. Osteosarcoma cells were cultured in 96 well plates at 5,000 cells/well (15,000 cells/cm²) and were allowed to adhere at 37 °C in 5% CO₂ for 24 hours using DMEM, 10% FBS, 1% penicillin/streptomycin, whereas healthy osteoblast cells were cultured in osteoblast basal media, supplement mix, and a 1% penicillin/streptomycin solution. The next day, the cells were treated with dextran coated ceria nanoparticles at 250 µg/mL, 500 µg/mL and 1000 µg/mL at pH 6. As a control group, cells were cultured in the pH 6 media only (no nanoceria), as well as regular media at pH 7.4 for comparison. Following 24 hours of incubation with nanoparticles, ROS measurements were performed. The carboxy-H₂DCFDA dye was dissolved in 50 µL DMSO (provided in the kit) to achieve a 10 mM final concentration. The solution was vortexed until the powder was completely dissolved. 10 mM of a carboxy-H₂DCFDA solution was further diluted to 25 µM in a warm Hank’s Balanced Salt Solution (HBSS/Ca/Mg) (Life Technologies, 14025126). After the nanoparticle treatment, cells were washed with warm HBSS/Ca/Mg two times and trypsinized for 15 minutes with 20 µL trypsin (0.083%). Following the trypsinization of the cells, 90 µL of 25 µM carboxy-H₂DCFDA was added to each well to label the cells. 100 µL of the cell and dye suspension were transferred into blank 96 well plates and were incubated at 37
°C for 30 min. The fluorescence measurements were taken every 5 minutes for 30 min using a SpectraMax M3 (MT05412) spectrophotometer at 495/529 nm.

### 3.2.7 Statistical analysis

Numerical data were analyzed using standard analysis of variance (ANOVA) followed by Student t-tests. All cell experiments were repeated three times in triplicate for all of the nanoparticle concentrations and pH values.

### 3.3 pH- Controlled Nanoceria Inhibition of Both Gram- Positive and Gram-Negative Bacteria Growth

In this section, experimental details of the dextran coated cerium oxide nanoparticles activity against the Gram- positive *Staphylococcus epidermidis* (*S. epidermidis*) and Gram- Negative *Pseudomonas aeruginosa* (*P. aeruginosa*) are given. Cerium oxide nanoparticles were synthesized and characterized as explained in the previous section.

#### 3.3.1 Cerium oxide nanoparticles in bacteria growth medium

In order to determine the hydrodynamic radius, and distribution of the colloidal nanoparticles, Dynamic Light Scattering (DLS) (Malvern Zetasizer Nano ZS) measurements were performed. Particle concentrations were prepared at 1000 μg/mL, dispersed in phosphate buffered saline (PBS) (Sigma Aldrich, P3813), and the pH of the solution was adjusted to either pH 6 or 9. To determine the surface charge of the
particles, zeta potential measurements were performed by DLS (Malvern Zetasizer Nano ZS) and the software provided by the manufacturer.

The oxidation states were measured via UV-visible absorption spectroscopy in order to observe the changes in the oxidation states in solution depending on the pH. Particles were dispersed in Tryptic Soy Broth (TSB) (Sigma Aldrich, 22092) at a 1000 μg/mL concentration and the pH of the solution was adjusted to pH 6 and pH 9 with acetic acid (mentioned in detail below). pH-adjusted TSB media was used as a reference to eliminate the absorption of TSB. Spectra for the cerium oxide nanoparticles were acquired using a SpectraMax M3(MT05412) at room temperature in polystyrene cuvettes.

3.3.2 pH adjustments

To conduct experiments at different pH values, for each nanoparticle concentration (250 and 500 μg/mL), the pH of the solution was adjusted to basic (pH 9) and acidic (pH 6) conditions. For this, after the nanoparticles were dispersed in the corresponding bacteria culture media at each concentration, an aqueous solution of 1 M to 10 M acetic acid (Sigma Aldrich, USA) was added to fix the pH values at the above-mentioned acidic values. For pH 9, an aqueous solution of 1 M sodium hydroxide (Sigma Aldrich, USA) was added dropwise. The pH of the solutions was measured with a S230 Seven Compact pH meter (Mettler, Toledo).
3.3.3 Bacterial culture and maintenance

Before bacterial experiments, for both *Pseudomonas aeruginosa* (American Type Culture Collection, 27853, Manassas, VA, USA) and *Staphylococcus epidermidis* (American Type Culture Collection, 12228, Manassas, VA, USA), a sterile 10 µL loop was used to withdraw bacteria from the prepared frozen stock, the bacteria were streaked onto a Tryptic Soy Broth (TSB) (Sigma Aldrich, 22092) Agar plate (Sigma-Aldrich, A1296), and the TSB agar plate was incubated for 20 hours, to form single bacterial colonies. For each experimental trial, a single bacterial colony of *P. aeruginosa* or *S. epidermidis* was selected and grown overnight in TSB media on a shaking incubator set at 200 rpm and 37 ºC. The pH (pH 9 and 6) and nanoceria concentrations (250 µg/mL, 500 µg/mL) of the solution were adjusted as previously mentioned in section 3.3.2. The overnight bacteria suspension was adjusted by OD$_{600}$ measurements and diluted to possess a final bacterial density of $10^6$ CFU/mL at each nanoparticle concentration. Two sets of controls were used for each experiment, one treated at the specific pH studied for each experiment, and one regular (pH 7.4) TSB media. The well plate was then incubated at 37 ºC inside a spectrophotometer under static conditions (SpectraMax Paradigm, Molecular Devices, Sunnyvale, CA) with OD$_{600}$ measurements taken every 2 minutes for 24 hours to measure the bacterial growth curves of the treatments.
3.3.4 Curve fitting

In order to analyze the kinetics of bacteria growth, three typical parameters derived from growth curves were estimated. The Gompertz growth model was fitted to the experimentally-collected growth curves using a Matlab Curve Fitting Toolbox, with the Gompertz equation given as a custom function to the Curve Fitting Toolbox. Parameters derived from growth curves are as follows: the lag time, \( \lambda \), x-axis intercept of the tangent, maximum specific growth rate, \( \mu \), denoted by the slope of the curve, and the maximum cell growth, \( A \), is the asymptote of the curve.\(^\text{154}\)

The Gompertz equation is given in Equation 1, and then rewritten with biological parameters (\( \mu \), \( \lambda \), and \( A \)) to substitute the mathematical parameters (Equation 2).

\[
y = a \cdot \exp \left(-\exp(b - cx)\right) \quad \text{(Equation 1)}
\]
\[
y = A \cdot \exp \left[-\exp \left(\frac{\mu_m \cdot e}{A} (\lambda - t) + 1\right)\right] \quad \text{(Equation 2)}
\]

3.3.5 LIVE/DEAD assay

A confocal microscope (Zeiss LSM 700, 63 X 1.4 N.A. Plan Apo oil objective, Zeiss, Oberkochen, Germany) with a 63X objective lens was used for imaging bacteria alone (control) and nanoceria treated (500 \( \mu \)g/mL) bacteria to distinguish viable cells from dead cells, and to observe any morphological changes with the help of a LIVE/DEAD BacLight Bacterial Viability Kit (L7007, Molecular probes, Invitrogen). Briefly, \( P. \) aeruginosa and \( S. \) epidermidis cells were treated with nanoceria in 5 mL
cultures in a shaking incubator for 5 hours and 30 minutes at 37 °C. To adhere the cells onto the surface, Cell Tak (Sigma Aldrich, St Louis, MO) was used to coat the surface of the chambered coverglass according to the instructions from the manufacturer, and left for Cell Tak to coat on the surface of coverglass for 20 minutes at room temperature. After 20 minutes, 400 µL of the cell solution was added onto the Cell Tak treated chambered coverglass and incubated for another 30 minutes at 37 °C under static conditions to allow cells to adhere onto the surface. After the cells attached, the media was aspirated from each well and replaced with 400 µL of the dye solution (1.5 µL/mL of each dye in saline solution). Cells were kept in the dark at room temperature for 15 minutes before the analysis.

3.3.6 Nanoceria-bacterial interactions observed by TEM

For TEM analysis of bacteria with nanoceria, 4 mL bacterial suspensions were exposed to the nanoceria at pH 9 and pH 6 for 6 hours in a shaking incubator at 37 °C. Cells were collected by centrifugation at 3000 rpm for 15 minutes in order to reduce the chance for artifacts, and concentrate the samples. Following centrifugation, pelleted cells were dispersed and fixed in 2.5% glutaraldehyde solution in a 0.2 M Sodium Cacodylate buffer (pH 7.4) (Electron Microscopy Sciences, 11652, Hatfield, PA) for 16 hours at 4 °C. The next day, cell morphology was visualized under the TEM.
3.3.7 Determination of reactive oxygen species (ROS) generation

Intracellular ROS generation in bacteria was measured using non-fluorescent 2’,7’ Dichlorofluorescin diacetate (Sigma Aldrich, D6883). Briefly, 1 mL of bacteria cultures from the untreated control and 500 µg/mL from the treated *P. aeruginosa* and *S. epidermidis* cells were incubated at pH 9 and pH 6 for 6 hours. Then, bacteria were centrifuged at 8500 rpm for 5 minutes. A bacteria pellet was re-suspended in 30 µg/mL of a dye solution in PBS, and left in a shaking incubator at 37 °C for 45 minutes. Upon entry of DCFH$_2$-DA into cells, an oxidation reaction between the non-fluorescent dye and intracellular ROS occurred, and the dye turned into a highly fluorescent compound called 2,7’ dichlorofluorescein. Cells were then centrifuged and re-suspended in 450 µl of fresh PBS, and fluorescence measured at an excitation of 485 nm, and emission of 528 nm in a black 96-well plate using SpectraMax M3 (MT05412; Molecular Devices LLC, Sunnyvale, CA).

