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Synthesis Of Biocompatible Antioxidant Polymer Coated Cerium Oxide Nanoparticles, Its Oxidase Like Behavior And Cellular Uptake

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SYNTHESIS OF BIOCOMPATIBLE ANTIOXIDANT POLYMER COATED CERIUM OXIDE NANOPARTICLES, ITS OXIDASE LIKE BEHAVIOR AND CELLULAR UPTAKE STUDIES

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Arts and Sciences at the University of Central Florida Orlando Florida

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Major Professor: J. Manuel Perez
ABSTRACT

Cerium oxide nanoparticles have been widely used for various applications such as catalytic converters for automobile exhaust, ultraviolet absorber, and electrolyte in fuel cells. Most recently, cerium oxide nanoparticles (nanoceria) have been employed as potent free-radical scavengers with neuroprotective, radioprotective, and anti-inflammatory properties. These properties of cerium oxide nanoparticles can open new vistas in medicine and biotechnology. The present study utilizes the water-based-wet-chemical method to synthesize biocompatible, stable and highly monodisperse polymer coated cerium oxide nanoparticles. Polymer coated cerium oxide nanoparticles possess all the characteristics of the uncoated cerium oxide nanoparticles. These nanoparticles were found to be effective as pH-dependent antioxidant giving cytoprotection to normal cell lines against hydrogen peroxide and nitric oxide radical but not to cancer cells. Moreover, cerium oxide nanoparticle also exhibits unique oxidase-like activity at acidic pH oxidizing a series of organic compound without the need of hydrogen peroxide. Based on these results, we have designed an immunoassay in which folate-conjugated cerium oxide nanoparticles provide dual functionality by binding to folate expressing cancer cells and facilitating detection by catalytic oxidation of sensitive colorimetric substrates (dyes). Finally, we have shown that the polymer coated cerium oxide nanoparticles shows distinct toxicity depending upon their subcellular localization based on uptake studies using DiI loaded-cerium oxide nanoparticles. In these results, we have found that cerium oxide nanoparticles entrapped into lysosomes are more toxic as opposed to when they are localized in the cytoplasm. Overall we propose that the polymer coated cerium oxide nanoparticles displays selective antioxidant property, oxidase-like activity, and cytotoxicity to biological systems depending upon its pH environment.
ACKNOWLEDGMENTS

I wish to express my sincere gratitude towards my advisor Dr. J Manuel Perez, for his support, guidance, thoughtful discussions and patience. Without his motivation and help, this research work would not have been possible.

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Words cannot express my deepest gratitude towards my parents and my brothers Ashish, Akash and Abhishek for their limitless sacrifices towards what I am today. I would like to give the fullest credit to my family members without whose love and support this thesis would never been realized.
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<th>Definition</th>
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<tr>
<td>AzBTS</td>
<td>2,2-azino-bis(3-ethylbenzothizoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>CDI</td>
<td>1,1’-carbonyldiimidazole</td>
</tr>
<tr>
<td>CeO₂</td>
<td>Cerium oxide</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNC</td>
<td>Dextran coated-nanoceria</td>
</tr>
<tr>
<td>DOPA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>EDA</td>
<td>Ethylene diamine</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immune sorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform-infra red spectroscopy</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
</tr>
<tr>
<td>HDF</td>
<td>Human dermal fibroblasts</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HRTEM</td>
<td>High Resolution Transmission Electron Microscopy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectroscopy</td>
</tr>
<tr>
<td>isDNC</td>
<td>In-situ dextran coated nanoceria</td>
</tr>
<tr>
<td>isPNC</td>
<td>In-situ polyacrylic acid coated nanoceria</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyltetrazolium bromide)</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxy succinamide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PNC</td>
<td>Polyacrylic acid coated nanoceria</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAED</td>
<td>Selected area diffraction pattern</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>swDNC</td>
<td>Stepwise dextran coated nanoceria</td>
</tr>
<tr>
<td>swPNC</td>
<td>Stepwise polyacrylic acid coated nanoceria</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3,5,5’-tetramethyl benzidine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
</tr>
<tr>
<td>ZP</td>
<td>Zeta Potential</td>
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CHAPTER 1 INTRODUCTION

1.1 Nanotechnology

Most people accept that nanotechnology was first introduced at a lecture entitled “There is plenty of room at the bottom”, delivered in 1959 by Noble laureate Richard Feyman at the California Institute of Technology’s American Physical Society Meeting.¹ It has not been until the last 10 years, that nanotechnology has seen revolutionary growth in different areas such as chemistry, physics, and biology. This growth and development has brought the Feyman’s dreams of playing with the individual atoms and molecules at scale of nanometers i.e. “Nanoscience” to reality²,³

Nanotechnology is an emerging and enabling technology carried out at the nanometer scale (1nm is $10^{-9}$m). It involves the manufacturing of chemical and biological systems at the nanoscale level. The impressive and enhanced physiochemical properties are noticed when the materials are manufactured and engineered at the nanoscale. Although, the potential of nanotechnology has been greatly applauded, novel properties of nanomaterials raise concerns about adverse effects on biological systems and environmental impact.

Surface area plays a key role in determining the properties of a nanomaterial. At the nanoscale, surface area increases exposing more atoms on the surface. These changes in dimensions not only increase the surface area but also significantly alter the other properties such as catalytic or chemical reactivity, electrical conductivity, optical properties and so on. At nanoscale, the surface energy of nanomaterials and their corresponding surface morphology such as surface defects also changes significantly, therefore contributing to alterations in their fundamental properties and reactive nature.
Although significant advances have been made during last years, nanotechnology is still at its infant stage. It needs further exploration as it is just carrying forward from the bench scale to the industrial or real applications. Future developments in nanotechnology promises great advancement in various areas ranging from electronic, materials, medicine, diagnostics, biotechnology and environment etc. Discovery of novel materials, techniques and phenomena are expected through innovations at the nanoscale. Furthermore, developments of new theoretical and experimental processes may lead to well advanced nanosystems or nanostructures with new unique property which may further improve human life.

1.2 Cerium oxide: Background and Motivation

Cerium is the second element in the lanthanides series in the periodic table which are known as the rare earth element. Most of the rare earth metals exist in their trivalent state; however cerium occurs in both trivalent (+3) as well as tetravalent (+4) state and it has the unique ability to switch readily between these two states.\(^{4,6}\) Cerium oxide is widely used for various applications such in catalytic converters, solid oxide fuel cells\(^7\), and as an excellent oxygen buffers.\(^8\) Because of its ability to alter its oxidation state readily (Ce+3 and Ce+4), cerium oxide creates oxygen vacancies or surface defects either by loss of electron or oxygen.\(^{4,5}\) These alteration in surface property or crystal structure depend upon various factors such oxygen partial pressure, temperature, and doping.\(^{4,5,9}\)

The chemical reactivity of cerium oxide nanoparticles (nanoceria) is for the most part similar to the reactivity of the bulk cerium oxide. However, the increased numbers of oxygen vacancy defects on the cerium oxide nanoparticles will improve the performance and the efficiency. For example, the antioxidant or free radical scavenging activity of cerium oxide nanoparticles and its
radioprotective capacity to normal cell line against the radiation has received much attention. Although, cerium oxide nanoparticles has been seen as a impressive antioxidant nanoparticles for therapeutic applications, very little consideration has been given to the fact that one needs to synthesize biocompatible cerium oxide nanoparticles with better stability and biocompatibility in physiological solutions. Here we report the synthesis of biocompatible polymer coated cerium oxide nanoparticles and their characterization. Furthermore, this polymer coated cerium oxide nanoparticles were evaluated for their free radical scavenging properties. Our original intent was to create a highly biocompatible polymer coated cerium oxide with all characteristics property of cerium oxide nanoparticles. The increased water solubility allowed for studies at different pH. Based on results we hypothesized that the polymer coated cerium oxide nanoparticles exhibits pH-dependent antioxidant property.

Polymer coated cerium oxide nanoparticles displays antioxidant property at physiological pH while at low pH loses its antioxidant activity. Here we propose cerium oxide nanoparticles behave as an oxidant. These studies include the oxidation of pH-sensitive colorimetric dyes in the range of pH 4.0 and higher pH. Results showed that cerium oxide nanoparticles were able to oxidize these dyes at acidic pH 4.0 while no reaction was seen at pH 7.0. Next, when compared with the other systems such as HRP/H₂O₂, cerium oxide nanoparticles mediated oxidation of these dyes does not require hydrogen peroxide. Based upon these findings, the oxidase-like enzymatic behavior of polymer coated cerium oxide nanoparticles was studied. We developed the cerium-oxide-nanoparticles-based-cellular-ELISA for monitoring the folate receptor on the lung carcinoma cell line, utilizing the oxidase like activity of cerium oxide nanoparticles.

Next in order to understand the cellular interactions of cerium oxide nanoparticles, cellular uptake and their corresponding toxicity studies were carried out. For this purpose cerium oxide
nanoparticle with different surface charge was synthesized. It was observed that cerium oxide nanoparticles with neutral surface charge were not toxic to either one of the cancer or normal cells. In contrast, positive nanoparticles exhibited significant toxicity to both cell lines, and negative nanoparticles were toxic to only cancer but not normal. Moreover different kind internalization was seen for both the cell lines with different surface charge particles.

The primary objective of this work is to synthesize biocompatible cerium oxide nanoparticles having antioxidant property; study their pH-dependent behavior in aqueous systems and cellular uptake since it could have important applications in the design of improved therapeutics.

