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THE ROLE OF SURFACE CHEMISTRY IN THE TOXICITY OF MANUFACTURED CERIUM DIOXIDE NANOMATERIALS TO CAENORHABDITIS ELEGANS

Emily Kay Oostveen
University of Kentucky, ekoostveen@gmail.com

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Emily Kay Oostveen, Student

Dr. Jason Unrine, Major Professor

Dr. Mark Coyne, Director of Graduate Studies

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THESIS

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By

Emily Kay Oostveen

Lexington, Kentucky

Director: Dr. Jason Unrine, Assistant Professor

Lexington, Kentucky

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ABSTRACT FOR THESIS

THE ROLE OF SURFACE CHEMISTRY IN THE TOXICITY OF MANUFACTURED CERIUM DIOXIDE NANOMATERIALS TO CAENORHABDITIS ELEGANS

Manufactured CeO_2 nanomaterials (CeO_2-MNMs) are used for a wide variety of applications including diesel fuel additives and chemical/mechanical planarization media. To test the effects of CeO_2-MNM surface coating charge on to model organism *Caenorhabditis elegans*, we synthesized 4 nm CeO_2 with cationic (DEAE-), anionic (CM-), and neutral (DEX) coatings. In L3 nematodes exposed for 24 hours, DEAE-CeO_2 induced lethality at lower concentrations than CM- or DEX-CeO_2. Feeding slightly decreased CeO_2 toxicity, regardless of coating. In L2 nematodes exposed for 48 hours with feeding, DEAE-CeO_2 caused lethality at the lower concentrations as compared to CM- and DEX-CeO_2. Sublethal effects were measured by observing reproduction and oxidative/nitrosative protein damage. Low concentrations of DEAE-CeO_2 induced similar reductions as CM- and DEX-CeO_2 that were two orders of magnitude higher. Using immunochemical slot blots to explore oxidative/nitrosative stress, no treatments produced significant changes in protein carbonyl or 3-nitrotyrosine formation; however, the statistical power of our assay was low. All treatments caused large but not statistically significant increases in protein carbonyl levels. DEAE-CeO_2 exposure caused a significant reduction in 4-hydroxy-2-nonenol levels. This research suggests that cationic coatings render CeO_2 significantly more toxic to *C. elegans* than neutral or anionic coatings.

KEYWORDS: ceria nanoparticle, nanotoxicology, ecotoxicology, model organism, redox chemistry

Emily Kay Oostveen

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Emily Kay Oostveen

Dr. Jason Unrine

Thesis Director

Dr. Mark Coyne

Director of Graduate Studies

20 June 2014

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1.1 Introduction

In recent years, manufactured nanomaterials (MNMs) have come to the forefront of technology. The American Standard Testing Methods (ASTM) defines MNMs as materials with at least one dimension in the range of 1-100 nm [1]. Between 2005 and 2009, more than 1,000 consumer products containing MNM-materials were available for purchase with more applications expected each year [2-4]. These products include semiconductors, catalysts, pigments, biomedical applications, textiles, and cosmetics to name a few [2, 5-8]. Because nanotechnology is consistently growing, intentional or accidental environmental release of MNMs is inevitable [5, 9]. The increase of the inclusion of MNMs in everyday products and the possibility of MNM exposure for humans and other organisms has led to an increase in the need for toxicological information [10].

Physiochemical properties of materials at the nano-scale may be significantly different than the same materials at the bulk scale (>100 nm). Likewise, because MNMs are <100 nm in at least one dimension, MNMs have greater surface area to volume ratio as when compared to their bulk counterparts. This exposes more atoms on MNM surface, implying the possibility of size-dependent characteristics [11, 12]. As more atoms are exposed, the likelihood of defects in crystal faces increases. This can enhance a MNM's reactivity. Additionally, the amount of reactive functional groups located on MNM surface potentially increases as particle size decreases [13]. As particle size decreases, the band gap between the valence and conduction bands also decreases [14]. Vossmeyer et al. found that as CdS quantum dots decreases in size from 39Å to 13Å , the band gap decreases from 4.5

to 2.5 eV [15]. Additionally, the melting point changes from 400°C to 1600°C. Likewise with decreasing particle size, CeO_2 MNMs shows increased electronic conductivity [16]. CeO_2 MNMs have been shown to decrease diesel fuel consumption by 5-8% by decreasing the activation energy required for combustion. Combustion temperatures can decrease by as much as 100°C with the addition of a CeO_2 MNM catalyst [17, 18].