To quantify the colony forming units (CFU) at each solution, following 6 hours of incubation, several dilutions of each solution were prepared. From each dilution, 5 * 10 µL drops were pipetted onto an agar plate and incubated for approximately 14 hours. CFUs were then counted manually. Total fluorescence was divided into CFU/mL in order to find the fluorescence per colony.
3.3.8 Statistical analysis

Numerical data were analyzed using standard analysis of variance (ANOVA) followed by Student t-tests. All cell experiments were repeated three times in triplicate for all of the nanoparticle concentrations and pH values.

3.4 pH-Dependent Cell Rescue Potential of Nanoceria for Cells Under Stress

In this section, the cell rescue potential of dextran coated cerium oxide nanoparticles was analyzed on human dermal fibroblast cells that were stressed with hydrogen peroxide. HDF cells are under constant oxidative stress as they are exposed to various exogenous ROS sources. Hence it is important that they survive in case of oxidative stress. As a well-established ROS modulator, nanoceria particles were pre-incubated with HDF cells and then exposed to high levels of oxidative stress via H₂O₂. The effect of pH and pre-incubation time was analyzed. Particles used in this study were synthesized and characterized as explained in the previous section.

3.4.1 Human dermal fibroblast culture

Human dermal fibroblasts (HDF) (Lonza, CC-2509) were cultured in DMEM supplemented with 10% FBS and a 1% P/S solution. Cells at passage numbers 4 to 7 were used in these experiments. Cells were incubated at 37 °C in air supplemented with 5% CO₂ until 80% confluency was achieved. Cells were enzymatically detached from the
surface of the T-75 using trypsin (0.5%, Gibco Life Technologies) and collected by centrifugation at 1100 rpm for 5 minutes.

When not in use, HDF cells were stored in 90% FBS and a 10% DMSO solution and kept in a cryofreezer. Prior to experiments, cells were thawed and dispersed in their own cell culture media. To remove the excess DMSO, cells were centrifuged at 1100 rpm for 5 mins.

3.4.2 pH adjustments

To conduct experiments at different pH values, at each nanoparticle concentration (250 μg/mL, 500 μg/mL and 1000 μg/mL), the pH of the solution was adjusted to neutral (pH 7) and acidic (pH 6) values.

To adjust the pH values, nanoparticles were dispersed in the corresponding cell culture media at each concentration; an aqueous solution of 1 M to 10 M acetic acid (Sigma Aldrich, USA) was added to fix the pH values at the above mentioned acidic pH values. The pH of the solutions was measured with a pH meter (S230 Seven Compact, Mettler Toledo).

3.4.3 Cytotoxicity (MTS) assays

CellTiter 96® AQ cell Proliferation Assays (MTS) were carried using DMEM, 10% FBS and a 1% penicillin-streptomycin solution for HDF cells. HDF cells were seeded into a 96 well plate at 5,000 cells/well (15,000 cells/cm²) and were
allowed to adhere at 37 °C in 5% CO₂ for 24 hours. 24 hours after seeding, the cells were pre-incubated with dextran coated ceria nanoparticles at 250 µg/mL, 500 µg/mL and 1000 µg/mL at pH 7 and pH 6 for 2, 4, and 8 hours. Following pre-incubation with nanoparticles, 1µL of 100 mM, 150 mM and 175 mM of H₂O₂ was added and incubated for another 24 hours to assess the rescue capability of dextran coated cerium oxide nanoparticles. As a control group, cells were cultured in the pH-adjusted media with corresponding H₂O₂ concentration (no nanoceria). Following 24 hours of incubation with nanoparticles, 200 µL of media were removed from each well and 200 µL of an MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3581 Promega) reagent (1:5 ratio with cell culture media) was added to each well and incubated for 3 hours. At the end of incubation, a color change from pink to dark brown was observed, and absorbance of each well was measured by a SpectraMax M3 (MT05412) at 490 nm.

Viability graphs (24 hours) were plotted by normalizing the data with respect to control group: cells treated with pH adjusted media without ceria, from the first day of each experiment.

To create a standard curve, we seeded serial dilutions of osteosarcoma cells and healthy osteoblast cells into a 96 well plate (50 000 to 0 cells/cm²) and were left to equilibrate for 2.5 h. After cells adhered, 200 µL of an MTS reagent (1:5 ratio with appropriate cell culture media) was added to each well and incubated for 3 h. Again, the absorbance of the solution was measured at 490 nm.
3.4.4 **Statistical analysis**

Numerical data were analyzed using standard analysis of variance (ANOVA) followed by Student $t$-tests. All cell experiments were repeated three times in triplicate for all of the nanoparticle concentrations and pH values.
CHAPTER 4

Results and Discussion

4.1 Utilization of Cerium Oxide Nanoparticles Against Cancer Cells

In the first section of Chapter 4, results regarding the utilization of dextran coated nanoceria particles are listed. First, results regarding a detailed study of the stability of the nanoparticles in cell culture media are given. Following the particle characterization, the nanoparticle dose and pH dependent toxicity results against cancer and healthy cells are given. An elaborate discussion about the mechanism of action of nanoceria and potential anti-cancer applications is given at the end of the first section of this chapter.

4.1.1 Characterization of dextran coated nanoceria

Even though nanosized materials have been utilized for a multitude of applications over several decades, toxicologists are still investigating their potentially hazardous health effects.\textsuperscript{155,156} The toxicity of nanoparticles are dependent on several factors including the nature and chemical composition of the nanoparticle core, synthesis process and unreacted reactants, size, shape, crystallinity, surface reactivity, solubility in aqueous media, and degree of aggregation among many other factors.\textsuperscript{157-159}

In terms of reducing their toxicity and enhancing their circulation time, surface coatings are now a prerequisite for nanoparticles. However, it is difficult to control the response of colloidal nanoparticles to their environment due to the complexity of
biological fluids. The nanoparticles are very sensitive to conditions like pH, ionic strength, concentration and temperature. Therefore, it is important to characterize the nanoparticles in biological fluids. When nanoparticles are dispersed in biological fluids, two different hypotheses have been reported. One possible scenario is that particles may be surrounded with a protein corona and other bio-macromolecules. Another possibility is that they may aggregate in kinetically driven processes through the formation of clusters.

Figure 15 shows the characterization results of the ceria nanoparticles. TEM micrographs of the ceria nanoparticles with the 0.1 M dextran coating demonstrated that particle sizes were 3-4 nm in diameter (Figure 15 a) and were spherical in shape.

X-ray photoelectron spectroscopy (XPS) measurements were performed to determine the relative atomic composition and the bonding state of the Ce on the surface of the nanoparticles. XPS confirmed the presence of Ce. 1 atomic % Ce was detected in the 0.1 M dextran coated cerium oxide samples. The sampling depth for the XPS is approximately 10 nm. Assuming a uniform particle sample and the ability to detect a small amount of Ce, suggests a layered structure with a dextran coating thickness of close to, but less than, 10 nm surrounding each particle. Figure 16 shows the four peaks of the Ce3d spectrum corresponding to the pairs of spin orbit doublets identified as Ce(III) oxides [Ce$^{3+}$ state].

UV-visible spectroscopy measurements were performed in order to elucidate the oxidation state of cerium oxide nanoparticles at different pH values in solution. Cerium
can strongly absorb the ultraviolet light at both oxidation states. Cerium (III) absorbance occurs between the 230 to 260 nm range, whereas cerium (IV) absorbs the light between the 300 to 400 nm range.\textsuperscript{50} Contrary to XPS results performed on dried particles immediately after synthesis, UV-visible absorption spectroscopy measurements showed the presence of Ce (IV) oxides at pH 6 and pH 7 in the DMEM solution as a single peak was obtained around 300 nm (Figure 17). The role of serum in the cell culture media on particle stability was investigated here by measuring the particle size by DLS at different FBS concentrations (Figure 15 b). These experiments were performed because it is anticipated that the proteins and the other macromolecules in cell culture media might actively adsorb on the surface of the nanoparticle. The results demonstrated that nanoparticle sizes in various amounts of FBS were larger than their non-serum containing samples. At lower FBS concentrations at 1% and 2.5%, the hydrodynamic radius of the particles increased to over 80 nm. However, as the FBS concentration increased to 5% and 10 %, the hydrodynamic radius reduced to 70 nm. As indicated in the literature, it was assumed here that the change in diameter of the nanoparticles can be explained by the formation of a protein corona around the particles, although this would need to be the focus of future studies.\textsuperscript{161,162}
Figure 15  (a) TEM micrograph of 0.1 M dextran coated nanoceria, (b) Size of DCN in DMEM media supplemented with (0, 1, 2.5, 5, 10 %) FBS and 1% P/S, (c) Size of DCN in the presence of osteosarcoma culture media (DMEM supplemented with 10% FBS 1% P/S) at different pH values, and (d) Zeta potential of DCN at different pH values. ¹denotes that particles were dispersed in osteosarcoma (OS) media, ²denotes that particles were dispersed in osteoblast (OB) media.
Figure 16 XPS Ce 3d peaks of cerium oxide nanoparticles. The Ce 3d spectra are composed of two core electrons 3d_{5/2} and 3d_{3/2} (labeled a and b) with multiplets (a' and a'', b' and b'').
The hydrodynamic diameter of the particle was measured as 40 nm when it was dispersed in a PBS solution (i.e. 0% FBS) at 1000 µg/mL. Then, an increase in the hydrodynamic diameter was observed as the FBS concentration increased to 1% (85 nm in diameter) and 2.5% (79 nm in diameter). Further increasing the FBS concentration to 5% and 10% showed a declining trend in the particle size, where it was measured to be around 65 nm for both concentrations. These findings can be interpreted as the formation of a protein corona around the particles, especially at lower FBS concentrations at short times. Regardless, particle size was still measured to be below 100 nm (Figure 15 b).