1.3 Organization

The primary focus of this dissertation/study is on the synthesis of biocompatible nanoceria and their application in biomedical fields. The dissertation can be divided into three major parts namely synthesis of nanoceria, its oxidase like behavior, and cytotoxicity studies. Chapter 2 shed light into the wet chemical method for the synthesis of biocompatible, stable and monodisperse polymer coated cerium oxide nanoparticles. Chapter 3 provides facts about the pH-dependent oxidase-like behavior and potential of nanoceria as a single reagent for cellular ELISA. Chapter 4 is focused on the cellular interactions, internalization, subcellular localization and toxicity of nanoceria with different cell lines.
CHAPTER 2: SYNTHESIS, CHARACTERIZATION AND ANTIOXIDANT ACTIVITY OF DEXTRAN COATED CERIUM OXIDE NANOPARTICLES

2.1 Introduction
Cerium oxide nanoparticles have been used as a potent free-radical scavengers with neuroprotective, radioprotective, and anti-inflammatory properties. Cerium oxide nanoparticles also have the unique property of being regenerative or autocatalytic. These results point to the possibility of engineering cerium oxide nanoparticles with selective antioxidant properties that promote cell survival under conditions of oxidative stress. However, most of these studies have been done with nanoparticles with poor water solubility or synthesized by procedures involving toxic solvents, therefore hindering their potential clinical use. Here we proposed a facile synthesis of monodisperse, water-soluble, and highly crystalline dextran-coated cerium oxide nanoparticles. We found that dextran coated cerium oxide nanoparticles show a unique pH-dependent antioxidant activity that could have important applications in the design of improved therapeutics and in tailoring its antioxidant properties.

2.2 Synthesis and characterization of dextran coated cerium oxide nanoparticles
Our synthetic procedure involves the alkaline-based precipitation of cerium oxide (Ce₂O₃:CeO₂) from a solution containing cerium salt and dextran. Briefly, an aqueous solution of cerium nitrate and dextran was added to an ammonia solution under continuous stirring. Upon formation of the cerium oxide nanocrystals, molecules of dextran coat the nanoparticles’ surface, preventing
further growth and resulting in dextran-coated cerium oxide nanoparticles or nanoceria (DNC) (Figure 2.1). The dextran-coated cerium oxide nanoparticle preparation is stable in phosphate-buffered saline (PBS) at concentrations of 40mM or higher for months. Dextran-coated cerium oxide nanoparticles demonstrates good water stability even after several heating cycles (70–80 °C) with no sedimentation upon centrifugation at 8000 rpm for 30 min. These characteristics make our water based synthetic method advantageous over organic-solvent based preparations,\textsuperscript{14–20} which are prone to aggregation when suspended in aqueous media.

Next, synthesized nanoparticles were characterized for surface morphology, shape, size, crystallinity, oxidation state and polymer coating. Transmission electron microscopy (TEM) analysis of dextran-coated cerium oxide nanoparticle shows the presence of discrete nanocrystals with an average size of 4 nm (Figure 2.2a), while high-resolution TEM (HRTEM) shows a crystalline nanoceria core (Figure 2.2b). A face-centered-cubic (fcc) core was identified by selected area electron diffraction (SAED; Figure 1a, inset) with lattice planes in good agreement with those obtained from an X-ray diffraction (XRD) pattern (Figure 2.2c).\textsuperscript{18} X-ray photoelectron spectroscopy (XPS) data indicates the presence of a mixed valence state in dextran-coated cerium oxide nanoparticle, giving the corresponding binding energy peaks for Ce\textsuperscript{3+} and Ce\textsuperscript{4+} (Figure 2.2d), and suggesting that the dextran coating does not affect its mixed valence state. In addition, dynamic light scattering (DLS) studies revealed a monodisperse size distribution for dextran-coated cerium oxide nanoparticle with an average diameter of 14nm (Figure 2.2e). Most importantly, the dextran-coated cerium oxide nanoparticle preparation can be concentrated in PBS by ultrafiltration, without precipitation of the nanoparticles (Figure 2.2f), and no difference in crystallinity, monodispersity, or mixed valency was found in dextran-coated cerium oxide nanoparticle preparations stored at 4 °C for over six months.
In addition, experiments with concanavalin A (Con-A), a protein with high affinity towards carbohydrates, were performed to further demonstrate the association of dextran with nanoceria. We hypothesized that a ConA-induced self-assembly of the nanoparticles would result if the nanoparticles are coated with dextran.22 As expected, DLS studies of dextran-coated cerium oxide nanoparticle after addition of ConA (10 mgmL⁻¹, final concentration) showed a time-dependent aggregation of the nanoparticle (Figure 2.3). Taken together, these results demonstrate the robustness of the dextran nanoceria preparations under physiologically relevant conditions and that the dextran is associated with the nanoparticles.

Figure 2-1. Schematic depicting the formation of stable cerium oxide nanoparticles.
Figure 2-2. Characterization of dextran-coated nanoceria. a) TEM image with corresponding SAED pattern (inset). b) Representative HRTEM image of a single crystal. c) XRD image showing the presence of (111), (220), (311), and (331) planes, typical of an fcc crystal. d) XPS analysis showing the presence of both Ce+3 and Ce+4 in the nanoceria preparation. e) DLS analysis showing a monodisperse nanoparticle preparation with an average hydrodynamic diameter of 10 nm. f) Photograph showing a 1) 10.0, 2) 20.0, 3) 30.0, and 4) 40.0mM aqueous solutions of dextran-coated nanoparticle.
Figure 2-3. Confirmation of the presence of dextran on nanoceria. Time-dependent DLS analysis of a DNC preparation upon incubation with Concanavalin A, showing an increase in the average hydrodynamic diameter due to concavalin-A-induced clustering of the dextran coating on DNC.
2.3 Autocatalytic activity determination of dextran coated cerium oxide nanoparticles

One of the most interesting properties of nanoceria is its autocatalytic behavior. The ability of these nanoparticles to reversibly switch from Ce\(^{3+}\) to Ce\(^{4+}\) is a key factor for their catalytic and biological applications as antioxidants. For these reason, we first tested whether the dextran coating compromised the nanoceria’s autocatalytic behavior. In these experiments, a solution of dextran-coated cerium oxide nanoparticles (2.54 mM) in PBS, pH 7.4, was incubated with 0.2 M hydrogen peroxide (H\(_2\)O\(_2\)) as an oxidizer. Upon addition, a rapid color change, from yellow to orange (Figure 2.4a), was observed, corresponding to a red shift in UV/Vis transmittance studies (Figure 2.4c), red curve, compared to a control sample (Figure 2.4c), black curve. This change in color is due to an increase in the ratio of Ce\(^{4+}\) to Ce\(^{3+}\) in the nanoceria core. During the following 10 days, as the H\(_2\)O\(_2\) decomposed from the nanoparticle suspension, the observed orange color started to disappear and the solution returned to its original yellow color (Figure 2.4a). Correspondingly, a gradual shift of the transmittance curve to its original control value was observed (Figure 2.4c), green, blue, and pink curves, respectively, demonstrating the reversibility of the system under normal physiological conditions. Subsequent addition of H\(_2\)O\(_2\) brought the color of the solution back to orange, which again started to disappear within the next ten days (Figure 2.4a). This reversible behavior under physiological conditions is essential for the antioxidant properties of nanoceria and its potential medical applications. Our results show that the dextran coating does not affect the autocatalytic properties of nanoceria, as hydrogen peroxide and peroxyl radicals can diffuse through the hydrophilic dextran coating and oxidize Ce\(^{3+}\) to Ce\(^{4+}\). Similar results were obtained at basic pH 8 and 9; however, intriguing results were obtained at acidic pH. Results show that at pH 4.0, dextran-coated cerium oxide nanoparticles does not exhibit a significant red shift upon addition of H\(_2\)O\(_2\), even though the color of the
solution has turned slightly orange (Figure 2.4b and 2.4d). Surprisingly, after 24 hours, the solution turned completely clear and a significantly large blue shift was observed in the transmittance curve (Figure 2.4d), green curve. After ten days, the solution remained clear and the addition of more H$_2$O$_2$ did not bring either the color of the solution back to orange or have any effect on the transmittance curve. Furthermore, once the pH of dextran nanoceria preparation was lowered to 4.0, its autocatalytic activity could not be reversed even upon raising the pH to 7.4, showing irreversibility at low pH. However, dextran-coated cerium oxide nanoparticles at pH 7.4 was able to recover (regenerate) and come back to its initial amount of Ce$^{+3}$ and Ce$^{+4}$, while the preparation at pH 4.0 remained the non-reversible at low pH. The mechanism for the nonreversible, low-pH poisoning of dextran nanoceria is not well understood at this time. It has been proposed that the antioxidant properties of nanoceria are due to the presence of mixed valence states (Ce$^{+3}$/Ce$^{+4}$) on the nanoparticles’ surface that allow for the scavenging of free radicals. During the scavenging process, Ce$^{+3}$ ions are converted to Ce$^{+4}$. The system is regenerated via a series of surface chemical reactions between ions in solution (such as H$^+$) and the Ce$^{+4}$ ions on the nanoparticle surface, where they are converted back to Ce$^{+3}$, and therefore allows for the scavenging of more free radicals. We hypothesize that the low-pH environment interferes with the cyclical regenerative or autocatalytic nature of dextran-coated cerium oxide nanoparticles due to the high concentration of protons (H$^+$) at low pH, and therefore inhibit the ability of nanoceria to scavenge more free radicals (Figure 2.5). This effect renders dextran nanoceria as an inefficient antioxidant at low pH.
Figure 2-4. Autocatalytic activity of dextran-coated nanoceria at physiological and acidic pH. Autocatalytic activity of dextran-coated nanoceria at physiological and acidic pH. A) Reversible color changes in a solution of dextran-coated nanoceria at pH 7.4, upon addition of hydrogen peroxide. B) Irreversible color changes in a solution of dextran-coated nanoceria at pH 4.0, upon addition of hydrogen peroxide. C) Transmittance spectra showing reversible autocatalytic behavior for DNC upon incubation with H₂O₂ at pH 7.4 (black: control, before addition of H₂O₂; red: immediately after addition of H₂O₂; green: 24 h, blue: 3 days, pink: 7 days). D) Transmittance spectra of nanoceria showing irreversible autocatalytic behavior for DNC upon incubation with H₂O₂ at pH 4.0. (black: control, before addition of H₂O₂; red: immediately after addition of H₂O₂; green: 24 h, blue: 3 days).
Figure 2-5. Hypothesized pH-dependent behavior of nanoceria. A facile water based synthetic technique for monodispersed and highly crystalline dextran-coated nanoceria (DNC) has been reported. The particles exhibit an effective antioxidant property for several living cell lines, which is found to be active at neutral pH and inactive in acidic microenvironment.
2.4 Determination of antioxidant or free radical scavenging activity of dextran coated cerium oxide nanoparticles:

2.4.1 Chronic treatment studies:

The excellent water stability and autocatalytic properties of dextran coated cerium oxide nanoparticles at physiological pH motivated us to perform cell studies. Multiple treatments (treatment at every 48 hours) and prolonged incubation of normal cardiomyocytes with dextran coated cerium oxide nanoparticles (1.0mM) at intervals of 48 h over a period of two weeks resulted in no significant changes in the growth pattern and morphology of the cells, as compared to control cells (Figures 2.6). More importantly, when the treated cells were harvested and replated in the presence of additional dextran coated cerium oxide nanoparticles, the cells proliferated similar to the control cells. These results indicate that the dextran nanoceria preparation does not alter cellular physiology even after chronic exposure, since no changes in morphology and proliferation of the cells were observed.
Figure 2-6. Chronic exposure experiment. Multiple treatments of DNC to cardiomyocytes. Multiple treatment of DNC to cardiomyocytes (d,e,f) does not affect the morphology and growth patterns of the cells in culture, as compared to non-treated control cells (a,b,c), even after the cells were harvested and replated at a lower density and allow to grow for a week (f). These results confirm the biocompatibility and nontoxicity of both preparations.