1.2 Environmental release, fate and transport of MNMs

Manufactured nanomaterials can be introduced into the environment either intentionally or unintentionally. Depending on the use pattern of the product, MNMs can enter the environment through aquatic, terrestrial, and aerial routes [12]. Both industrial and domestic practices can lead to MNM environmental introduction through aquatic routes. Aquatic contamination can occur through both wastewater production and normal wear of consumer products [19, 20]. CeO_2 MNMs commonly used for polishing semiconductors pass through wastewater treatment plants [5]. Ag MNMs have been shown to be released from textile based products like socks, medical masks, and toys when washed with tap water [21]. In wastewater treatment plants, MNMs are thought to primarily partition to sewage sludge, eventually being applied to soil as a fertilizer [22], suggesting that terrestrial systems may sustain a higher likelihood for contamination than aquatic systems [19, 23]. MNMs have also been observed to be released from outdoor products including textiles and paints when exposed to weathering conditions, passing directly into the environment [24]. Additionally, MNMs may be intentionally introduced into terrestrial environments; for example, nano-zero valent Fe has been shown to remediate organic and inorganic pollutants in ground water [5].

Lastly, MNMs could be deposited to soil and water from airborne deposition. CeO_2 MNMs are added to diesel fuel to help make fuel consumption more efficient [7, 17]. Similarly to tetraethyl lead ($(CH_3 CH_2)_4 Pb$), CeO_2 MNMs can enter the atmosphere through diesel exhaust and be deposited on surrounding soils and surface waters [25]. Incineration of MNM-containing products such as capacitors and filters could also lead to similar airborne deposition [5].

As the presence of MNMs increases in everyday products, both intentional and unintentional environmental contamination becomes more likely. Gottschalk et al. developed a predictive model based on a probabilistic material flow analysis from the perspective of MNM-based products in sewage sludge [9, 26]. This model includes both MNMs in sewage sludge and waste water. The model predicts a consistent increase in environmental concentrations of TiO_2 MNMs, ZnO MNMs, Ag MNMs, carbon nanotubes, and fullerenes. MNM presence in sediment and sludge treated soil was predicted by the model to double every three years [9].

Ultimately, MNMs from both aquatic and terrestrial environments have the potential to interact with various components of environmental media and undergo chemical, physical, or biological transformations [27]. Naturally occurring organic matter, organisms, and mineral components have potential to interact with MNMs. In the case of CeO_2 MNMs, the sorption of soil organic matter (SOM) may alter the ratio of Ce (III)/Ce (IV) [28]. Collin et al. found that CeO_2 MNMs had decreased toxicity when coated with SOM [29]. Other chemical modifications MNMs could undergo include sulfidation, dissolution, and photoreactivity [30, 31]. Interaction with their environment could cause MNMs to aggregate, potentially affecting their toxicity [32, 33].

1.3 Mechanisms of MNM toxicity

Numerous studies have investigated the toxicity of MNMs in biota. Oxidative stress caused by the production of reactive oxygen species (ROS) is often considered to play a primary role in the mechanisms of toxicity [34-39]. Many ROS are free radicals. Free radicals are short lived, highly reactive compounds containing an unpaired electron [40]. Due to the instability created by a missing electron, free radicals either donate or abstract an electron from the first molecule with which they collide. This begins a self-perpetuating chain reaction; rarely do free radicals first collide with each other, thus pairing all electrons [41]. Common examples of oxygen based free radicals include superoxide ($O^{\cdot 2-}$), hydroxyl radicals ($OH^{\cdot}$), and perhydroxyl radicals ($O_2H^{\cdot}$) [42]. Hydrogen peroxide (H_2O_2) is also a potent ROS despite containing a satisfactory number of electrons [42]. Nitric oxide ($^{\cdot}NO$) is a well-known reactive nitrogen species (RNS). While not a free radical, peroxynitrite ($ONOO^-$) is also a RNS [41, 42]. Because these compounds are also intermediates of biochemical pathways, organisms have natural defenses against ROS and RNS damage [43]. Enzymes such as catalase, superoxide dismutase, and glutathione peroxidase neutralize ROS and RNS [43]. Likewise vitamins C and E help mitigate oxidative and nitrosative damage [44]. When an organism's natural defenses against ROS and RNS are overwhelmed, the organism is said to experience oxidative stress.