After observing the effect of the FBS amount on the diameter of the nanoparticles, pH dependent particle sizes were also examined. As shown in Figure 15 c, particle size...
changed with respect to the pH of the solution. In an acidic environment (pH 6), particle size was 29 nm and at a physiological pH (pH 7), particle size was 45 nm. Lastly, in a basic environment (pH 9), particles were 50 nm in diameter. To observe the effect of pH on the surface charge of the 0.1M dextran coated cerium oxide nanoparticles, zeta potential measurements were performed. The zeta potential of the 0.1 M dextran coated ceria nanoparticles was 0.58 mV with a deviation of +/- 2.27 mV at physiological pH values (pH 7) (Figure 15 d). It was observed that at different pH values, the surface charge of the particles changed as well. At pH 6 in healthy osteoblast and osteosarcoma media, the surface charge of the particles were 16.68 mV and 17.52 mV, respectively. At pH 9, in osteosarcoma media, the zeta potential was measured as 6.32 mV. The surface charge of the nanoceria was positive at pH 6 and pH 9 and near a neutral zeta potential at pH 7.

Besides the physical and chemical properties of nanoparticles, reactive oxygen species (ROS) generation is one of the most well known methods related to nanoparticle toxicity.\textsuperscript{156,165} The exact mechanism of ROS generation by nanoparticles still remains unclear, but it is known that nanoparticles of various sizes and chemistries attack the mitochondria (a redox active organelle of cells) which may change ROS production, and possibly cause an alteration in antioxidant activity.\textsuperscript{156} Of course, this mechanism of toxicity is being exploited for cancer cell treatment to increase their death without using chemotherapeautic agents that also kill healthy cells.
Nanoparticle interactions with cells *in vitro* need to be evaluated to understand their mechanism of biological influence induced by nanoparticles. Here, 0.1 M dextran coated sub 5 nm cerium oxide nanoparticles were synthesized in a 30% ammonium hydroxide solution, when they were dispersed in cell culture media at various pH values and concentrations (Table 2). It is believed that the catalytic activity of nanoceria stems from oxygen defects or delocalized electron density on the surface of the particles. A low reduction potential of the Ce$^{3+}$/Ce$^{4+}$ (~1.52 V) redox couple makes regeneration of the surface and catalytically active sites feasible for extended time periods. Thus, by altering the pH of the environment (i.e., ion balance), a change in the catalytic activity can be created.

<table>
<thead>
<tr>
<th>Nanoparticle Concentration (µg/mL)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>7.8</td>
</tr>
<tr>
<td>50</td>
<td>8.3</td>
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<tr>
<td>100</td>
<td>8.7</td>
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<td>250</td>
<td>9.2</td>
</tr>
<tr>
<td>500</td>
<td>9.5</td>
</tr>
<tr>
<td>1000</td>
<td>9.8</td>
</tr>
</tbody>
</table>

### 4.1.2 Cellular responses to dextran coated nanoceria treatments at different pH values

Initially, the cytotoxicity of nanoceria on osteosarcoma cells was studied at 5 different pH values: pH 3, pH 5, pH 6, pH 7, and pH 9. Results from pH 3 and 5 were not
reported, because, including the control groups, none of the cells were alive at any nanoparticle concentration. The addition of acetic acid and sodium hydroxide did not have any effect on cell growth for both osteoblast and osteosarcoma cells at pH 6, pH 7, and pH 9. They were used to change the pH of the culture environment.

4.1.2.1 Dextran coated nanoceria toxicity on osteosarcoma cells at pH 9

As shown in Figure 18 a, at the end of the first day, osteosarcoma cell viability did not change at any ceria nanoparticle concentration (10 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL) compared to the control group at pH 9. After 3 days, at low nanoparticle concentrations (10-250 µg/mL), no toxic effect of nanoceria was observed. Slight increases in cell viability were obtained due to cell proliferation. However, cell viability when treated with 500 µg/mL was statistically less compared to the control group (160% compared to 140%, respectively). After 5 days, a very similar trend in terms of cell viability at low nanoparticle concentrations (10-100 µg/mL) was observed after 3 days. For higher concentrations, cell viability when treated with 250 µg/mL ceria nanoparticles reduced to 140% and when treated with 1000 µg/mL, cell viability reduced to 75%. After day 3, a drastic change was observed at 500 µg/mL, but the cytotoxic effect of nanoceria shifted to lower concentrations of 250 µg/mL at the end of the 5th day.
4.1.2.2 Dextran coated nanoceria toxicity on osteosarcoma cells at pH 7

Osteosarcoma cell viability also showed a time and dose dependent response to nanoceria treatments at pH 7. At the end of the first day, cell viability was not significantly different at any nanoparticle concentration (10 µg/mL to 1000 µg/mL) compared to the control, when cells were treated with media at pH 7. A similar cell response was observed for pH 9 as described above. After 3 days of treatment, cell viability decreased to 180% when exposed to 500 µg/mL and then gradually decreased to 125% when exposed to 1000 µg/mL dextran coated ceria nanoparticles. After 5 days, osteosarcoma cells showed a statistically significant decrease compared to the control group when they were treated with 250 µg/mL ceria nanoparticles. Cell viability gradually decreased from 425% to 80% when exposed to 1000 µg/mL ceria nanoparticles compared to controls (Figure 18 b).
Figure 18 Toxicity effect of 0.1 M DCN against osteosarcoma cells at various concentrations after 1, 3 and 5 days of treatment at (A) pH 9 and (B) pH 7. N=3, Data = mean +/- SD and *p < 0.05 compared to controls at the same time period and **p < 0.05 at the same DCN concentration at the different time points. Control groups are only cells treated with media at (A) pH 9 and (B) pH 7.
4.1.2.3 Dextran coated nanoceria toxicity on osteosarcoma cells at pH 6

An enhancement in nanoceria’s cytotoxicity was observed as the pH was reduced to below physiological pH values and closer to that of tumor values. After 1 day of nanoparticle treatment at pH 6, cell viability decreased to 75% when exposed to 250 µg/mL of ceria nanoparticles; when exposed to 500 µg/mL of ceria nanoparticles, cell viability decreased to 55% and at 1000 µg/mL, cell viability decreased to 50% (Figure 19).

The most significant observation at pH 6 involved a shorter treatment time at a certain concentration for killing osteosarcoma cells. In addition, a lower dose of nanoparticles was shown to have a higher efficiency in killing osteosarcoma cells at pH 6 compared to pH 9 and pH 7 after 1 day of treatment. After 3 days, a statistically significant reduction in cell viability was observed at 100 µg/mL and no cell growth was observed at 1000 µg/mL of ceria nanoparticles. A similar trend was observed after 5 days of treatment at 100 µg/mL and 1000 µg/mL of ceria nanoparticles as shown in Figure 19.
Figure 19 Toxicity effect of 0.1 M DCN against osteosarcoma cells at various concentrations after 1, 3 and 5 days of treatment at pH 6. N=3, Data = mean +/- SD and *p < 0.05 compared to controls at the same time period and **p < 0.05 at the same DCN concentration at the different time points. Control groups are only cells treated with media at pH 6.

When first day cytotoxicity results were compared at pH 7 and 9, it was observed that there were no significant differences compared to controls at any concentration. However, at pH 6, cell death started at a 250 µg/mL dextran coated cerium oxide concentrations for the first day. At the end of 3 days of nanoparticle treatment, the IC-50 value for pH 7 was 1000 µg/mL and was greater than 1000 µg/mL for pH 9 (Table 3). After 5 days, it was found to be greater than 250 µg/mL for both pH 7 and 9. 3 days after treating osteosarcoma cells at pH 6, the IC-50 value was found to be greater than 50 µg/mL; after 5 days this value was 100 µg/mL. Among all of the pH values, nanoceria
showed the most toxic behavior at a pH of 6, meaning the highest numbers of osteosarcoma cell death.