2.4.2 Cell viability assays against Hydrogen peroxide induced oxidative stress:
Next, we investigated if DNC would protect normal cells against oxidative damage. In these experiments, we exposed cardiomyocytes (Figure 2.7a) to high levels of H$_2$O$_2$ (0.2 M), and monitored cell viability. As expected, when cells were exposed to H$_2$O$_2$, most of them died. In contrast, when cells were pre-incubated with DNC before H$_2$O$_2$ incubation, no significant changes in cell viability were observed as compared to untreated cells or cells treated with nanoceria alone. Similar results were observed using a non-transformed human dermal fibroblast
The observed nonreversible poisoning of dextran nanoceria at pH 4.0 could have important implications in cancer therapy. It is well known that most tumors have acidic microenvironments due to high rates of glycolysis and lactic acid production, known as the Warburg effect.\textsuperscript{23,24} Since generation of oxygen radicals (oxidative stress) occurs in both tumor and healthy tissue during radiotherapy, DNC could facilitate pH-dependent preferential protection of healthy tissue. We hypothesized that upon treatment with dextran nanoceria, cancer cells would not be protected against H\textsubscript{2}O\textsubscript{2}-induced oxidative stress, due to their acidic microenvironment. Here we suggest a possible role of the acidic tumor microenvironment, which might contribute to the low-pH inhibition of nanoceria’s protective activity. To this end, we treated lung (A-549) and breast cancer (BT-474) cells with DNC before administering H\textsubscript{2}O\textsubscript{2} (0.2 M), and as expected no protection against oxidative damage was observed (Figure 2.7c and 2.7d). A-549 and BT-474 cancer cell lines have been reported to have upregulated glycolitic activity and production of lactic acid, which results in an acidic microenvironment.\textsuperscript{25,26} As lung and breast tumors are among the most prevalent and aggressive carcinomas, requiring extensive radiotherapy and chemotherapy, DNC can provide long-term cytoprotection to nontransformed surrounding cells, minimizing adverse side effects. Furthermore, dextran nanoceria provides similar protection against other biologically relevant radicals, such as nitroxyl (NO\textsuperscript{·}) (Figure 2.8).
Figure 2-7. Effect of dextran nanoceria on normal and cancer cell line against H$_2$O$_2$-induced toxicity. DNC protects normal cardiomyocytes (a) and human dermal fibroblasts (b) against
hydrogen peroxide induced oxidative stress. In contrast, no protection is observed in lung carcinoma cells (c) and breast carcinoma (d) treated with DNC. All data points represent the average of three measurements with standard errors ranging between 1.0 to 2.0% (too small to graph).

Figure 2-8. Dextran nanoceria protects cardiomyocytes from nitroxy radical (*NO) induced.

2.5 Experimental section:

2.5.1 Synthesis and characterization of DNC:

A solution containing 5.0 mL of 1.0 M cerium (III) nitrate (Aldrich, 99%) and 10.0 mL of 1.0 M dextran T-10 (Amersham Bioscience) was added dropwise to a 30.0 mL ammonium hydroxide
solution (Sigma Aldrich, 30%) and stirred for 24 h at 25 °C. The color of the solution changed from light yellow to dark brown, indicating the formation of stabilized dextran-coated ceria nanoparticles. Afterwards, the solution was centrifuged at 4000 rpm for two 30 min cycles to settle any debris and large agglomerates. Finally, the centrifuged solution was purified from free dextran and concentrated using an Amicon cell (YM 30 K; Millipore Inc.) and stored at 4 °C. The concentration of cerium ion present in the nanoparticle preparation was determined by inductively coupled plasma mass spectroscopy (ICP-MS) using an acid digestion technique at Army Edgewood Chemical and Biological Center. TEM was performed by mounting a drop of DNC on a holey carbon coated copper 400-mesh grid (2SPI, USA). Images were taken in a FEI TECNAI F30 microscope operating at 300 kV. A Rigaku D/MAX XRD instrument with CuKa1 radiation was used for XRD analysis and measurements were recorded at a scan rate of 0.25 degree min\(^{-1}\). XPS was performed in a Physical Electronics 5400 ESCA spectrometer with a base pressure of 109 Torr and MgKa X-ray radiation at a power of 200 W. Dynamic light scattering (DLS) studies were performed by analyzing dilute solutions of DNC preparation using a PDDLS/Cool Batch 40T, PD2000 DDLS. UV/Vis spectroscopic measurements were performed using solutions of DNC in a Varian Cary 300 Bio UV/Vis spectrometer. Fluorescence spectroscopy studies were done using a Nanolog HORIBA JOBIN YVON spectrometer. FT-IR experiments were performed in a Perkin Elmer Spectrum 100 FT-IR spectrometer using samples spotted on plastic cover slips.

2.5.2 Autocatalytic activity assay:
The autocatalytic activity was assessed by treating a 2.54 mM solution of DNC with 0.2 M of \(\text{H}_2\text{O}_2\) solution (Acros, 30%). The percentage transmittance was then monitored upon addition of
hydrogen peroxide at 0 h, 1 day, 3 days, 7 days, and 10 days. As a control, a solution of nanoparticles without hydrogen peroxide was used.

2.5.3 Cell studies:
Cardiomyocytes (H9c2), human dermal fibroblasts (BJ), lung cancer (A-549) and breast cancer cells (BT-474) were obtained from ATCC, USA. Cardiomyocytes and human dermal fibroblasts cells were grown in Eagle’s Minimal Essential medium supplemented with fetal bovine serum (10%), sodium pyruvate, L-glutamine, penicillin, streptomycin, amphotericin B, and nonessential amino acids. Lung-cancer cells were grown in Kaighn’s modification of Ham’s F12 medium (F12K) supplemented with fetal bovine serum, L-glutamine, streptomycin, amphotericin B, and sodium bicarbonate. Breast-cancer cells were grown in modified Dulbecco’s medium supplemented with fetal bovine serum (10%), L-glutamine, streptomycin, penicillin, and amphotericin B. All cell lines were maintained at 37 °C, 5% CO₂ in a humidified incubator.

Chronic exposure experiments:
Cardiomyocytes were seeded into 6-well plates at 50000 cells per well. After 24 h, the cells were treated with DNC (1.0 mM) and incubated for 48 h. The medium was then removed, the cells were washed with PBS buffer, and again treated with the same concentration of DNC. After cells were confluent they were harvested and replated into new flask. A similar procedure was repeated for a period of two weeks.

Evaluation of DNC protection against H₂O₂-induced cytotoxicity:
Cells were seeded in 96-well plates at a density of 3000 cells per well and incubated with DNC (1 mM) and then with hydrogen peroxide (0.2 M) for 2 h. Then, 0.5 mM of MTT (3-(4,5-
dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) was added followed by incubation for 24 h. After 24 h, the resulting crystals were dissolved in 20 mL of isopropyl alcohol and the absorbance at 570 nm was recorded using a plate reader (Bio-TEK, Synergy HT Multidetection Microplate reader). These experiments were performed in triplicate.

**Evaluation of DNC protection against nitric oxide-induced cytotoxicity:**

Cells were seeded in 96-well plates at a density of 3000 cells per well and incubated with DNC (1.0mM) and then with a 500 mM solution of S-nitrosoglutathione (GSNO; Sigma). The cells were then incubated for 24 h. Next, 0.5mM of MTT was added, followed by incubation for 24 h. Afterwards, the resulting crystals were dissolved in 20 mL of isopropyl alcohol and the absorbance at 570 nm was recorded using a plate reader (Bio-TEK, Synergy HT Multidetection Microplate reader). These experiments were performed in triplicate.

**2.6 Conclusion:**

In summary, these results show that our biocompatible dextran nanoceria exhibit excellent, pH-dependent antioxidant properties for improved cancer therapeutics, as they provide cytoprotection from free radicals to normal cells but not to cancer cells, which are typically in an acidic environment. Dextran-coated iron oxide nanoparticles have been successfully developed to exhibit enhanced biocompatibility and used as long circulating MRI contrast agents.\textsuperscript{27-32} Therefore, the adoption of the dextran nanoceria in animal and potentially human clinical studies is possible. Taken together, our results support the imminent potential use of DNC as smart, pH-
dependent therapeutic agents and devices that selectively protect healthy tissue from free radicals, which might be elevated during chronic inflammation or upon radio or chemotherapy.
CHAPTER 3: STUDY OF pH-DEPENDENT BEHAVIOR OF POLYMER COATED CERIUM OXIDE NANOPARTICLES, ITS OXIDASE LIKE ACTIVITY AND POTENTIAL APPLICATION IN CELLULAR ELISA

3.1 Introduction:

Unique catalytic activities have been reported for nanoscale materials in recent years. These size-dependent properties, which are often absent in the bulk materials, are the basis for the design of novel catalysts with multiple applications in energy storage, chemical synthesis, and biomedical applications. In previous chapters, we discussed that cerium oxide nanoparticles (nanoceria) possess antioxidant activity at physiological pH values. Furthermore, the synthesis of biocompatible dextran-coated cerium oxide nanoparticles and its enhanced stability in aqueous solution has been recently reported.