Different mechanisms for the creation of ROS, and consequently oxidative stress, from MNMs have been explored [45]. Defects in crystal planes and more exposed surface area on MNMs can lead to the free radical formation by creating active electronic configurations [10, 46]. This can stimulate the donation or abstraction of an electron from oxygen-based molecules. Metal oxide MNMs like ZnO, Fe_2O_3, or CeO_2 may be subject to

Fenton chemistry, a series of reactions that produce oxygen-based free radicals through redox cycling [46-48]. Phototoxicity can occur when UV light causes an electron to split from the surface of MNMs, leaving behind an unpaired electron [30, 49]. Xiong et al. found that TiO_2 MNMs released a large number of hydroxyl radicals upon photoactivation, significantly damaging mouse macrophage cell lines at concentrations as high as 1000 μg/L [50]. ZnO MNMs and SiO_2 MNMs have also been shown to release ions with UV activation [51, 52].

Additionally, dissolution of unstable MNMs could release toxic ions [31]. Some of the toxicity caused by Ag MNMs is suspected to be due to ion release [53, 54]. Levard et al. showed that when Ag MNMs are sulfidized, consequently decreasing dissolution into Ag ions, toxicity to *Daphnia magna*, *Caenorhabditis elegans*, *Lemna minuta*, and *Fundulus heteroclitus* was significantly decreased as compared to controls [55]. CeO_2 MNMs are highly stable in suspension; dissolution into ions has generally not been shown to play a significant role in its toxicity [56]. However, a recent study showed that, 30 nm CeO_2 MNMs were bioprocessed to form an ultrafine second generation CeO_2 MNM (1-3 nm) in Dawley rat liver. These ultrafine particles have been shown to possess more antioxidant potential due to a higher occurrence of surface defects where Ce was in the +III oxidation state [57].

Lastly, MNMs can denature macromolecules such as proteins [58-60]. Many examples of MNMs causing oxidative stress in a variety of organisms can be found in the literature. Heng et al. found a dose dependent increase in ROS levels when exposing human epithelial cell lines to ZnO MNM [61]. Juvenile *Cyprinus carpio* were found to accumulate more ZnO MNMs than bulk ZnO after a 30-day exposure to 50 mg/L. The suspected mechanism of toxicity was oxidative stress as implied by gill and liver biomarker responses

[62]. SiO_2 MNM induced dose-dependent cell death and oxidative damage to human skin and lung epithelial cell lines (A431 and A549 respectively). Doses ranged from 25-200 μg/L [63]. Wu et al. observed an increase in ROS formation in nematode *Caenorhabditis elegans* with exposure to three MNMs (30 nm ZnO, TiO_2, and SiO_2) [64].ROS formation was recorded with a laser scanning confocal microscope at 488 nm of excitation wavelength and 510 nm of emission filter. Redox status was quantified by measuring the fluorescence of CM-H_2CFDA dye when oxidized. When the relative fluorescence intensities were semi-quantified as relative fluorescent units, the damage significantly correlated with mortality, growth rates, reproduction rate and locomotion of the nematodes. Hsuet et al. suggested that oxidative stress from mercaptosuccinic acid (MSA)-capped quantum dot exposure caused decreased reproduction in *C. elegans* [65]. Park et al. suggested that a single exposure to 100 mg/kg dose of single-walled carbon nanotubes intratracheally can induce oxidative stress by activating immune responses in rats [66, 67]. Multi-walled carbon nanotubes similarly induced oxidative stress in mice, leading to mesothelioma [67, 68]. Fullerenes, spherical carbon-based MNM, have both an antioxidant and a prooxidant potential [69]. In addition to scavenging ROS, fullerenes have been shown to induce oxidative stress in human astrocytes, liver carcinomas, dermal fibroblasts, macrophages, and red blood cells [69-71].