4.1.2.4 Dextran coated nanoceria toxicity on healthy osteoblast cells at pH 6 and pH 7

As shown in the previous sections, the most effective pH for killing osteosarcoma cells was pH 6, which is the approximate pH of the bone tumor microenvironment. In order to utilize nanoceria as a therapeutic agent for bone cancer at pH 6, observing the response of healthy osteoblasts to the same conditions is crucial. For this reason, osteoblasts were also treated with dextran coated cerium oxide nanoparticles at pH 6. After the first day of treatment, no statistical difference was observed at any nanoparticle concentration (10 µg/mL to 1000 µg/mL) compared with the control group. After 3 days of treatment, cell viability for each ceria nanoparticle concentration was roughly half of the control group and no statistical difference was observed between them. As Figure 20 indicates, statistically significant decreases were observed between 250 µg/mL to 1000 µg/mL in a time dependent manner. After 5 days of treatment, cell viability dropped from 60% (at 10 µg/mL) to 18% (at 1000 µg/mL) as shown in Figure 20.
Figure 20 Toxicity effect of 0.1 M dextran DCN against osteoblast cells at various concentrations after 1, 3 and 5 days of treatment at pH 6. \(N=3\), Data = mean +/- SD and \(*p < 0.05\) compared to controls at the same time period and \(**p < 0.05\) at the same DCN concentration at the different time points. Control groups are cells treated with media at pH 6.

In addition to pH 6 results, the effect of nanoceria on healthy osteoblasts at pH 7 (as a model of the physiological pH environment) was also evaluated (Figure 21). The results indicated that nanoceria at pH 7 did not show any toxicity after 1 day of treatment to healthy osteoblast cells. After 3 and 5 days of nanoceria treatment, a significant decrease (albeit small) in healthy osteoblast viability was observed for 250 µg/mL. Compared to nanoceria treatment at pH 6 to healthy osteoblast cells, at pH 7, a lower toxicity was observed. In general, little to no toxicity was observed for healthy osteoblasts exposed to nanoceria at any nanoparticle concentration or time. At pH 6, after
3 and 5 days of treatment, a significant decrease started at the lowest nanoparticle concentration, which was 10 µg/mL.

![Bar chart with data points](image)

**Figure 21** Toxicity effect of 0.1 M dextran DCN against healthy osteoblast cells at various concentrations after 1, 3 and 5 days treatment at pH 7. N=3, Data = mean +/- SD and *p < 0.05 compared to control at the same time period and **p<0.05 at the same DCN concentration at the different time points. Control groups are cells treated with media at pH 7.

As reported here for bone tumor applications, for all pH conditions, the toxicity of the nanoceria needs to be evaluated not only based on dose but also in a time dependent manner on both healthy osteoblasts and osteosarcoma cells.

After determining an optimal pH value that killed bone cancer cells when exposed to ceria nanoparticles, we then determined the cytotoxicity of ceria nanoparticles against
healthy bone cells. At the end of the first day of nanoceria treatment at pH 6, there was no significant difference in healthy bone cell viability compared to controls at any nanoparticle concentration (10 µg/mL – 1000 µg/mL). Cell viability started to decrease for healthy bone cells as well as after 3 days of treatment, but was much less compared to bone cancer cells, even though bone cancer cells grew much faster compared to healthy bone cells. 3 days after treating osteoblasts at pH 6, no difference among nanoparticle concentrations was observed. When osteosarcoma cells were treated at the same condition, this value was found to be greater than 50 µg/mL. After 5 days, at pH 6, the osteosarcoma IC-50 value was found to be 100 µg/mL, whereas it was greater than 500 µg/mL for healthy osteoblasts (Table 3). This provides the first data of an optimal concentration of nanoceria (between 100 and 500 µg/mL) for the selective killing of osteosarcoma over healthy osteoblasts.

Table 3 IC-50 values of osteosarcoma cells at pH 6, 7, and 9 and healthy osteoblast cells at pH 6 and pH 7 after 3 and 5 days of nanoparticle treatment.

<table>
<thead>
<tr>
<th>Days</th>
<th>Osteosarcoma</th>
<th>Osteoblast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 9</td>
<td>pH 7</td>
</tr>
<tr>
<td>3</td>
<td>1320 µg/mL</td>
<td>1000 µg/mL</td>
</tr>
<tr>
<td>5</td>
<td>385 µg/mL</td>
<td>197.5 µg/mL</td>
</tr>
</tbody>
</table>
In addition to data for healthy osteoblasts and osteosarcoma cells at pH 6, the IC-50 values at a physiological pH (pH 7) for cancer and healthy cells were compared. After 3 and 5 days of treatment for healthy osteoblast cells at pH 7, IC-50 values were observed well above 1000 µg/mL. However, in the presence of osteosarcoma cells after 5 and 3 days of treatment, the IC-50 values were 250 µg/mL and 1000 µg/mL, respectively.

Although pH 6 was found to be an efficient condition for killing osteosarcoma cells in the presence of ceria, significant differences were found at pH 7 for the selective killing of cancer over healthy bone cells in a dose and time dependent manner.

4.1.3 ROS generation of dextran coated nanoceria on osteosarcoma and osteoblast cells at pH 6

According to the highest efficiency in killing osteosarcoma cells as shown in Figure 19, the dose range of nanoparticles studied in this experiment was limited to between 250 µg/mL to 1000 µg/mL at one day. Two control groups (pH 7.4 and pH 6) were used in this experiment. To understand the effect of acetic acid for the generation of ROS, the control group at pH 6 was added to the experimental data set. As shown in Figure 6, there was no ROS generation at pH 6.
The generation of ROS was observed at 250 and 500 µg/mL nanoceria concentrations with a similar trend (150%) when cultured with osteosarcoma cells. At 1000 µg/mL, a drastic change in ROS generation was examined around 180%. In healthy osteoblast cultures, there was no significant ROS generation (Figure 22).

![Graph showing ROS generation](image)

**Figure 22** ROS generation of 0.1 M dextran DCN against osteosarcoma and healthy osteoblast cells at various concentrations after 1 day of treatment at pH 6. N=3, Data = mean +/- SD and *p < 0.05 compared to controls and **p < 0.05 compared to 1000 µg/mL. Control groups are cells treated with media at pH 6.

Previously, Perez *et al.* reported optimal antioxidant activity at physiological and basic pH values, whereas at acidic pH, nanoceria acted like an oxidase. In other words, the higher cytotoxic activity of nanoceria at lower pH values was explained as ‘oxidase-
like’ activity. In Figure 22, osteosarcoma cells also showed an increase in ROS generation when treated with a 250-1000 µg/mL nanoparticle dose range at pH 6 as opposed to non-significant changes in ROS generation for healthy osteoblast cells. Thus, a higher toxic activity of nanoceria at pH values of 6 may also be explained by an enhanced ‘oxidase-like’ activity in nanoceria. Although acidic cellular environments lead to a preferential uptake of nanoceria into cancer cells, the mechanism of uptake is still controversial. Wason et al. explained the activity of nanoceria as the producer of H$_2$O$_2$ in an acidic environment (similar with the cancer microenvironment) and as a scavenger of H$_2$O$_2$ in a neutral environment.

Another factor related with cytotoxicity is cellular uptake and sub-cellular localization of the particles. This localization depends on surface charge of the nanoparticles, charge of the cellular membrane (negatively charged) and pH of the cellular organelles such as lysosomes (which are acidic) and cytoplasm (which is at a neutral pH in normal cells). Due to the negative charge of the cell membrane, highly positively charged nanoparticles will attack the cell membrane intensively through electrostatic interactions.

Due to this possible interation, zeta potential studies were performed here. In the current studies, the zeta potential of the 0.1 M dextran coated ceria nanoparticles was measured at 0.58 mV with a deviation of +/- 2.27 mV at physiological pH values (Figure 15 d). At pH 9, in osteosarcoma media, the zeta potential was measured at 6.32. At acidic
pH values (pH 6), the zeta potential of the particles increased up to 17 mV, both in osteosarcoma and osteoblast media. The highly positive surface charge of the particles at pH 6 may enhance the cellular uptake of the nanoparticles compared to other pH values. In addition to the change in surface charge, a change in size was also observed in a pH dependent manner (Figure 15 c). Since the particles are smaller in diameter at pH 6, their cellular uptake may be easier than their larger counterparts at pH 7 and pH 9, although this remains to be studied.

Figure 1 Schematic of cerium oxide nanoparticle interactions with the tumor microenvironment with changing pH.
In this study, the cytotoxic effect of nanoceria on killing osteosarcoma cells was demonstrated for the first time at different pH values. Previously, different sizes and surface coatings for nanoceria have been studied for lung, pancreas, breast and ovarian cancer cells showing various level of toxicity. However, as previously declared by Park et al. and others, different cell types or cellular microenvironments have a various effect on the cytotoxicity exerted by the same nanoparticles. The exact mechanism of cytotoxicity is not fully understood at this moment. However, there are several differences in cellular physiology that may be related with nanoceria’s antioxidant effects that have yet to be exploited.