Nanoceria has pH-dependent antioxidant activity which are enhanced at physiological pH and lost at acidic pH. Therefore we asked the question; if cerium oxide nanoparticles behave as an oxidant at low pH. Herein, we report that nanoceria has an intrinsic oxidase-like activity at acidic pH values, as it can quickly oxidize a series of organic substrates without any oxidizing agent (e.g. hydrogen peroxide). The observed activity is not only pH-dependent but is also dependent on the size of the cerium oxide nanoparticles as well as the thickness of the polymer coating. On the basis of these findings, we have designed an immunoassay in which folate-conjugated cerium oxide nanoparticles provide dual functionality by binding to folate expressing cancer cells and facilitating detection by catalytic oxidation of sensitive colorimetric substrates (dyes). The unique pH-dependent oxidase-like activity of cerium oxide nanoparticles in aqueous media
makes them a powerful tool for a wide range of potential applications in biotechnology and environmental chemistry.

3.2 Assessment of the pH dependent oxidase-like behavior of polymer coated cerium oxide nanoparticles by oxidation of pH-sensitive colorimetric dyes

In these studies, we first investigated if a dextran coated cerium oxide nanoparticles preparation could facilitate the oxidation of a series of organic dyes at low pH values. In these experiments, we selected 3,3’,5,5’-tetramethylbenzidine (TMB) and 2,2-azinobis(3-ethylbenzothizoline-6-sulfonic acid) (AzBTS), which upon oxidation develop either a blue (TMB) or green (AzBTS) color in aqueous solution. These dyes are typically used as horseradish peroxidase (HRP) substrates in various bioassays, and most recently they have been used to demonstrate the peroxidase-like activity of iron oxide nanoparticles. However, in these peroxidase-catalyzed reactions, hydrogen peroxide (H₂O₂) is required as the electron acceptor or oxidizing agent. In contrast, we have found that dextran coated cerium oxide nanoparticles catalyzes the fast oxidation (within minutes) of both TMB and AzBTS in the absence of hydrogen peroxide, as judged by the appearance of the characteristic color upon addition of the dyes to citrate-buffered solutions (pH 4.0) of the nanoparticles (Figure 3.1a) and by the corresponding UV/Vis spectrum (Figure 3.2a and Figure 3.2b). Meanwhile, at pH 7.0, no significant oxidation of TMB or AzBTS was observed, even in the presence of hydrogen peroxide or upon overnight incubation, as judged by the absence of color development upon addition of nanoceria at this pH value (Figure 3.1b).
Figure 3-1 Photographs shows production of oxidation product at pH 4.0 upon addition of nanoceria to TMB, AzBTS (a), and no color reaction at pH 7.0 (b).
Figure 3-2 Graph showing the characteristics UV-visible peak for oxidation product of TMB (a) and AzBTS (b).
Furthermore, pH-dependent studies of the DNC-catalyzed oxidation of TMB or AzBTS show that as the pH value of the buffered solution increases from pH 4.0 to 7.0, the ability of DNC to oxidize the dye decreases (Figure 3.3a and 3.3b). These results suggest that dextran coated cerium oxide nanoparticles behaves as an oxidation catalyst in a pH-dependent manner and performs optimally at acidic pH values.

![Figure 3-3 Nanoceria mediated oxidation of TMB (a) and AzBTS (b) is pH dependent.](image)

Figure 3-3 Nanoceria mediated oxidation of TMB (a) and AzBTS (b) is pH dependent.
To further verify the ability of cerium oxide nanoparticles to behave as an oxidation nanocatalyst, we chose dopamine (DOPA), a catecholamine difficult to oxidize at low pH values. Results showed that dextran coated cerium oxide nanoparticles facilitated the oxidation of DOPA in citrate buffer (pH 4.0) within minutes, producing the characteristic orange color corresponding to aminochrome, one of the major oxidation products of DOPA (Figure 3.4a). The formation of aminochrome by dextran coated nanoceria was confirmed by UV/Vis studies, which show the appearance of the characteristic band at 475 nm (Figure 3.4b). However, in the absence of DNC, no apparent oxidation of DOPA occurs at pH 4.0, even after days of incubation. This result is in contrast to that for DOPA solutions in water or citrate buffer pH 7.0, in which DOPA slowly autoxidizes, developing the characteristic reddish-brown color after overnight incubation. Taken together, our results indicate that DNC is able to catalyze the oxidation of various organic molecules at acidic pH values.
Figure 3-4 Nanoceria promoted oxidation of DOPA. Orange color aminochrome (a) and UV-profile of oxidized DOPA to aminochrome (b).
3.3 Effect of size on the catalytic property of cerium oxide nanoparticles:

It has been well established that the catalytic properties of nanomaterials often depend upon the size of the nanocrystal.\textsuperscript{44} However, studies on the effect of the thickness of a polymer coating surrounding the nanoparticles are less common. This motivated us to study whether the nanoceria catalyzed oxidation of these dyes is also dependent on nanoparticle size and on the thickness of the polymer coating. Our previously reported dextran-coated nanoceria preparation was synthesized by an in-situ procedure,\textsuperscript{39} in which the dextran (10 kDa) is present in solution at the time of the initial formation of the cerium oxide nanocrystals. Under these conditions, the polymer influences both the nucleation and the growth of the initial nanocrystal, resulting in nanoparticles with a small nanocrystal core surrounded by a thin polymeric coating. In the case of dextran coated cerium oxide nanoparticles, we have obtained nanoparticles with a cerium oxide core of 4 nm surrounded by a thin coating of dextran for a total nanoparticle size (hydrodynamic diameter) of 14 nm. Meanwhile, a stepwise procedure in which the polymer is added at a specific time after initial formation of the nanocrystals can be adopted for the synthesis of nanoceria. This method has been reported for the synthesis of polymer-coated iron oxide nanoparticles and yielded nanoparticles with a thicker polymer coating than those from the in-situ process.\textsuperscript{45} Furthermore, slightly larger nanocrystal cores are also obtained using this method. Therefore, to study the effect of the polymeric coating thickness on the catalytic activity of nanoceria, we synthesized dextran coated cerium oxide nanoparticles using a stepwise method. In this method, the dextran polymer was added 60 seconds after initial formation of the nanocrystals, to yield a stepwise dextran coated cerium oxide nanoparticle (swDNC) nanoparticle preparation with an average hydrodynamic diameter of 100 nm, approximately ten
times bigger than the dextran coated cerium oxide nanoparticles obtained with the in-situ method (isDNC). Moreover, another set of polymer-coated ceria nanoparticles was synthesized using poly(acrylic acid) (1.8 kDa). The use of a polymer with a smaller molecular weight in the synthesis of polymer-coated nanoceria is advantageous, because it allows the formation of nanoparticles with an even thinner coating than those obtained with dextran (10 kDa) using either the in-situ or the stepwise method. Dynamic light scattering experiments show that for the in situ nanoceria preparations coated with poly(acrylic acid) (isPNC), the average hydrodynamic diameter of the nanoparticles was 5 nm; whereas for the stepwise preparation (swPNC), a value of 12 nm was obtained Figure 3.5a-d. As expected, smaller nanoparticles with a thinner polymer coating were obtained using the 1.8 kDa poly(acrylic acid) polymer.
Figure 3-5 Hydrodynamic diameter distributions. (a) in-situ dextran coated nanoceria (isDNC), (b) stepwise dextran coated nanoceria (swDNC), (c) in-situ polyacrylic acid coated nanoceria (isPNC), and (d) stepwise polyacrylic acid coated nanoceria (swPNC).
Next, we used these preparations of nanoceria to perform various kinetic studies and to assess the effect of the coating thickness and nanoparticle size on the catalytic activity of nanoceria. Results show that nanoceria’s ability to oxidize TMB varies with nanoparticle size in the order isPNC (5 nm)>swPNC (12 nm)>isDNC (14 nm)>swDNC (100 nm). Interestingly, the nanoparticles with a thin poly-(acrylic acid) coating (isPNC) have a higher catalytic activity than those with a thicker dextran coating (swDNC) (Figure 3.6a). This result might be attributed to the fact that nanoceria with a thin and permeable poly(acrylic acid) coating can facilitate the transfer of molecules to and from the nanoceria core surface faster than a thicker coating. Similar experiments were performed with AzBTS, which shows similar behavior to TMB (Figure 3.6b).

Figure 3-6 Nanoceria-promoted oxidation of TMB (a) and AzBTS (b) is size dependent.
3.4 Determination of Steady-State kinetics of cerium oxide nanoparticles mediated oxidation of dyes

In order to understand the oxidase-like behavior of cerium oxide nanoparticles, we determined the steady-state kinetic parameters for the nanoceria-catalyzed oxidation of TMB. Typical Michaelis–Menten curves were obtained for both PNC and DNC (Figures 3.7a-d) and (Figure 3.8a-d). Results show that as the hydrodynamic diameter of the nanoparticles increases, lower values for the Michaelis constant Km and reaction rate Vmax are obtained (Table 3.1a). Similar results were observed with AzBTS. The fact that the nanoceria preparation with the smallest hydrodynamic diameter and thinnest coating (isPNC) displays the fastest kinetics (contrary to swDNC) suggests that the thickness of the polymer coating plays a key role in the rate of oxidation of the substrate. Furthermore, kinetic studies of nanoceria (isPNC) at various pH values indicate faster kinetics at acidic pH values (Km=3.8, Vmax=0.7) and much slower kinetics at neutral pH values (Km=1.3, Vmax=0.1) (Table 3.1b), as expected. These results contrast with those obtained using the enzyme HRP or iron oxide nanoparticles, for which slower kinetics are reported even in the presence of hydrogen peroxide. Better values were observed with the cerium oxide nanoparticles when evaluated against the HRP/H2O2 system.42
Table 3.1 Nanoceria’s oxidase-like kinetics are both size (a) and pH (b) dependent.

a)

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b)