1.4 Manufactured nanomaterials

Cerium dioxide (CeO_2), a metal-oxide compound containing one of the more plentiful rare-earth elements, is highly valued for its catalytic activity mediated through the ability of Ce to transition reversibly between Ce (III) to Ce (IV). This capability is enhanced in CeO_2 MNMs [28, 72, 73]. CeO_2 MNM crystal structures can accommodate a combination

of Ce (III) and Ce (IV), but primarily contain Ce (IV) [28]. As particle size decreases, the capability of CeO_2 MNMs to transition between redox states increases [8, 74]. The oxygen storage capacity of CeO_2 MNMs illustrates this further. CeO_2 MNMs can either reversibly abstract or donate oxygen from surface molecules with which it comes into contact [75]. The high abundance of oxygen vacancies resulting in Ce at the surface in the Ce (III) state contributes to the observed catalytic properties [28, 76]. CeO_2 MNMs are already used in fuel catalysts, oxygen sensors, energy storage devices, and more [77-81]. CeO_2 MNMs primary use by tonnage is as a semiconductor, glass or mirror polishing agent [82-84]. There is also potential for use in medical antioxidant therapies [85-87].

In organisms, CeO_2 MNMs has potential to behave as both a prooxidant and an antioxidant. Some research has proposed that CeO_2 MNMs behave as a biological antioxidant, making it advantageous for use in preventative medicine. Korsvik et al. found that 3-5 nm CeO_2 MNMs both mimicked and competed *in vitro* with superoxide dismutase (SOD), an enzyme that specifically neutralizes O_2^- radicals into O_2 and H_2O_2 [88]. Pirmohamed et al. suggest that CeO_2 MNMs also mimic catalase, another biological antioxidant enzyme that neutralizes hydrogen peroxide [72]. Similarly, other *in vitro* studies conclude that CeO_2 MNMs are not highly toxic. Singh et al. found that normal human keratinocyte cells (HaCat) were not affected negatively at concentrations as high as 0.1 mg/L [89]. Park et al. observed no toxic effects when exposing human epithelial cell lines to diesel exhausted containing CeO_2 MNM supplementation [17]. *In vivo* studies have been completed as well. Niu et al. explored the potential of using 7 nm CeO_2 MNMs to prevent cardiovascular damage, specifically left ventricular shortening, in MCP-1 rats; they found that this was feasible when using 15 nmol exposures intravenously twice a week for two weeks

[87]. Chen et al reported that 5 nm CeO_2 MNMs scavenged reactive compounds in rats with doses as high as 1.0 μM/kg, helping prevent retinal disorders [90].

Other studies have focused on potential adverse effects of CeO_2 MNM exposure. Some studies found that the redox properties of CeO_2 MNMs cause negative effects. Park et al. observed increased ROS levels, decreased glutathione levels, cell death, and an increase in natural oxidative stress defenses in cultured human lung epithelial cells [35]. Yokel et al. found that 31 nm CeO_2 MNMs induced oxidative stress in rats at intravenous doses ranging from 50 to 750 mg/kg [36]. They observed an increase in 4-hydroxy-2-nonenal in rat hippocampus 20 hours after exposure with immunochemical slot blot assays. Nalabotu et al. also observed toxic responses in rats; with intravenous injections of up to 7.0 mg/kg CeO_2 MNMs, rat liver weight was noticeably reduced [91]. Roh et al. found that 15 nm CeO_2 MNMs caused both significant mortality and decreased fecundity in *Caenorhabditis elegans* [92]. They exposed *C. elegans* to both 15 nm and 45 nm CeO_2 MNMs at 1 mg/L; the smaller particles induced more negative responses, suggesting a size dependent effect. Thill et al. found that CeO_2 MNMs speciation is modified when in contact with *Escherichia coli* [93]. They observed significant amounts of CeO_2 MNMs interacting with the bacteria's outer membrane. Van Hoecke et al. studied the acute and chronic toxicity of 14, 20, and 29 nm CeO_2 MNMs to a green algae and two crustaceans (*Pseudokirchneriella subcapitata, Daphnia magna*, and *Danio rerio*). No significant acute toxicity was observed for all three organisms. The LC_{50} of the growth rate of *P. subcapitata* was affected after chronic exposure to doses between 7.6 and 28.8 mg/L, depending on particles size. The EC_{50} for *D. magna* reproduction ranged from 20.5 to 42.7 mg/L depending on the size of CeO_2 MNMs [94]. In both cases, the smaller CeO_2 MNMs required lower doses to cause toxicity.