Among all of the pH values, dextran coated nanoceria (+4) enhanced killing of osteosarcoma cells at pH of 6. The enhanced toxicity could be explained by the smaller hydrodynamic diameter and the positively charged surface of the nanoparticles at pH 6. Nanoceria was found to be much less toxic to healthy osteoblasts at pH 6. ROS generation results show that nanoceria treatment causes significant ROS generation in osteosarcoma cells, whereas it does not generate any ROS in osteoblast cells. An efficient dose range after three days of treatment of osteosarcoma cells was between 250 – 1000 µg/mL, whereas it exerted minimum toxicity to healthy osteoblasts at the same concentration.
4.2 Preventing Bacterial Infection Using Nanoceria

In this section, dextran coated cerium oxide nanoparticles were tested against Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria to see whether these particles can be engineered to prevent bacteria growth. Similarly with the previous section, this section starts with characterization of nanoceria in tryptic soy broth (TSB), the bacteria culture media, and phosphate buffered saline (PBS). Following the material characterization, results of the effect of particles on bacterial growth are given along with an analysis of the bacteria growth curve parameters. To elucidate the mechanism of action of the particles, microscopic studies and ROS generation per bacteria colony forming unit was conducted. A detailed discussion on how these nanoparticles affect bacterial growth is given at the end of this section of the chapter.

4.2.1 Characterization of Dextran Coated Cerium Oxide Nanoparticles

The toxicity of nanoparticles can be ascribed to many factors, such as size, shape, crystallinity and surface chemistry of the particles, chemicals used in the synthesis of the particles, etc. Maintaining the size of the nanoparticles in suspension is an important challenge that is hard to achieve in the absence of a surface coating. Macromolecules are commonly used to provide steric hindrance in colloidal systems and they also enable nanoparticles to be soluble in aqueous environments; thus, in this study,
dextran (a neutral, hydrophilic, biocompatible, and biodegradable, branched polysaccharide) served in this capacity.\textsuperscript{168}

An aqueous solution of cerium salt and dextran was thoroughly mixed and added drop-wise into ammonium hydroxide. The color change from light yellow into dark brown within 24 hours was indicative of particle formation (Figure 13).

0.1 M dextran coated cerium oxide nanoparticles were synthesized by an alkaline-based precipitation method. In order to confirm the size of these particles, Transmission Electron Microscopy (TEM) was performed. TEM micrographs suggested that the particles had a uniform size distribution and were sub 5 nm in spherical particle form. However, the behavior of particles may vary in colloidal form, as particles may aggregate in a kinetically driven process through the formation of clusters. Thus, it is important to analyze nanoparticles in solution. The hydrodynamic radius and size distribution of the particles were measured via DLS after dispersing them in PBS at pH 9 and pH 6. Particles were found to be smaller (15.5 nm) in diameter at basic pH values than at acidic pH values (24 nm). The size difference under two conditions may be a factor that affects the antibacterial activity of the particles. Particles with a smaller hydrodynamic diameter at pH 9 could penetrate the thick cell membrane of bacteria more easily. Table 4 shows the comparison of hydrodynamic radius of the particles in TSB and DMEM media.
Table 4 Comparison of hydrodynamic radius of nanoceria at different biological fluids at different pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>TSB Media</th>
<th>DMEM Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>24.5 nm</td>
<td>28.78 nm</td>
</tr>
<tr>
<td>9</td>
<td>15.5 nm</td>
<td>50.24 nm</td>
</tr>
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</table>

The physiochemical properties of the synthesized nanoceria particles were analyzed using a variety of techniques. TEM micrographs of the nanoceria demonstrated that particle sizes were 3-4 nm in diameter (Figure 24 a) and were spherical in shape. First, the size distribution (Figure 24 b) and surface charge (Figure 24 c) of the colloidal nanoceria particles were measured in PBS at different pH values (pH 6 and 9). While the size distributions of the particles were uniform at both pH values, the particle size at pH 9 was smaller (15.5 nm in diameter) compared to the ones at pH 6 (24.5 nm in diameter). As the pH of the environment changed from acidic (pH 6) to alkaline (pH 9), the zeta potential of the particles went from negative (-8.75 ± 4.17 mV) to positive (11.76 ± 3.54 mV). Table 5 shows the comparison of zeta potentials of the particles in TSB and DMEM media.

Table 5 Comparison of zeta potential of nanoceria at different biological fluids at different pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>TSB Media</th>
<th>DMEM Media</th>
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</thead>
<tbody>
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<td>6</td>
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<td>9</td>
<td>11.76 mV</td>
<td>6.32 mV</td>
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</tbody>
</table>
UV-visible spectroscopy measurements were performed in order to elucidate the oxidation state of cerium oxide nanoparticles in bacteria growth media adjusted to have different pH values (Figure 24 d). While cerium can strongly absorb ultraviolet light at both oxidation states, cerium (III) absorbance occurs between the 230 nm to 260 nm range, whereas cerium (IV) absorbs light between the 300 to 400 nm range. At pH 6 and pH 9 in solution, cerium oxide nanoparticles were found as Ce (IV) oxides (as a single peak was obtained at 300 nm).
Figure 24 Nanoparticle characterization. a) Transmission Electron Micrographs of 0.1 M dextran coated cerium oxide nanoparticles. b) Hydrodynamic radius of 0.1 M dextran coated cerium oxide nanoparticles dispersed in PBS at pH 6 and pH 9. c) Zeta potential of 0.1 M dextran coated cerium oxide nanoparticles dispersed in PBS at pH 6 and pH 9. d) UV-visible absorption spectroscopy of 0.1 M dextran coated cerium oxide nanoparticles in TSB media at pH 6 and pH 9.

It has been reported that the antibacterial activity of cerium oxide nanoparticles is highly associated with surface charge and redox ability of cerium ions. First, to elucidate what the surface charge of these particles are, and whether they change between different pH values, zeta potential measurements were performed. Results suggested that surface charge of the particles varied depending on the pH. At pH 6, nanoceria particles possessed a negative surface charge, whereas at basic pH values (pH 9), the surface charge of the particles was positive. The change in the pH can alter the surface charge of the nanoceria particles as shown here, and potentially affect the affinity of the nanoparticles to bacteria. Both for Gram-positive and Gram-negative bacteria, the cell membrane is negatively charged, but the magnitude of the charge varies from strain to strain. The surface charge of different bacteria studies were previously studied by Gottenbos et al. In this study, the zeta potential of S. epidermidis (HBH2 102) was found to be –8 mV and P. aeruginosa (AK1) was found to be –7mV. An attraction between the positively charged nanoceria particles at pH 9 and the bacteria cell membrane may be one of the reasons that could explain why nanoceria particles possess enhanced
antibacterial activity. In contrast, nanoceria particles at pH 6 obtained a negative charge (-8.75 mV), which may lead to a repulsive interaction between bacteria and the nanoparticles.

4.2.2 Growth of Gram-positive and Gram-negative Bacteria in the Presence of Dextran Coated Nanoceria Treatments at Different pH Values

The antibacterial activity of dextran-coated nanoceria was evaluated against a Gram-negative bacteria (*P. aeruginosa*) and a Gram-positive bacteria (*S. epidermidis*) bacteria in terms of a dose, time and pH dependent response. Bacterial experiments were performed at 250 µg/mL, and 500 µg/mL nanoparticle concentrations, at both pH 6 and 9. Lower nanoparticle concentrations (10 µg/mL, 50 µg/mL, and 100 µg/mL) were also tested under the same conditions, but not reported as there was no difference in bacterial growth in comparison to non-treated control groups.

4.2.2.1 Antibacterial Efficacy of Nanoceria against *Pseudomonas aeruginosa*

At an acidic environment (pH 6), *P. aeruginosa* growth was not affected by nanoceria at 250 µg/mL (Figure 25 a). A 2 hour delay in lag time (λ) was seen in the 500 µg/mL treated samples. No significant change was observed in the calculated growth rate (µ) for both concentrations. All treatments were compared to an untreated control grown in pH 6 conditions.
At basic environments (pH 9), nanoceria was more efficient in killing *P. aeruginosa* in comparison to an acidic environment. The lag phase was significantly delayed as particle concentrations increased from 250 µg/mL to 500 µg/mL. Bacteria began to grow exponentially after 4 hours when treated with 250 µg/mL, and 9 hours when treated with 500 µg/mL. After 6 hours of culture with 500 µg/mL nanoceria at pH 9, a 2 log reduction compared to untreated controls was found (Table 6). At both pH values, the untreated control group displayed a 2 hour and 20 minute lag time, indicating that the pH of the media did not affect the initial growth of the bacteria. According to the Gompertz model curve fitting results in Figure 25 b, the calculated growth rate was significantly higher with the 250 µg/mL and 500 µg/mL treatment compared to controls (bacteria cultured at pH 9 media). Even though growth rates showed an increase with longer delays in lag phase growth, the nanoceria treatment at pH 9 was shown to be more effective against *P. aeruginosa.*
Figure 25 Bacterial growth inhibition and growth rate ($\mu$) and lag phase ($\lambda$) comparison. Proliferation of $10^6$ CFU/mL of Gram-negative bacteria *P. aeruginosa* (a, b) was measured over 24 hours in the presence of different concentrations of cerium oxide nanoparticles at pH 6 (a) and at pH 9 (b). Values represent the mean +/-SEM, N=3. Corresponding Gompertz Model curve fitting parameters of bacteria in every condition was calculated. Values represent the mean +/-SEM, N=3 and *p<0.05 compared to the untreated control.
4.2.2.2 Antibacterial Efficacy of Nanoceria against Staphylococcus epidermidis

The growth of Gram-positive bacteria, *S. epidermidis*, was affected more by the nanoceria treatment when compared to Gram-negative *P. aeruginosa*. In an acidic environment (pH=6), the exponential growth of the control group (bacteria cultured in pH 6 conditions) started after 4 hours. The *S. epidermidis* growth rate and lag phase was similar when treated with 250 µg/mL compared to controls (Figure 26 a). Even though bacteria growth started after the 11th hour when treated at 500 µg/mL, a 7 hour delay in comparison to controls, *S. epidermidis* growth (A) was significantly lower through 24 hours due to the nanoceria treatment.