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<tr>
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<td>1.3</td>
<td>0.10</td>
</tr>
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</table>
Figure 3-7 Steady state kinetics of nanoceria mediated oxidation of TMB at various pH, (a) pH 4.0, (b) pH 5.0, (c) pH 6.0, and (d) pH 7.0.
Figure 3-8 Steady state kinetics of nanoceria mediated oxidation of TMB using different size nanoceria preparation, (a) in-situ polyacrylic acid coated nanoceria (isPNC), (b) stepwise polyacrylic acid coated nanoceria (swPNC), (c) in-situ dextran coated nanoceria (isDNC), and (d) stepwise dextran coated nanoceria (swDNC).
3.5 Oxidase like activity of cerium oxide nanoparticles and application in cellular ELISA

The enzyme horseradish peroxidase (HRP) has been widely used in biotechnology, particularly in ELISA for the detection of biomarkers and targets using substrate such as TMB and AzBTS which produce characteristics color signal upon oxidation easily detected by spectrophotometric methods. In traditional ELISA, an HRP-labeled secondary antibody is utilized to assess the binding of a specific primary antibody to a particular target or surface receptor (Figure 3.9a). This binding event is assessed by the ability of HRP to oxidize a chromogenic substrate such as TMB in the presence of hydrogen peroxide. In traditional ELISA, the high rate of negative results is mainly attributed to 1) the instability of the antibodies that, when denatured, do not bind effectively to their target, 2) the instability of HRP that, when denatured, loses its peroxidase activity, and 3) the instability of hydrogen peroxide which, upon prolonged storage, decomposes and losses its ability to oxidize the substrate TMB in the presence of HRP. Since nanoceria has the intrinsic ability to oxidize chromogenic dyes without the need of hydrogen peroxide, we hypothesized that a nanoceria-based detection system could be approach would be more robust than current HRP-based assays, as no hydrogen peroxide would be needed (Figure 3.8b). The oxidase-like activity of the nanoceria, by itself, should facilitate the oxidation and corresponding color development. Using this assay, we can perform an immunoassay and identify the presence and concentration of a target faster and cheaper than using traditional ELISA. For this purpose, nanoceria coated with poly(acrylic acid) (isPNC) was conjugated to folic acid using click chemistry (Figure 3.10), and conjugation was confirmed by presence of characteristics emission peak for folic acid (Figure 3.11).46-49
Figure 3-9 Comparison of traditional ELISA (a) and nanoceria-based ELISA (b). In traditional ELISA, an HRP antibody is utilized as secondary antibody that, upon hydrogen peroxide treatment, facilitates the oxidation of TMB, resulting in color development. In nanoceria based ELISA, the oxidase-like activity of nanoceria facilitates the direct oxidation of TMB without the need of HRP or hydrogen peroxide.
Figure 3-10 Synthesis of folate-conjugated cerium oxide nanoparticles.

Figure 3-11 Fluorescence emission profile of folate-conjugate cerium oxide nanoparticles. Graph exhibits the characteristics peaks for both cerium oxide nanoparticles and folic acid in folate conjugated particles confirming the that surface of nanoceria is functionalized with folic acid.
We used the folic acid because it is the ligand to folate receptor, which is over expressed in many tumors and cancer cell lines.\textsuperscript{50,51} We hypothesized that a nanoceria conjugate with folic acid instead of an antifolate receptor antibody will make a more robust nanoprobe for our immunoassay. Experiments were performed using the lung cancer cell line (A-549), which overexpresses the folate receptor.\textsuperscript{50,51} In control experiments, cardiac myocytes (H9c2) that do not overexpress the folate receptor were used.\textsuperscript{52} In our first set of experiments, either A-549 or H9c2 cells (6000 cells) were incubated with an increasing amount of folate–cerium oxide nanoparticles in a 96-well plate for three hours and subsequently incubated with TMB (0.04 mm) for 30 minutes; product formation was monitored at 652 nm using a microtiter plate reader. As expected, a folate–nanoceria-dependent binding was observed for the lung carcinoma cell line (A-549) compared to cardiac myocytes (H9c2), as judged by an increase in absorbance at 652 nm with increasing amount of folate–nanoceria (Figure 3.12). In another set of experiments, an increasing number of folate-positive lung carcinoma cells (1500 to 6000 cells) were treated with a constant amount of folate–ceria (5.0 mm). Results show an increase in the formation of TMB oxidation product (652 nm absorbance) with increasing number of A549 cells (Figure 3.13). This result was expected, as an increasing number of A549 cells translate into an increasing number of surface folate receptors available for binding to the folate–ceria nanoparticles. These results demonstrate the utility of cerium oxide nanoparticles as a detection tool, which arises from their dual functionality. Nanoceria-based assays outperform traditional sandwich ELISA, which requires hydrogen peroxide and an additional step to introduce an antibody carrying horseradish peroxidase (HRP antibody) to allow detection.
Figure 3-12 Nanoceria-mediated ELISA detection of folate receptor expressing cells. Folate-nanoceria associating with A-549 cells, which overexpress the folate receptor, effectively oxidizes TMB.

Figure 3-13 Nanoceria-based immunoassay is sensitive to the number of folate-positive cells (folate-receptor-expressing cells A-549).
3.6 Experimental section:

3.6.1 Synthesis of isPNC and isDNC

A solution containing 1M cerium (III) nitrate (Aldrich, 99%) solution (2.17g in 5.0 mL of water) was mixed separately with 0.5 M solution of polyacrylic acid (PAA) (Sigma) or 1.0M dextran (Sigma). Under continuous stirring, mixture was added to 30.0 mL ammonium hydroxide (Sigma Aldrich, 30%) solution. Afterwards the mixture was stirred continuously for 24 h. At this point the solution has changed from a light yellow to a deep brown color indicating the formation of stabilized dextran and PAA coated-nanoceria. The preparation was then centrifuged at 4000 rpm for two 30 minute cycles to settle down any debris and large agglomerates. The supernatant solution was then purified from free PAA or dextran and concentrated using 30K Amicon cell (Millipore Inc.).

3.6.2 Synthesis of swPNC and swDNC

For stepwise synthesis, a solution containing 1M cerium (III) nitrate (2.17 g in 5.0 mL of water) was added continuous stirring to 30.0 mL ammonium hydroxide solution. Then, after one minute 0.5 M solution of PAA or 1.0 M dextran solution was added and allowed to stir for 3 h. Purification and subsequent step were the same as in-situ method.

3.6.3 Characterization

Dynamic light scattering (DLS) studies were performed by analyzing dilute solutions of nanoceria preparation using a PDDLS/Cool Batch 40T, PD2000 DDLS. UV-visible spectroscopic measurements were done using solutions of nanoceria in a Varian Cary 300 Bio
UV-visible spectrometer. FT-IR experiments were performed using vacuum dried samples of nanoceria and spectra were recorded on Perkin Elmer Spectrum 100 FT-IR spectrometer.

3.6.4 Oxidation Studies and kinetic analysis

For oxidation studies, tetramethyl benzidine (TMB), 2,2-azinobis-(3-ethylbenzothizoline-6-sulfonic acid (AzBTS) and dopamine (DOPA) were purchased from Sigma, USA. Steady-state kinetic assays were carried out in 96 well plate using nanoceria (5.0 µM) at room temperature with TMB and AzBTS in citrate buffer of pH 4.0. Reactions were monitored at 652 nm for TMB and 405 nm for AzBTS in time scan mode using a Bio-TEK, Synergy HT Multidetection Microplate reader. Color reactions were observed immediate upon addition of nanoceria to substrate (TMB or AzBTS). To study the steady-state mechanism of the reaction, further experiments were carried out using above conditions with varying concentrations of TMB or AzBTS while nanoceria concentration was kept constant (5.0 µM). The kinetic parameters were calculated based on $V_{\text{max}}$ is maximal velocity, $[S]$ concentration of substrate and $K_m$ Michaelis constant. In addition to above studies, simple UV profile of all three dyes were done to confirm the type of oxidation product.

3.6.5 Synthesis of folate-conjugated cerium oxide nanoparticles:

**Synthesis of propargylated cerium oxide nanoparticles:**

The carboxylated cerium oxide nanoparticles (0.005 mg of ceria) were taken in an eppendorf tube containing 2.0 mL of MES buffer (pH = 6.0), to this were added EDC (12.3 mg, 10 mmol) and NHS (7.5 mg, 10 mmol) and stirred for 3 minutes. To the resulting reaction mixture, propargyl amine (3.6 mg, 10 mmol) in 0.25 mL of DMF was added and incubated at room
temperature for 4 h. The final reaction mixture was purified by dialysis using 6-8 K molecular weight cut off dialysis bag, against deionized water and phosphate buffered saline (PBS) solution.

**Synthesis of Aminopropylazide:**

Chloropropyl amine (2.0 g, 21.5 mmol) and sodium azide (4.19 g, 64.5 mmol) were taken in a 50 mL round bottom flask containing 25 mL of distilled water and heated at 75 °C for 18 h. The reaction mixture was concentrated via a rotavapor using high vacuum, and 1.2 g of KOH was added to it and then extracted by using diethyl ether. Subsequently, the reaction mixture was dried over anhydrous sodium sulphate and concentrated. Then, the mixture was purified through flash column chromatography using 4% ethyl acetate in petroleum ether as an eluent, in order to obtain the pure aminopropylazide.

Yield: 1.45 g (68%). $^1$H NMR (300 MHz, CDCl$_3$, $\delta$ ppm): 1.25 (bs, 2H), 1.82 (m, 2H), 2.81 (t, 2H), 3.36 (t, 2H). IR (CHCl$_3$): 3305, 2942, 2087, 1665, 1434, 1372, 1258, 1241, 1076, 1026, 818, 761 cm$^{-1}$.

**Synthesis of azide-functionalized folic acid:**

1,1'-carbonyldiimidazole (CDI) (9.0 mg, 0.054 mmol) was taken in an eppendorf tube containing folic acid (20.0 mg, 0.045 mmol) in dry DMF (1 mL) and incubated for 2 h at 35 °C. To this solution we added aminopropylazide (5.0 mg, 0.049 mmol) in dry DMF (75 µL) and incubated it for 30 h at room temperature. The reaction mixture was then centrifuged and washed to remove excess starting materials. Finally, we dissolved the azide-functionalized folic acid in 1 mL of DMF. The presence of a band at 2091 cm$^{-1}$ in the IR spectrum and a UV absorbance at 353 nm confirmed the formation of azide-functionalized folic acid.
Synthesis of folate functionalized cerium oxide nanoparticles (Click chemistry):

The propargylated cerium oxide nanoparticles (0.013 g, 3 x 10^-3 mmol) in bicarbonate buffer (pH = 8.5) were taken to an eppendorf containing catalytic amount of CuI (0.05 µg, 3 x 10^-10 mmol) in 125 µL of bicarbonate buffer, mixed for 25 seconds. To this was solution azide-functionalized folic acid (0.002 g, 4 x 10^-2 mmol) in DMSO was added and the reaction was incubated at room temperature for 12 h. The final reaction mixture was purified by dialysis using 6-8 K molecular weight cut off dialysis bag, against deionized water and phosphate buffered saline (PBS) solution. The purified functional cerium oxide nanoparticles were stored in refrigerator for further characterization.