Observed differences in toxicity could arise from differences in properties among CeO_2 MNMs. Particle size is a strong determining factor due to differences in available surface area with smaller particles having larger surface area [48]. The dose could also cause differing observations. As the dose increases, prooxidant effects might dominate more than antioxidant. Lastly, most of the research above does not take into account any aspect of CeO_2 MNMs beyond the CeO_2 MNM core. The surface coating of CeO_2 MNM is a largely ignored factor in many of these studies. Yet because of the observed differences in the toxic potential of CeO_2 MNMs, further study is needed to understand potential hazards, or lack thereof, in the environment. We have chosen to study these effects using the model organism *Caenorhabditis elegans*.

1.5 Surface coating

When synthesizing colloidal suspensions of MNMs, a surface coating is typically included to increase colloidal stability and decrease aggregation [12, 95]. Additionally, these coatings can affect the chemical and physical properties of the MNM core [96]. Rarely are MNMs synthesized in colloidal suspension without some form of coating and functionalization to optimize desired properties. For example, fullerene particles have a well-documented inability to be dispersed in polar solvents [69, 71, 97, 98]. The hydrophilicity of fullerenes can be increased by conjugating the surface with surfactants or polymers, rendering them more usable in applications.

Understanding how surface coating affects MNM interaction with its environment is an aspect of nanotoxicology that is often overlooked. But because the surface coating is the

portion of the MNMs that first comes into contact with the external environment, it is vital to investigate its role [99]. One chemical property of interest is that of molecular charge. The charge of MNM surface—positive, negative, or neutral—can vastly change MNM behavior. Cell membranes have a net negative charge, which can repel anionic MNMs and attract cationic MNMs. Upon contact with the membrane, MNMs can induce cellular damage through absorbing to or damaging membrane [59, 100]. Goodman et al. illustrated charge dependent properties when reporting that positively-charged 2 nm Au MNMs were moderately toxic to Cos-1 (kidney) cell lines, red blood cells, and *Escherichia coli* while negatively-charged Au MNMs of the same size were significantly less toxic [101]. Chen et al. also found that cationic MNMs are more likely to induce nanoscale holes in the lipid bilayers of living cell membranes [102].

Wang et al. explored the relationship between positively-charged enzyme lysozymes from chicken egg whites and CeO_2 MNMs with surface coatings of differing charges. They found that negatively-charged CeO_2 MNMs adsorbed the most strongly to lysozyme, neutral CeO_2 MNMs the second most, and positively-charged CeO_2 MNMs the least [103]. Asati et al. found that positively-charged CeO_2 MNMs induced more toxicity than neutral or negatively-charged CeO_2 MNMs in human H9c2, HEK293, A549, and MCF-7 cell lines, which have net negatively-charged membranes [104]. Additionally, they reported that cell damage depends heavily on where CeO_2 MNMs localizes in the cell; CeO_2 MNMs are more toxic if they partition into lysosomes as opposed to residing in the cytoplasm or not passing through the membrane at all. Positively-charged CeO_2 MNMs were found to enter lysosomes while neutral and negatively-charged CeO_2 MNMs did not [104]. When exposing cancer cell lines, though, Asati et al. reported that negatively-charged CeO_2 MNMs internalized into the cell membrane more readily. He et al. further showed that the toxicity

of CeO_2 MNMs relies heavily on electrochemical properties of its surface coating. By altering exposure media to add either a positive or negative charge to CeO_2 MNM's surface, toxicity to *E. coli* was also altered. Positively-charged CeO_2 MNMs caused membrane disruption while negatively-charged CeO_2 MNMs induced no toxic effects [105]. In addition to interaction with cell membranes, CeO_2 MNMs have been shown to interact with proteins. Patil et al. found that positively-charged CeO_2 MNMs absorbed to bovine serum albumin (BSA) more so than negatively-charged CeO_2 MNMs. The researchers also exposed adenocarcinoma lung cells; negatively-charged CeO_2 MNMs absorbed more readily into the cells [106]. From these studies, one can infer that there is a relationship between CeO_2 MNM toxicity and the chemical properties of its surface coating.