The alkaline environment affected the growth of *S. epidermidis*. The exponential growth of *S. epidermidis* started after 8 hours when bacteria were cultured in pH 9 media. Even though a significant reduction was not observed in the growth rate, the lag phase shifted for another 9 hours when treated with 250 µg/mL (Figure 26 b). Figure 26 b also denotes that no bacteria growth was observed with the 500 µg/mL nanoceria treatment within 24 hours. All treatments were compared to an untreated control grown in pH 9 conditions.
Figure 26 Bacterial growth inhibition and growth rate ($\mu$) and lag phase ($\lambda$) comparison. Proliferation of $10^6$ CFU/mL of Gram-positive bacteria *S. epidermidis* (a, b) was measured over 24 hours in the presence of different concentrations of cerium oxide nanoparticles at pH 6 (a) and at pH 9 (b). Values represent the mean +/-SEM, N=3. Corresponding Gompertz Model curve fitting parameters of bacteria in every condition was calculated. Values represent the mean +/-SEM, N=3 and *p<0.05 compared to the untreated control.
Even though there are some contradictory reports on whether nanoceria is a free radical scavenger or not, its effects on the modulation of ROS levels are now well established.\textsuperscript{103, 124, 157} The source of the catalytic behavior of nanoceria stems from the presence of a number of large surface defects, which are mainly oxygen deficiencies in the lattice.\textsuperscript{124} The presence of these vacancies is responsible from the change in the local electronic stability and the valance state of the particles. Whether cerium atoms are in the +3 or in +4 oxidation state, it has a direct impact on their catalytic activity, thus, it may affect nanoceria’s antibacterial properties. Nanoceria with a higher Ce\textsuperscript{+3}/Ce\textsuperscript{+4} ratio is known to have more surface oxygen vacancies, and possess more superoxide dismutase (SOD) mimetic activity, which helps to fight diseases associated with oxidative stress. In contrast, nanoceria particles with a lower Ce\textsuperscript{+3}/Ce\textsuperscript{+4} ratio has more catalase mimetic activity and these particles possess more anticancer and antibacterial properties.\textsuperscript{84} UV-Vis measurements revealed that there was a single absorption peak between 300 – 400 nm when nanoceria was distributed in the TSB media at pH 6 and pH 9, which corresponds to the absorbance of cerium at a +4 oxidation state. Thus, the higher ratio of Ce\textsuperscript{+4} in solution at both pH values may be the reason for the presently observed enhanced antibacterial properties.

The physiological and structural differences between \textit{S. epidermidis} and \textit{P. aeruginosa} bacteria strains resulted in different bacteria responses to the pH change in the present study even without the presence of the nanoparticles. These differences may
stem from the differences in bacteria membrane structure and metabolic differences
based on their respective energy generation. At a neutral pH (pH 7), *P. aeruginosa*
started growing after 2 hours and 20 minutes (data not shown), and as the pH of the
media was changed to 6 or 9, the lag time without the particles did not change. Similar
with lag phase, no significant difference was observed in the growth rate between pH 6,
pH 7, and pH 9. On the other hand, for *S. epidermidis*, even though the bacteria growth
started after 4 hours at pH 7 and pH 6 (data not shown), at pH 9, the bacteria growth
started after 8 hours. Also, the growth rate for *S. epidermidis* treated at pH 9 media was
significantly lower in comparison to the pH 6 and pH 7 media. This denotes that a basic
pH had some inhibitory effect on *S. epidermidis* growth without the nanoparticles.

<table>
<thead>
<tr>
<th></th>
<th>pH 9</th>
<th>pH 6</th>
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<tbody>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
<td><em>S. epidermidis</em></td>
</tr>
<tr>
<td>Control</td>
<td>1000000000</td>
<td>880000000</td>
</tr>
<tr>
<td>500 µg/mL</td>
<td>8333333.33*</td>
<td>825396.83*</td>
</tr>
</tbody>
</table>

*Table 1 Colonization (CFU/mL) of *P. aeruginosa* and *S. epidermidis* after 6 hours in
culture with 500 µg/mL nanoceria at pH 9 and pH 6. Values represent the mean, N=3,
*p <0.05 compared to untreated control.*

4.2.3 Microscopy Study of Bacteria Treated with Nanoceria

In Figure 27 the proliferation and the morphology of *P. aeruginosa* (Figure 27 a)
and *S. epidermidis* (Figure 27 c) were visualized under confocal microscopy after 6 hours
of culture with nanoceria and compared with non-treated control groups. While a
reduction in the cell number was observed for *S. epidermidis* when treated with nanoceria at pH 9, both a reduction in cell number and drastic morphological changes were observed for *P. aeruginosa*. The ratio of live to dead cells did not change after nanoceria treatment of *S. epidermidis* which implies that nanoceria has a bacteriostatic effect rather than a bactericidal effect.

On the other hand, *P. aeruginosa* treated with nanoceria, showed an increase in number of dead cells as well as in cell length compared to the control (bacteria cultured in a pH 9 environment) after 6 hours. This phenomenon is also known as filamentation which is a marker of cellular stress observed mostly in Gram-negative cells.\textsuperscript{171} Filamentation of *P. aeruginosa* was also shown under the TEM in Figure 27 b in the presence of nanoceria treatment. Along with filamentation, TEM images also showed thinning and loss of the outer membrane in Gram-negative *P. aeruginosa* which may be another way of nanoceria administering its antibacterial efficacy.

TEM analysis of *P. aeruginosa* was also performed at pH 6 in order to see whether nanoceria causes any morphological changes when treated with ceria at pH 6 (Figure 28 a and b). No morphological change was observed in Gram-negative *Pseudomonas* with nanoceria treatment at pH 6.
Figure 27 LIVE/DEAD staining and TEM imaging. The proliferation and morphology of a $10^6$ CFU/mL culture of Gram-negative bacteria *P. aeruginosa* (a and b) and Gram-positive bacteria *S. epidermidis* (c) was visualized after 6 hours at pH 9 both with and without nanoceria treatment.

![Figure 27](image)

Figure 28 TEM images. The morphology of a $10^6$ CFU/mL culture of Gram-negative bacteria *P. aeruginosa* was visualized after 6 hours at pH 6 both with (a) and without (b) nanoceria treatment.

Another controversial aspect of ceria is whether it can be internalized by cells or not. Some previous research reported that the internalization of nanoceria does not occur in organisms like bacteria and algae due to their thick cell walls. However, in human and animal cell lines and tissues, it has been reported that nanoceria can be internalized. The uptake of nanoparticles usually involves two steps: 1) particle binding to the cell membrane and 2) internalization of the particles. The first step is governed by electrostatic interactions between the cell membrane and the particles. When
ceria is internalized, the toxicity has been found to be associated with lysosomal injury and oxidative stress.\textsuperscript{173} For non-internalized ceria, toxicity seems to be exerted by direct contact of nanoceria to bacteria and algae cell walls. The proposed mechanism(s) of actions are membrane disruption, ROS generation and interference with the nutrient transport functions.\textsuperscript{174} Here, in our studies, we did not observe direct interference of particles with the bacteria cell membrane in \textit{P. aeruginosa}. However, complete rupture of the outer membrane in Gram-negative bacteria was observed in TEM images along with a drastic increase in the size of the bacteria.

The vast majority of studies looking into antibacterial properties of nanoceria have been performed with \textit{E. coli} as it is the most commonly studied microorganism.\textsuperscript{113,175} Relatively fewer studies were performed against more clinically relevant bacteria like \textit{P. aeruginosa}.\textsuperscript{118,120} Even fewer groups studied the morphological changes in the bacteria after the treatment.\textsuperscript{115,121} Here, we observed a significant morphological change in gram-negative \textit{P. aeruginosa} after 6 hours of nanoceria treatment at pH 9. The length of \textit{P. aeruginosa} increased drastically, and bacteria showed clumping due to particle exposure, also known as filamentation. \textit{P. aeruginosa} without the nanoceria treatment was 3-5 \textmu m in length, whereas ceria treated counterparts length increased to above 30 \textmu m. Filamentation occurs as a result of bacteria not being able to divide, thus, generating excessive cellular stress. Elevated ROS levels were also shown at pH 9. Although never reported before for nanoceria, there are a few reports on morphological changes caused
by filamentation in Gram-negative bacteria \((E. \text{coli})\) after metal oxide nanoparticle exposure (such as CdO, ZnO, and TiO\(_2\))\(^{176}\). Here, in our case, nanoceria had a clear effect on \(P. \text{aeruginosa}\) at basic pH values which resulted in cell stress leading to cell death. This study represents the first to elucidate such a mechanism of bacteria death via the use of nanoceria.