3.6.6 Immunoassays:

Cardiomyocytes (H9c2) and lung cancer (A-549) were obtained from ATCC, USA. Cardiomyocytes cells were grown in Eagle’s Minimal Essential medium supplemented with fetal bovine serum (10%), sodium pyruvate, L-glutamine, penicillin, streptomycin, amphotericin B, and nonessential amino acids. Lung-cancer cells were grown in Kaighn’s modification of Ham’s F12 medium (F12K) supplemented with fetal bovine serum, L-glutamine, streptomycin, amphotericin B, and sodium bicarbonate. All cell lines were maintained at 37 °C, 5% CO_2 in a humidified incubator. Cells were plated in 96 well plate at density of 6000 cells per well and after 24 h incubation treated with different concentration of folate-nanoceria particles (0.5 µM to 5.0 µM). Particles were allowed to incubate for 3 h. Afterwards cells were washed with PBS buffer and 50 µL of buffer was added to the plate. Afterwards, cells were treated with 50 µL of TMB (0.04 mM) and allowed to develop the blue color. Then blue color reaction was measured at 652 nm on a plate reader. In another set of experiment, similar immunoassay were carried out.
using increasing number of lung carcinoma cells (1500 to 6000 cells) keeping the concentration of folate-cerium oxide constant (5.0 μM)

3.7 Conclusions:
In conclusion, we report that ceria nanoparticles possess unique oxidase-like activity, as they can facilitate the fast oxidation of organic dyes and small molecules in slightly acidic conditions without the need of hydrogen peroxide. When compared to other systems that require peroxides or proteins (such as oxidases and peroxidases), our polymer-coated nanoceria is a more robust and water-soluble redox nanocatalyst, as it is not susceptible to denaturation or decomposition. Furthermore, conjugation with targeting ligands makes nanoceria an effective nanocatalyst and detection tool in immunoassays. Taken together, these results demonstrate that this unique aqueous oxidase-like activity of nanoceria can be used in a wide range of new potential applications in biotechnology, environmental chemistry, and medicine.
4.1 Introduction:

Nanomaterials with unique magnetic, luminescent and catalytic properties are being engineered for a wide range of biomedical applications ranging from imaging, diagnostics and treatment. However, nanomaterials greatest strength, which relies primarily on the enhanced physical and chemical characteristics that mater, exhibits at this scale, has the potential to be its greatest liability. Potentially harmful interactions can occur between nanomaterials and living systems, including humans. For that reason, nanomaterials must be engineered from nontoxic, biocompatible and biodegradable components or components that have minimal and in some cases beneficial properties. An inflammatory response is a parameter that is often investigated to assess the effect that nanomaterials have with living systems. Exposure of titanium oxide nanoparticles to rodents results in airway inflammation and interstitial fibrosis.53-57 Similarly, carbon nanotubes, quantum dots, buckyballs, and silver nanoparticles are known inducer of pro-inflammatory response in animals.58

Cerium oxide nanoparticles are unique because they exhibit anti-inflammatory properties, scavenging reactive oxygen species, mimic superoxide dismutase activity, and preventing cardiovascular myopathy.11-13 As discussed in previous chapters, nanoceria displays optimal antioxidant properties at physiological pH, whereas it behaves as an oxidant at acidic pH. This selective behavior of nanoceria could have important implications in understanding why
nanoceria gives selective cytoprotection to normal cell but not to the cancer cell during radiation treatment or oxidative stress. In addition, the nature of the polymer coating surrounding the cerium oxide core could play a critical role in determining its antioxidant versus oxidant properties. We also reasoned that the cytotoxicity of cerium oxide nanoparticles could depend upon the localization either inside (intracellular) or outside (extracellular) the cells. If inside then it could be depend on whether the nanoparticles are localized in subcellular organelles such lysosomes (which are acidic) or distributed in the cytoplasm (which is at neutral pH in normal cells). In addition, the acidic microenvironment in most tumor might affect the nanoceria’s antioxidant activity sensitizing the tumor against radiation therapy.

In this work, we report the surface charge dependent cell internalization and cytotoxicity profile of cerium oxide nanoparticles in normal versus malignant cell lines. We selected normal H9c2 cardiac myocytes and malignant A549 lung carcinoma for our studies as they exhibit different cytoplasmic microenvironment. A549 cell lines have been reported to have upregulated glycolysis and production of lactic acid which result in an acidic microenvironment. In contrast, H9c2 cardiac myocytes possesses neutral microenvironment as they have normal cellular metabolism. Results showed that nanoceria coated with a positively charged polymer internalized in both cell lines and localize preferentially in their lysosomes, resulting in cytotoxicity. In contrast, nanoceria coated with a negatively charged polymer only internalized into lung carcinoma cell lines and exhibits toxicity as they localized in lysosomes. No significant internalization or toxicity of negatively charged nanoceria was seen in normal cells. Surprisingly, the nanoceria coated with a neutral polymer exhibit no toxicity to the normal cells or cancer cell line as they preferentially localize in the cytoplasm and not the lysosomes of these cell lines. Taken together, our results suggest that the internalization and subcellular localization of
polymer coated cerium oxide nanoparticles plays a critical role in their toxicity profile. Furthermore, these results suggest that the surface coating of nanoceria can be engineered to modulate its differential cytotoxicity behavior in cancer versus normal cells.

4.2 Synthesis and characterization of polymeric DiI labeled cerium oxide nanoparticles:
For our studies, we first synthesized polymer coated cerium oxide nanoparticles of various surface changes. To accomplish this, we coated nanoceria with polyacrylic acid (PAA), aminated-PAA, or dextran (Figure 4.1). These diverse coatings introduce different surface charge to the nanoparticles (-ve, +ve and neutral) which was corroborated by zeta potential measurements (Figure 4.2a). FT-IR spectrum of these nanoparticles further confirmed their surface functionality (Figure 4.2b) and dynamic light scattering studies revealed stable and monodisperse nanoparticle preparations within range of 5 to 15 nm (Figure 4.2c). Moreover, transmission electron microscopy (TEM) data showed that although the synthesized nanoparticles have different polymer coating, they possess same nanocrystal core size of ~3 to 4nm which rule out the possibility that nanocrystal core size may be affect nanoceria cellular interactions (Figure 4.2d).

To be able to track the localization of the nanoceria within the cell, we labeled the nanoparticles by encapsulating a hydrophobic cyanine dye (DiI) into the available hydrophobic microdomains inside the polymer coatings (PAA and Dextran) of cerium oxide nanoparticles following a solvent diffusion method previously reported to label PAA and dextran coated iron oxide nanoparticles (Figure 4.1).60 This approach to introduce a fluorescent multimodality to polymer coated cerium oxide nanoparticles has the unique advantage that it does not compromises the solubility of the nanoparticles in aqueous media or reduces the number of available functional groups on the nanoparticle surface. We selected DiI since it possesses a high extinction
coefficient, high stability (resistance to quenching) under confocal microscopy, and is widely employed in the biomedical field for cell labeling. Successful encapsulation of DiI into cerium oxide nanoparticles was confirmed by observing a in the emission spectra of the encapsulated Dil (584 nm) compared to free Dil (592) (Figure 4.2e). These Dil encapsulated nanoparticles display good aqueous stability over long periods of time without significant release of the dye in aqueous medium.

**4.3 Differential Cellular Interaction of Polymeric Cerium Oxide Nanoparticles with different Surface Charge:**

Confocal microscopy was carried out to study the cellular uptake and intracellular distribution of Dil labeled cerium oxide nanoparticles. In these experiments, PAA (-), aminated PAA (+), and dextran (neutral) coated nanoceria (1.0 mM) were incubated with A549 lung carcinoma and H9c2 cardiomyocytes to assess their charge dependent internalization, localization and toxicity profile. First, and to rule out the possibility of potential artifacts due to fixation, live cell imaging after incubation PAA, dextran and aminated PAA nanoceria was carried out (Figure 4.3). Figure 4.4 shows the difference in the behavior of differently charged nanoparticles. Results show that aminated nanoparticles bearing positive charge were internalized by both cell lines at 37 °C and exhibits a more punctuate type of internalization suggesting an endosomal uptake and lysosomal localization (Figure 4.4a and 4.4d). Surprisingly, negatively charged carboxylated nanoparticles were uptaken by the malignant lung carcinoma cell line (A-549) but not by normal cardiac myocytes (H9c2) (Figure 4.4b and 4.4e). In the malignant A549 cells, a lower degree of internalization of the negatively charged nanoceria (PAA coated) is observed compared to the positively charged (aminated PAA coated) nanoceria; however their internalization results in a more punctuated localization of the nanoparticles suggesting that in this cell line more of the
negatively charged carboxylated nanoparticles localized into the endosome. In contrast, neutral dextran nanoceria internalized in both cell lines judged by the presence of a diffuse intracellular fluorescence pattern (Figure 4.4c and 4.4f), suggesting that these nanoparticles are mostly internalized into cytosol with a very less fraction confined to endosomal compartment. Taken together, the above results points to the fact that the surface chemistries on polymer coated nanoceria surface dictate their differential internalization and localization in malignant versus normal cells.
Figure 4-1 Schematic representation of the synthesis of surface functionalized cerium oxide nanoparticles and dye encapsulation. Cerium oxide nanoparticles with different surface charge were synthesized using water based wet chemical method. Carbodiimide chemistry was employed to modify the surface of the carboxylated nanoceria. DiI was encapsulated using modified solvent diffusion method.
Figure 4-2 Characterization of cerium oxide nanoparticles. (a) Zeta potential measurement of cerium oxide nanoparticle with different surface functional group (negative, neutral and positive), (b) FT-IR spectrum confirming the amination of PAA-coated cerium oxide nanoparticles, characteristics peak are shown (c) Nanocrystal core size calculated from the TEM images, and (d) Fluorescence emission spectra of the Dil encapsulated nanoceria and that of free dye in solution.
Figure 4-3: Real time live cell confocal imaging. Cationic polymeric nanoceria is being uptaken in real time by lung carcinoma which shows the internalization is not due the artifacts introduced by fixation procedure. Similar results were obtained with anionic polymeric, neutral nanoceria, and also with cardiac myocytes.