1.6 Caenorhabditis elegans

In addition to being a model organism for developmental biology, *Caenorhabditis elegans* has been accepted as an emerging model organism in ecotoxicological testing in both aquatic and soil environments [107]. Responses to stress and basic physiological processes are relatable to human biology, lending importance to *C. elegans* as a model organism for both human and ecological studies [108]. Additionally, fully described and invariant developmental program, ease of care, short generation times, small and clear bodies, fully mapped genome, and ease of genetic manipulation all make this soil dwelling nematode an ideal organism for exploring CeO_2 toxicity in terrestrial systems [109].

Caenorhabditis elegans is a detritivorous nematode that primarily resides alongside their bacterial food source in decaying plant matter [108, 110]. *Caenorhabditis elegans* grow through

four larval stages (L1-L4) before reaching adulthood approximately three days after hatching from eggs. Molting and a stage of lethargy are indicative of the nematodes progressing to the next developmental stage. When an environment is unfavorable, nematodes at L1 and L2 developmental stages can enter into a dormant dauer stage, which is characterized with a thicker cuticle, closed mouth parts, and little to no movement [111]. Exit from this dauer stage depends on the individual nematode and is encouraged by warmer temperatures and readily available food resources [111]. Upon reaching adulthood, nematodes are self-fertilizing hermaphrodites. Approximately 0.01% of *C. elegans* are facultative males, providing small amounts of genetic variation. Most hermaphrodites are capable of producing 250-300 offspring while sexual reproduction with a male nematode may yield as many as 1,000 offspring. In the laboratory, the Bristol (N2) strain of *C. elegans* are often raised on K-agar seeded with *E. coli* OP-50 strain, a uracil auxotrophic mutant strain [110]. N2 is the reference laboratory strain of *C. elegans*, meaning there are no intentional genetic mutations [109].

The potential negative effects of MNMs on *C. elegans* have been fairly extensively studied. Using Ag NPs, Roh et al. found decreased reproductive rates [112]. Roh also suggested that oxidative stress is the cause of toxicity by observing increased levels of antioxidant enzymes. Additionally, Meyer et al. observed both increased mortality and reduced growth rates when *C. elegans* were exposed to Ag MNMs [113]. On the other hand, Kim et al. showed that Pt MNMs acts as a SOD/catalase mimetic by increasing antioxidant defenses *C. elegans* at 0.5 mM. They quantified O_2^- scavenging by measuring formazan formation in conjunction with a SOD assay kit [114]. The nematodes were then analyzed with fluorescence methods for lipofuscin formation. Wan et al. explored the toxicity of three common metal oxide MNMs: TiO_2, ZnO, and Al_2O_3. They found that all three MNMs negatively affected the nematodes, especially their reproductive capabilities [115]. Tsyusko et

al. explored Au MNM toxicity using toxicogenomic approach and *C. elegans* mutant strains. At 5.9 mg/L (the LC_{10}), Au MNM interference with different biochemical pathways suggested unfolded protein responses and endoplasmic reticulum stress [58]. Studies involving CeO_2 MNMs also observed toxicity to *C. elegans*. Zhang et al. found evidence to demonstrate that CeO_2 MNMs caused detrimental oxidative stress at environmentally relevant concentrations (1 nM, 127 ng/L) [116]. Oxidative stress was measured in terms of resistance to juglone exposure. Juglone induces oxidative stress in *C. elegans* [117]. With the addition of CeO_2 MNMs, the nematodes were more sensitive to juglone's effects, i.e. increased ROS formation. This was measured with lipofuscin formation using fluorescence methods. This study claims to be the first to find adverse MNM-driven effects at realistically low doses to *C. elegans*, but does not account for toxicity induced by the surface coating alone. The CeO_2 MNM tested was coated with hexamethylenetetramine (HMT), which is of unknown toxicity to *C. elegans*. Porta et al. observed no carcinogenesis in rats and mice exposed to 0.5 or 1 % HMT in drinking water for up to 104 weeks [118]. In beagle dogs, Hurni et al. observed no reproductive or teratological effects [119]. More recently, Schrimer et al. also report the LC_{50} of HMT to be 354.8 mM to fathead minnows [120].

1.7 Research objectives

The objective of this research was to explore the relationship between surface coating charge and CeO_2 MNM toxicity to *C. elegans*. By using CeO_2 MNMs with neutral, positively-, or negatively-charged dextran-based surface coatings, we investigated effects on nematode mortality and reproduction. Furthermore, biomarkers of oxidative and nitrosative

stress (protein carbonyls, protein-bound 3-nitrotyrosine, and protein-bound 4-hydroxy-2-nonenal formation) were measured to evaluate oxidative damage to proteins.