4.2.4 ROS Generation of Nanoceria at pH 9 and pH 6

Elevated amounts of ROS cause oxidative stress within cells, eventually leading to cell death. ROS generation is known to be a prominent mechanism of cell death when cells are treated with nanoparticles. In order to see whether nanoceria treatment at pH 9 led to the generation of ROS or not, intracellular ROS generation of both Gram-negative and Gram-positive bacteria was measured (Figure 29 a and b). Since 500 µg/mL of nanoceria at pH 9 was the most efficient treatment in inhibiting bacteria growth for both bacteria strains, ROS generation was detected under these conditions. Additionally, to be able to correlate these results with TEM and confocal images, incubation time was kept the same at 6 hours. As shown in Figure 30 a and b both \(P. \text{aeruginosa}\) and \(S. \text{epidermidis}\) cells showed elevated amounts of ROS generation per colony compared to the untreated control. ROS generation per colony increased by 150 fold for \(P. \text{aeruginosa}\) and 120 fold for \(S. \text{epidermidis}\) when treated with nanoceria.
Figure 29 ROS generation. Reactive oxygen species generation of Gram-negative bacteria \textit{P. aeruginosa} (a) and Gram-positive bacteria \textit{S. epidermidis} (b) per colony after treatment with 500 µg/mL nanoceria at pH 9 for 6 hours. Values represent the mean +/-SEM, N=3 and *p<0.05 compared to the untreated control.

Similarly, generation of ROS was also analyzed for both bacteria after 6 hours in culture with nanoceria at pH 6 (Figure 30). Results suggest that nanoceria does not cause any ROS generation for both \textit{P. aeruginosa} and \textit{S. epidermidis} when treated at pH 6 compared to the non-treated controls.
Figure 30 ROS generation. Reactive oxygen species generation of Gram-negative bacteria *P. aeruginosa* (a) and Gram-positive bacteria *S. epidermidis* (b) per colony after treatment with 500 µg/mL nanoceria at pH 6 for 6 hours. Values represent the mean +/-SEM, N=3.

In this study, the idea was to enhance the antibacterial activity of nanoceria by altering the pH of the local environment (i.e., ion balance), thus changing the catalytic activity of the particles. A simple experimental approach was employed to compare the antibacterial activities of nanoceria at different pH values. As expected, bacteria growth decreased with increasing nanoparticle concentrations. Particles at pH 9 delayed the lag phase and reduced the total amount of bacteria for both strains more than particles at pH 6 with a reasonable presumption that once electrostatic interactions govern the proximity of particles to cells, nanoceria elevated bacteria ROS levels leading to their death. Different growth curves were observed between Gram-positive and Gram-negative bacteria. For both of the pH values, pH 6 and pH 9, Gram-positive bacteria *S. epidermidis*
showed a longer delay in bacterial growth compared to the control group and *P. aeruginosa* growth curves under the same conditions. This study, thus, showed that ceria nanoparticles possess outstanding antibacterial efficacy at basic pH values (i.e., pH 9), especially for *S. epidermidis*, indicating that they can be integrated with an approach to locally increase pH to reduce bacterial infection, either with or without a medical device.

**Figure 31** Interaction between positively and negatively charged nanoceria particles and a positively charged bacteria membrane.
4.3 **Cytoprotective Properties of Nanoceria**

Particles used in this study were the same sub 5 nm particles used in the earlier studies. A detailed characterization of the particles as synthesized and then when distributed in mammalian cell culture media is given at section 4.1.1 Characterization of dextran coated nanoceria. The aim of this study is to understand whether ceria nanoparticles (+4) can be utilized as cytoprotective agents for human dermal fibroblast cells (HDF) when cells are under oxidative stress.

4.3.1 **Cytotoxic effect of hydrogen peroxide on HDF cells**

Initial cytotoxicity assessment was performed with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to see whether what H\textsubscript{2}O\textsubscript{2} concentration is required to effectively kill (or reduce the viability of) HDF cells. Based on the results of the dose response assay in Figure 32, 750 µM and higher H\textsubscript{2}O\textsubscript{2} concentrations significantly reduced the viability of HDF cells. To have a stronger oxidizing effect on the HDF cells, the concentration range that was chosen to work with was 1 mM, 1.5 mM and 1.75 mM.
Figure 32 Toxicity effect of $\text{H}_2\text{O}_2$ against human dermal fibroblast cells at various concentrations after 1 day treatment. Data = mean +/- SEM and *p < 0.05 compared to control (labeled “Media”).

4.3.2 Pre-incubation of HDF cells with nanoceria particles at pH 6 and 7

While the HDF cells are constantly exposed to exogenous sources of ROS (as UV light), they also play a vital role in the wound healing process. In this section, the goal was to understand whether nanoceria could be engineered to rescue HDF cells from the harming effects of ROS. To elucidate this, an experiment was designed where HDF cells were pre-incubated with dextran coated nanoceria at different concentrations and pH values.
Initially, HDF cells were pre-incubated with nanoceria at different concentrations (250 µg/mL, 500 µg/mL, 1000 µg/mL) and for different times (2 hours, 4 hours, and 8 hours) at pH 6 and pH 7 separately. Following the pre-incubation with the particles, cells were stressed with different amounts of H₂O₂ (1 mM, 1.5 mM and 1.75 mM) and their viability was assessed 24 hours after exerting the stress.

In Figure 33, cells were stressed with different amounts of H₂O₂ following the 8 hours of pre-incubation with nanoceria. When compared to control groups at relevant H₂O₂ concentrations, we observed that the initial treatment with particles enhanced the killing capacity of H₂O₂. In all of the treatments there was a reduction in the HDF viability due to nanoceria pre-incubation.
Figure 33 Toxicity of $\text{H}_2\text{O}_2$ at various concentrations against human dermal fibroblast cells at various nanoparticle concentrations when they were preincubated with ceria nanoparticles for 8 hours at pH 6. N=3, Data = mean +/- SD and *p < 0.05 compared to respective $\text{H}_2\text{O}_2$ only concentration.

On the other hand, when HDF cells were pre-treated with nanoceria at pH 7 for 8 hours, and then exposed to oxidative stress with $\text{H}_2\text{O}_2$, we observed that their viability increased compared non-ceria treated control groups at the respective $\text{H}_2\text{O}_2$ concentration. Viability of the cells did not change with the increasing nanoceria concentration after 8 hours of treatment.
Figure 34 Toxicity of H$_2$O$_2$ at various concentrations against human dermal fibroblast cells at various nanoparticle concentrations when they were preincubated with nanoparticles for 8 hours at pH 7. N=3, Data = mean +/- SD and *p < 0.05 compared to relevant H$_2$O$_2$ only concentration.

In agreement with the literature, nanoceria showed a cytotoxic effect at pH 6. HDF cells pre-incubated with nanoceria and then exposed to H$_2$O$_2$ showed much less viability compared to control groups: HDF cells just treated with H$_2$O$_2$ at the same dose. The viability of the HDF cells were around 90 %, 70 %, and 18 % after 24 hours of treatment with 1mM, 1.5 mM and 1.75 mM H$_2$O$_2$, respectively. When cells were pre-incubated with 250 µg/mL nanoceria particles at pH 6 for 8 hours and then exposed to 1 mM, 1.5 mM and 1.75 mM H$_2$O$_2$, the viability of the cells decreased to 65 %, 30 %, and 8
% respectively. Similarly, after 500 µg/mL pre-incubation with nanoceria for 8 hours, cell viability significantly decreased to 55 %, 30 %, and 8% 24 hours after the addition of respective H₂O₂ amounts. For the highest nanoparticle concentration, 1000 µg/mL, there were no viable cells 24 hours after the H₂O₂ addition.

On the other hand, HDF cells pre-treated with nanoceria at pH 7 for 8 hours showed an increase in their viability compared to cells only stressed with H₂O₂. The viability of the cells treated with 1 mM, 1.5 mM and 1.75 mM H₂O₂ was 90 %, 70 %, and 18 %, respectively. When cells were pre-incubated with 250 µg/mL nanoceria at pH 7 for 8 hours and then exposed to 1 mM, 1.5 mM, and 1.75 mM H₂O₂, the viability of the HDF cells increased to 110 %, 100 %, and 85 %, respectively. Similarly, with 500 µg/mL pre-incubation under the same conditions, the viability of cells increased to 105 %, 85 %, and 85 % compared to relevant H₂O₂ concentrations. Finally, with 1000 µg/mL nanoceria pretreatment for 8 hours at pH 7, an increase to 90 %, 85 %, and 85 % was observed for the 1 mM, 1.5 mM, and 1.75 mM H₂O₂ treatment, respectively.

The difference in nanoceria’s cytotoxicity with changing pH can be supported with examples from the literature. Earlier, Perez et al. reported that nanoceria particles act like oxidizing agents at acidic pH values and oxidize organic components without the need of any H₂O₂. In this case, nanoceria may be acting synergistically with H₂O₂ to oxidize and kill the HDF cells or by using H₂O₂ as its substrate turns it into highly toxic hydroxide anion and then exerts toxicity to cells. Whereas at pH 7, higher viability of
nanoceria pre-treated cells was observed suggesting that nanoceria might be acting like catalase and turn H$_2$O$_2$ into H$_2$O and O$_2$.