Figure 4-4. Uptake of cerium oxide nanoparticles. Confocal microscopy show that aminated nanoceria (+ve) is being uptaken by both cell lines lung carcinoma and cardiac myocytes while carboxylated nanoceria (-ve) are being uptaken only by lung carcinoma cell lines. Positive nanoparticles internalize more and seem to be localized into particular cell organelle such as
lysosomes. Negative nanoparticles show less internalization but surprisingly mostly into lysosomes. While neutral particles exhibit more diffuse kind of internalization and all over the cytoplasm.

4.4 Mechanism of Cellular Uptake (Active Endocytic Process Blockage):
To further explore the mechanism of cellular internalization and to verify if an endocytic pathway is responsible for the internalization of polymer coated nanoceria, we performed experiments at 4°C or in the presence of 2-deoxyglucose or sodium azide, known inhibitors of the endocytic pathway. At 4°C active internalization of molecule by an endocytic/pinocytic mechanism is blocked. After incubating cells with the various DiI labeled nanoceria preparations at 4°C, we observed very negligible nanoparticle internalization (Figure 4.5a, 4.5b and 4.5c). None of the polymer coated nanoceria entered either of the cell lines studies suggesting endocytosis as a possible mechanism of internalization in all cases. Experiments performed in the presence of inhibitors at 37°C confirm our results (Figure 4.5d, 4.5e and 4.5f). Both cell lines incubated with nanoparticles in presence of inhibitors did not show any detectable internalized DiI labeled polymeric nanoceria as expected. These results confirm that our polymeric coated nanoceria were actively uptaken by an endocytic process by both lung carcinoma and cardiac myocytes.
Figure 4-5. Uptake studies where active internalization processes were blocked (Determination of internalization mechanism). No internalization of aminated or carboxylated nanoparticle is seen in either of the cell line in presence of inhibitors, 2-deoxyglucose and sodium-azide which blocks active internalization. Similarly, internalization of nanoparticles is abrogated at 4 °C. These results suggest that the uptake of nanoparticles is mediated by endocytosis since endocytic processes are inhibited by both inhibitors and at 4 °C.
4.5 Intracellular Distribution of Polymeric Cerium Oxide Nanoparticles (Lysosomal Staining or co-localization studies):

To corroborate that some of the polymer coated nanoceria preparations localize to the endosomal compartment after internalization, we treated the cells for 20 minutes with Lysotracker, a lysosome specific dye, after incubation for 3 hours with the various nanoceria preparations. Lysotracker is a green fluorescent dye that stains lysosomes (acidic) compartment of cells with excitation/emission spectra at 504/511 nm. Co-localization was performed to see if internalized nanoceria DiI (red) and lysosomes (lysotracker, green) overlap. Experiments showed that co-incubation of DiI label aminated-PAA coated nanoceria and lysotracker in A549 lung carcinoma cells results in co-localization of both the lysotracker and DiI label nanoceria by confocal microscopy (Figure 4.6a, 4.6b and 4.6c). In the case of H9c2 cardiac myocytes, a different distribution of lysotracker and DiI label nanoceria was observed which suggests that nanoparticles are distributed into both cytosolic and lysosomal compartments of the cell (Figure 4.7a, 4.7b and 4.7c). In contrast, even though less internalization of carboxylated-nanoceria is observed in A549 lung carcinoma, the internalized nanoparticles co-localize mostly with the lysotracker dye, indicating mostly lysosomal localization and no cytoplasm localization (4.6d, 4.6e and 4.6f). Meanwhile, when the dextran coated nanoceria was used instead, a diffuse distribution of internalized nanoparticle (DiI signal) was observed with very less co-localization with the lysotracker signal in both cell lines (Figure 4.6g, 4.6h, 4.6i, 4.7d, 4.7e and 4.7f). This indicates that the dextran coated nanoceria localizes mainly to the cytosol. These results suggest that engineered ceria nanoparticles show different intracellular distribution into both cancer and normal cell lines depending upon surface charge.
Figure 4-6. Nanoparticles cell localization, lysosomal staining (Lung carcinoma). Upon internalization nanoparticles fluorescently labeled DiI were found in colocalized into lysosomes. Most of the internalized nanoparticles were co-localized with lysotracker (overlay). Dextran nanoceria exhibits less co-localization with lysotracker.
Figure 4-7. Nanoparticles cell localization, lysosomal staining (Cardiac myocytes). Aminated nanoceria (red dye) were found to co-localize less with lysotracker in normal cell line compared to lung carcinoma. Also, dextran-nanoceria shows even less lysotracker co-localization compared to aminated nanoceria. However most the nanoparticles in both cases were found in the cytoplasm.

4.6 Intracellular Distribution Dependent Cytotoxicity of Polymeric Cerium Oxide Nanoparticles:

We have shown in earlier sections that different surface charge nanoceria vary in their cellular interaction with the normal and cancer cell lines. Nanoceria also exhibits different sub-cellular localization into cytoplasm and lysosomes. In order to see if nanoceria sub-cellular distribution (lysosomal or cytoplasm) can have any effect towards nanoceria cytotoxic behavior, cytotoxicity (MTT) assay were performed. Interestingly, we found that the nanoparticles which were internalized by the cell and were entrapped into lysosomes such as aminated (lung carcinoma and
cardiac myocytes) and carboxylated (lung carcinoma) showed toxicity to both lung carcinoma and cardiac myocytes (Figure 4.8a and 4.8b) Aminated nanoparticles were more toxic to lung carcinoma and cardiac myocytes since they were found significantly inside the lysosome upon internalization. Carboxylated nanoparticles were toxic only to lung carcinoma but less compare to aminated given that they are less internalized into lysosomes. While dextran (neutral) nanoparticles did not show any toxicity to either of cell lines since they were found into lysosomes in significantly low amount. Similar experiments were carried out in presence of inhibitors (2-deoxy glucose and sodium azide) and as expected no cytotoxicity was observed because inhibitors block the uptake of nanoparticles (Figure 4.9a and 4.9b). Hence, these results demonstrate that localization of nanoceria into lysosomes (acidic cell compartment) as opposed to localization into cytoplasm leads to its cytotoxicity.

Figure 4-8. Cytotoxicity of cerium oxide nanoparticles. Graph shows that aminated nanoparticles are more cytotoxic to both cell lines while carboxylated nanoparticles are cytotoxic to only lung carcinoma not to cardiac myocytes. However, dextran nanoceria don’t show any toxicity to either of cell lines
Figure 4-9. Cytotoxicity of cerium oxide nanoparticles in presence of inhibitors showing no toxicity.

4.7 Comparison with iron oxide nanoparticles:

The polymer coated iron oxide nanoparticles has been well established for various in-vitro and in vitro applications such as MRI contrast agents, and drug delivery vehicle.\textsuperscript{64-67} These reports demonstrate that polymeric iron oxide nanoparticles are non-toxic to living systems; in fact they are currently used in clinic to image liver metathesis and metastatic lymph nodes.\textsuperscript{68} To rule out the possibility that the toxicity profile observed with the studied polymer coated nanoceria is due to the nature of the polymer coating, we performed experiments with iron oxide nanoparticles coated with PAA, Aminated PAA or dextran coated iron oxide nanoparticles. Confocal microscopy studies indicate that aminated-iron oxide nanoceria was being uptaken by lung carcinoma as well as cardiac myocytes (Figure 4.10a and 4.10c). In contrast, carboxylated iron oxide nanoparticles didn’t show any detectable signal under confocal microscopy in either of cell lines (Figure 4.10b and 4.10d). In addition to these, very disperse kind of internalization is seen as compare to the punctuate kind in case of polymeric nanoceria. These results rule out the
possible localization of polymeric iron oxide into lysosomes. Next, MTT assay were carried out with polymeric iron oxide nanoparticles to confirm any toxicity. As expected, polymeric iron oxide nanoparticles didn’t show any toxicity even when they are internalized by both normal and cancer cell lines (Figure 4.11a and 4.11b). This behavior for iron oxide is expected as iron oxide does not possess oxidant activity at low pH and clearly indicate that it is the intrinsic oxidase-like behavior of cerium oxide nanoparticles which is responsible for its cytotoxicity, especially when they localized into the acidic cell compartment such as lysosomes.

Figure 4-10. Uptake of polyacrylic acid coated iron oxide nanoparticles. Confocal showed that only aminated-PAA iron oxide nanoparticles were uptaken by lung carcinoma and cardiac myocytes.
4.8 Experimental section:

4.8.1 Synthesis of PAA-coated ceria

Preparation of PAA-coated ceria involves the use of cerium (III) nitrate and polyacrylic acid (PAA). 1M cerium (III) nitrate (Aldrich, 99%) solution (2.17 g in 5.0 ml of water) was mixed with 0.5 mM solution of PAA (Sigma). Under continuous stirring, the mixture was added drop wise to 30.0 ml ammonium hydroxide (Sigma Aldrich, 30%) solution. Afterwards mixture stirred continuously for 24 h, at this point the solution has changed from a light yellow to a deep brown color. Next, preparation centrifuged at 4000 rpm for two 30-minute cycles to settle down any debris and large particles. Centrifuged solution was then purified from free PAA by
ultrafiltration using 30K molecular weight cut-off Amicon filter (Millipore Inc.). Dextran coated nanoceria was prepared in similar manner except dextran was used instead of PAA.

4.8.2 Amination of PAA-coated ceria

For amination, 5.0 mL of PAA-coated ceria (0.060mg/mL) was treated with EDC (30.0 mg in 500 uL of 0.1 M MES buffer pH 6.0) for one minute and reacted with n-hydroxy succinamide (22.0 mg in 500 uL of 0.1 M MES buffer pH 6.0) for 3 minutes. To the solution mixture, ethylene diamine (EDA) (20 mg in DMSO) was added in drop wise and the solution mixture was stirred for 3 hrs. Then mixture was purified to get rid of excess EDA using the amicon 30K membrane.

4.8.3 DiI encapsulation in PAA-coated ceria and aminated- PAA-coated ceria

In order to encapsulate DiI in PAA-coated ceria and aminated preparation, to 4.0 mL of both PAA-coated cerium preparation (0.060mg/mL) 200.0 uL of DiI (6.0 uL of DiI solution (1.0 mM) in 1.2 mL of DMSO) was added in dropwise on votex (1000 rpm) at room temperature. Afterwards, preparation was dialyzed in water to remove any free DiI and finally dialyzed overnight in phosphate buffer saline (PBS) to get final preparation in PBS.

4.8.4 Characterization

Dynamic light scattering (DLS) studies were performed by analyzing dilute solutions of all preparation using a PDDLS/Cool Batch 40T, PD2000 DDLS. Fluorescence spectroscopy studies were done using a Nanolog HORIBA JOBIN YVON spectrometer. Zeta potential measurements were performed using Malvern Zetasizer using disposable zeta cell. FT-IR experiments were performed in a Perkin Elmer Spectrum 100 FT-IR spectrometer using vacuum dried sample.
4.8.5 Cell Studies

Cardiomyocytes (H9c2) and lung cancer (A-549) were obtained from ATCC, USA. Cardiomyocytes cells were grown in Eagle’s Minimal Essential medium supplemented with fetal bovine serum (10%), sodium pyruvate, L-glutamine, penicillin, streptomycin, amphotericin B and non-essential amino acids. Lung cancer cells were grown in Kaighn’s modification of Ham’s F12 medium (F12K) supplemented with fetal bovine serum, L-glutamine, streptomycin, amphotericin B and sodium bicarbonate. All cell lines were maintained at 37 ºC, 5% CO₂ in a humidified incubator.

4.8.6 Cellular Uptake of Nanoparticles

Cells were seeded in petridishes and incubated with Carboxylated-Dil-nanoceria and Aminated-Dil-nanoceria for three 3 hrs. Then cells were washed with PBS buffer and fixed using 10% formalin in PBS buffer. Afterwards, cells were incubated with the DAPI for 10 minutes. Then again washed and visualized under the confocal microscope.

4.8.7 Inhibition of Uptake or Inhibitors

a) For inhibitors studies cells were treated with the inhibitors, sodium-azide (10 mM) & 2-dexoyglucose (50 mM) for 30 minutes. Afterwards, cells were incubated with Carboxylated-Dil-nanoceria and Aminated-Dil-nanoceria particles for three hours. Fixation and subsequent treatment with DAPI were similar to the internalization. b) A similar experiment was done except instead of treating cells with inhibitors cells were allowed to incubate with nanoparticles at 4 C.
4.8.8 Lysosome Labeling or Lysotracker Staining

After treatment with the nanoparticles for three hours cells were washed and incubated for 20 minutes with lysotracker (35 nM). Fixation and subsequent treatment with DAPI were similar to the previous experiment.

4.8.9 Cell viability Assays

Cells were seeded in 96-well plates at a density of 3,000 cells per well and incubated with (1.0 mM) carboxylated and aminated-nanoceria for 3 hrs. Then, 0.5 mM of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma,USA) was added followed by incubation for 24 h. After 24 h, the resulting crystals were dissolved in 20 μL of isopropanol and the absorbance at 570 nm was recorded using a plate reader (Bio-TEK, Synergy HT Multidetection Microplate reader). These experiments were performed in triplicates.

4.9 Conclusions:

The toxicity of nanomaterials depends on various factor including the nature of the nanoparticle core, mode of synthesis, chemical composition (purity, crystallinity), surface structure (surface reactivity, surface groups, inorganic or organic coatings), solubility, shape, and aggregation etc. Not much research has been done in the effect of polymeric surface coating and surface charge on the uptake, localization and toxicity of nanomaterials. This is highly important as some nanomaterials, such as cerium oxide, display either a beneficial (antioxidant) or toxic (oxidant) effect depending on the pH of the surrounding environment. We showed that the polymer coated nanoceria display different level of toxicity depending upon a surface charged dependent cellular uptake and subcellular localization. We found that when polymer coated nanoceria internalize
and localize mainly in the lysosomes it become toxic due to the acidic nature of this organelle; whereas, when it localizes mainly in the cytoplasm, less toxicity is observed. As expected, polymer coated nanoceria is not toxic when it does not internalize into the cell. The internalization and eventual localization of the nanoparticles within the cell depends greatly on the surface charge of the polymeric coating on nanoceria and the type of cell used (malignant versus normal). Here, cationic polymer coated nanoceria has been more toxic as these nanoparticles were uptaken by both cell lines (cancer and normal cell lines), and they localize mostly into the lysosomes. In fact, cationic polymer coated nanoceria are comparatively more toxic to lung carcinoma contrary to cardiac myocytes. Previously, it has been shown that cationic nanoparticles enters cell either by inducing transient holes/poration into cell membranes, a process associated with cytotoxicity.\textsuperscript{70} But cationic polymer coated nanoceria still exhibit toxicity although uptaken by endocytic mechanism. This proves the fact that it is the nanoceria’s intrinsic oxidase-like property which is responsible for cytotoxic property. Anionic polymer coated nanoceria were internalized into lung carcinoma cells and not in cardiac myocytes. Although a lower degree of internalization was observed with the anionic nanoparticles as compared to their cationic counterparts, almost all of them were found to be present in the lysosomal compartments of lung carcinoma. This explains why anionic polymer coated nanoceria elicits cytotoxicity to lung carcinoma while it is non-toxic to cardiac myocytes. Alteration in surface charge to neutral (dextran coated nanoceria) leads to drastic change in cellular penetration/interaction of neutral polymer coated cerium oxide nanoparticles. A very disperse and diffuse kind of intracellular distribution was seen throughout both cell lines (cancer and normal) with very few nanoparticles localized into lysosomes, which explains the minimal toxicity of these nanoparticles.
In summary, we believe that highlighting the role of surface charge and cell type in cellular interaction of polymer coated nanoceria will shed new light on understanding of molecular interactions/localization that leads to toxicity of nanoceria.
Cardiac Myocytes

Lung Carcinoma

Cell Membrane

Lung Carcinoma (A-549)
- Endocytosis
  - Antioxidant nanoparticles
  - Lysosome (pH 4.5)
  - Oxidant nanoparticles
- Cytotoxic

Cardiac Myocytes (H9c2)
- Endocytosis
  - Antioxidant nanoparticles
  - Lysosome (pH 4.5)
  - Oxidant nanoparticles
- Cytotoxic
Figure 4-12. Schematic representation of nanoparticles cell internalization, localization and hypothesized mechanism for their toxicity. As shown in schematic aminated nanoceria are found more internalized and entrapped mostly in lysosome in lung carcinoma. pH of microenvironment of lung carcinoma and lysosome is acidic enough for nanoceria to exhibit oxidase like activity. This might be the reason why aminated nanoceria are more cytotoxic compared to carboxylated which are less toxic to lung carcinoma. However, aminated nanoceria are also toxic to cardiac myocytes since they are found both in lysosomes and cytoplasm in cardiac myocytes. Carboxylated nanoparticles don’t show any internalization in cardiac myocytes hence any toxicity. Dextran nanoceria shows very diffuse kind of internalization and are less confined to lysosomes which do not alter nanoceria property and hence dextran nanoparticles not toxic to either of cell lines.
CHAPTER 5: CONCLUSIONS

Taken together, our results suggest that we have successfully synthesized a highly stable and water soluble polymer coated cerium oxide nanoparticles preparation that retains the unique antioxidant/radical scavenging properties of uncoated cerium oxide nanoparticles. Our aqueous nanoceria preparation was synthesized using non-toxic materials and without the use of surfactants or high temperature methods etc. Polymer coated cerium oxide nanoparticles shown great stability in physiological solution over long periods of time without any agglomeration, precipitation or loss of activity. Taking advantages of their enhanced aqueous stability we performed a series of experiments, not possible with previously synthesized methods. First, we performed pH-dependent experiments that showed an interesting phenomenon that nanoceria do not exhibits reversibility between Ce$^{3+}$ and Ce$^{4+}$ states at pH 4.0 while being regenerative at physiological pH 7.0. This observation could explain why nanoceria protects the normal cells (acting as antioxidant) and do not protect cancer cell (acting as oxidant). Second, we established the pH-modulated dual antioxidant/oxidant behavior of cerium oxide nanoparticles. We found that nanoceria exhibit an oxidase-like activity, by studying the oxidation of chromogenic dyes such TMB and AzBTS without the need of hydrogen peroxide at low pH. Third, internalization studies were carried out to see if the observed dual antioxidant/oxidase-like property can have any effect on cytotoxic behavior of nanoceria when it interact or penetrate the cell. The internalization experiments demonstrated that the polymeric cerium oxide nanoparticles do behaves differently when inside or outside the cells. They are non-toxic when they are outside. When they are inside, their localization in different cell organelles such lysosomes or cytoplasm dictate their cytotoxicity. Results demonstrated that localization of nanoceria into lysosomes...
(acidic cell compartment) as opposed to localization into cytoplasm is responsible for their cytotoxicity.

In summary, we found up with some of the new interesting properties of cerium oxide nanoparticles yet not discovered before such as oxidase-like behavior or pH-triggered dual antioxidant/oxidant behavior. All findings made in this dissertation open up new vistas for cerium oxide nanoparticles applications that include the development of antioxidant and anti-inflammatory coatings in prosthetic devices and tissue engineering, and their use as building blocks in supramolecular materials. Moreover, oxidase-like behavior can be employed to design as more robust and water-soluble redox nanocatalyst, as it is not susceptible to denaturation or decomposition. This unique aqueous oxidase-like activity of nanoceria can also be used in a wide range of new potential applications in biotechnology, environmental chemistry, and medicine.
REFERENCES