Here, further viability studies were performed by varying the pre-incubation time with nanoceria. Cells were pre-incubated with nanoceria at pH 7 for 2, 4 and 8 hours and then exposed to 1.75 mM H$_2$O$_2$. In Figure 35, the cytoprotective effect of nanoceria was plotted by varying the preincubation time. For all nanoparticle concentrations, there was no rescue ability of nanoceria when cells were preincubated with the particles for 2 hours. However, as the pre-incubation time increased to 4 and 8 hours, we observed an increase in cell viability, suggesting that time has an effect on the rescue effect on the ceria.
Figure 34 Time dependent toxicity of 1.75 mM H$_2$O$_2$ against human dermal fibroblast cells at various nanoparticle concentrations when they were preincubated with DCN for 2, 4 and 8 hours. N=3, Data = mean +/- SD and *p < 0.05 compared 0 hour preincubation and same concentration.

The results showed that ceria treated cells were able to recover from the oxidative damage/cytotoxicity exerted by the drugs which suggests that ceria nanoparticles may act as antioxidants when engineered right. Further studies needs to be completed in order to elucidate the full potential of cerium oxide nanoparticles cytoprotective properties.
Chapter 5
Conclusions and Recommendations

The current thesis describes whether the role of the local environment in which the colloidal cerium oxide particles are suspended in, has an effect on tuning physiochemical properties (like particle size, surface charge, colloidal stability, optical properties, etc.) for various medical applications. Subsequently, these changes in physiochemical properties translate into variations in their biological activity that leaves us room to engineer them to be better tumor targeting molecules, anti-microbial agents or anti-oxidants.

The most noticeable remarks of this thesis are listed as follows:

• Dextran coated sub-5nm cerium oxide nanoparticles can be synthesized successfully by a simple alkaline-based precipitation method. The amount of dextran has an impact on the size, shape, and toxicity of the particles. Particles coated with 0.1 M dextran were smaller in size and spherical in shape compared to particles with 0.01 M dextran coating. Thus, 0.1 M dextran coated ceria was used for further biological analysis.

• Physiochemical properties of particles (like particle size and surface charge) were analyzed in the cell culture media and shown to be dependent on pH and the ingredients of the solution in which the particles were dispersed. When particles were dispersed in cell culture media and the pH was adjusted to pH 6, particles
attained a surface charge of 16.68 mV and their colloidal diameter was 28.78 nm. On the contrary, the particles had a surface charge of -8.75 ± 4.17 mV and were 24.5 nm in diameter when dispersed in bacteria culture media at pH 6. A similar trend was observed for particles at pH 9. In mammalian cell culture media, particle size was found to be 50.24 nm and the surface charge was 6.32 mV versus in bacteria culture media, surface charge was 11.76 ± 3.54 mV and the hydrodynamic radius was 15.5 nm.

- Particles delivered in pH 6 cell culture media were more effective in killing cancerous osteosarcoma cells in comparison to particles delivered at pH 7 and pH 9. The fact that particles at pH 6 had stronger electrostatic interactions with the negatively charged cancer cell membrane could dictate the mechanism of action.

- Nanoceria delivered at pH 6 was selectively toxic to cancerous cells as they showed minimal toxicity to healthy osteoblast cells under the same conditions. ROS generation results were in agreement with the viability results. When both cell lines were treated at pH 6, elevated ROS generation was observed in osteosarcoma cells in comparison to osteoblast cells.

- Under basic conditions, particles were much more efficient in prohibiting bacterial growth due to their smaller hydrodynamic diameter and positively charged surfaces in bacteria culture media at pH 9.
• Additionally, nanoceria was found to be more effective in killing *S. epidermidis* at both pH values in comparison to *P. aeruginosa* at 250 and 500 µg/mL.

• At pH 9, a reduction in the cell number of *S. epidermidis* was observed while keeping the ratio of live to dead cells constant with treatment. This implies that nanoceria has a bacteriostatic effect rather than a bactericidal one. *P. aeruginosa* had both a reduction in cell number and immense morphological changes known as filamentation when treated with nanoceria at pH 9.

• Enhanced antibacterial activity of nanoceria at pH 9 was supported by the elevated ROS amounts at this pH. ROS generation per colony was 150 fold higher for *P. aeruginosa*, and 120 fold higher for *S. epidermidis* after 6 hours of nanoceria treatment at pH 9.

• 6 hours treatment with pH 6 nanoceria particles did not cause significant ROS generation compared to control groups for both bacteria strains.

• Human dermal fibroblast cells were rescued when they were pre-incubated with 0.1 M dextran coated nanoceria before being exposed to H₂O₂ only at pH 7.

• At pH 6, ceria nanoparticles enhanced the cytotoxicity effect of H₂O₂.

• Rescuing capacity changed with respect to pre-incubation time. Maximum efficacy was observed after 8 hours of pre-incubation at pH 7.
This thesis essentially signifies that biological activities of nanoparticles can be engineered by tuning their physiological properties and among all nanoparticles, cerium oxide nanoparticles have a lot of potential to be used in biological systems.

Even though this study enlightened how modulating the pH can enhance cerium oxide nanoparticle’s biological properties, there is still room for enhancing our understanding of better-designed materials. In that sense, results from this study can lay the foundation for future scientists to advance the field.

Initial studies show that cerium oxide nanoparticles have superior properties like regenerating their catalytic activity when they are smaller than 5 nm. Hence, a surface coating is essential to preserve the particle size. Additionally, the thickness of the surface coating is also important; the thickness has to be thin enough such that particles do not lose their catalytic activity. Here, we used dextran coating for that purpose, which worked efficiently in maintaining the size and catalytic activity. However, due to the increase in the hydrodynamic radius in biological fluids, an interaction of dextran with molecules/proteins in serum and proteins in cell culture media may occur, and some of the dextran on the surface of the particles may be solvated in the media leaving the particle surface. Thus, cerium oxide nanoparticles can be functionalized with macromolecules/proteins that can covalently bond to the surface of the particles such that they will preserve their size in biological fluids with minimal interaction with the biological molecules.
Secondly, our studies show that nanoceria can be engineered to effectively kill the cancerous osteosarcoma cells while protecting the healthy osteoblast cells by escalating the reactive oxygen levels in the cells. While cell numbers support that cerium oxide can be further studied for the treatment of osteosarcoma, its effects on cell functions like alkaline phosphatase activity and mineralization of bones are equally important and should be studied to understand the effect of the particles on cell functions.

The enhanced efficiency of nanoceria at acidic pH values in killing bone cancer cells can only be judged up to a certain level *in vitro*. The true test of applicability takes place *in vivo*. Therefore, an animal study using cerium oxide nanoparticles should be performed. Developing an understanding of where nanoceria would be accumulated *in vivo* is essential. Proper animal models are key factors in successful pharmaceutical and medicinal experiments. Reducing the number of animals used for ethical and financial reasons, and maximizing the data obtained is essential. The most suitable and commonly used animal model to test the biodistribution of any pharmaceutical agent or nanoparticle is a mouse. Hence, biodistribution assay of cerium oxide nanoparticles should be performed in a mouse model.

Cerium oxide nanoparticles also show enhanced antibacterial activity against the most common hospital acquired infections as *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* at pH 9. pH 9 seems high to be used in the human body, but these particles can be used as the surface coating material in various settings including
medical devices. Thus, integration of cerium oxide nanoparticles into metals and polymers should be studied along with the antibacterial activity of cerium oxide coated surfaces. Additionally, the effect of cerium oxide on biofilm growth would also be an important subject to dwell into. During the formation of biofilms, bacteria generate exopolysaccharides that antibiotics cannot penetrate through. With such promising results against bacterial growth, the effect of nanoceria can be further studied on biofilm formation. The outcome of whether ceria can penetrate inside the extracellular matrix, and prevent the biofilm formation is unknown and deserves further analysis.
APPENDIX

Viability assay results in terms of cell density were shown below.

Figure: Toxicity effect of 0.1 M DCN against osteosarcoma cells at various concentrations after 1, 3 and 5 days treatment at (A) pH 9 and (B) pH 7. N=3, Data = mean +/- SD and *p < 0.05 compared to control at the same time period and **p<0.05 at the same DCN concentration and different time period. Control groups are cells treated with media at either (A) pH 9 and (B) pH 7.
Figure: Toxicity effect of 0.1 M DCN against osteosarcoma cells at various concentrations after 1, 3 and 5 days treatment at pH 6. N=3, Data = mean +/- SD and *p < 0.05 compared to control and **p<0.05 at the same DCN concentration different time period. Control groups are cells treated with media at pH 6.
Figure: Toxicity effect of 0.1 M dextran DCN against osteoblast cells at various concentrations after 1, 3 and 5 days treatment at pH 6. N=3, Data = mean +/- SD and *p < 0.05 compared to control and **p<0.05 at the same DCN concentration different time period. Control groups are cells treated with media at pH 6.
Figure: Toxicity effect of 0.1 M dextran DCN against osteoblast cells at various concentrations after 1, 3 and 5 days treatment at pH 7. N=3, Data = mean +/- SD and *p < 0.05 compared to control and **p<0.05 at the same DCN concentration different time period. Control groups are cells treated with media at pH 7.
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