Chapter 2: The role of surface chemistry in the toxicity of cerium dioxide manufactured nanomaterials to *Caenorhabditis elegans*

2.1 Introduction

The American Standard Testing Methods (ASTM) defines manufactured nanomaterials (MNM) as materials with at least one dimension in the range of 1-100 nm [1]. As compared to bulk counterparts (>100 nm), MNM can display size dependent properties. A greater surface area-to-volume ratio exposes more atoms on the surface, which can increase MNM interaction with its environment [12, 45]. MNM are incorporated into products such as semiconductors, catalysts, pigments, biomedical applications, textiles, and cosmetics to name a few [2, 5-8]. Because the production and use of MNMs is consistently growing, intentional or accidental environmental release of MNMs is inevitable [5, 9]. The increased potential for exposure to MNMs in humans and other organisms has necessitated gaining an understanding their toxicity [10].

CeO_2 MNMs are valued for their catalytic properties. They are used for such applications as diesel fuel additives, chemical/mechanical polishing agents in glass and semiconductor production, in energy storage devices, and many others [72, 73, 77-79]. CeO_2 MNM also has potential in medical applications as a therapeutic antioxidants [36, 79, 85-87]. During use or disposal of these products, CeO_2 MNMs could enter the environment through wastewater treatment effluent, sewage sludge biosolids or in diesel exhaust [17, 24]. Because CeO_2 MNMs can easily accept or donate electrons, they have been shown to have both prooxidant and antioxidant properties [72, 73]. For example, CeO_2 MNM has been shown to mimic catalase and superoxide dismutase [72, 88], while other studies have found that CeO_2 MNMs can induce oxidative stress in a variety of different species such as

Caenorhabditis elegans, Escherichia coli, *Pseudokirchneriella subcapitata, Daphnia magna, Danio rerio*, Sprague Dawley rats, and Fisher rats [35, 36, 92-94, 121].

In recent years, *C. elegans* has emerged as a model organism for ecotoxicology for many reasons including ease of maintenance in the laboratory, short generation times, and a fully mapped genome with associated functional genomic tools [108-110]. *Caenorhabditis elegans* has been used fairly extensively as a model organism in nanotoxicology, including studies of CeO_2 MNMs [122]. For example, Roh et al. observed decreased reproduction in *C. elegans* exposed to 15 and 45 nm CeO_2 MNMs at 1 mg/L [92]. Zhang et al. reported decreased lifespan in *C. elegans* exposed to 8.5 nm CeO_2 MNMs at environmentally relevant concentrations (1, 5, 10, and 100 nM) [116]. They concluded that the toxicity was caused by oxidative stress. However, these particles were coated with hexamethyleneteramine (HMT) and there were no controls to determine if the coating alone elicited similar toxicity or if free coating molecules existed in the exposure suspensions.

Surface coatings, in the form of small ligands or polymers, are often applied to increase colloidal stability of MNMs [12, 95]. Because the coating is the first component of MNMs to come into contact with an organism, it is vital to understand how coatings affect MNM bioavailability and toxicity [99]. Charge is one of the most important properties of coating molecules. Collin et al. found that the charge of coatings had an extremely important influence on the toxicity where CeO_2 MNMs with positively-charged coatings were two orders of magnitude more toxic than CeO_2 MNMs with neutral or negative coatings [29]. They also found that Ce was reduced from the (+IV) to the (+III) oxidation state *in vivo* by *C. elegans*. The charge of the coatings influenced the degree of reduction. Positively-charged particles were reduced to a lesser extent than neutral or negatively-charged particles [29]. Asati et al. also found that CeO_2 MNM toxicity is influenced by

surface coating charge [104]. Positively-charged CeO_2 MNMs were more toxic to A549 and MCF-7 cancer cell lines than negatively-charged CeO_2 MNMs at a dose of 1.0 mM. The increased toxicity of positively-charged particles is likely explained by the fact that they are electrostatically attracted to biological membranes than negatively-charged particles [101, 102]. Wu et al. showed that positively-charged CeO_2 MNMs are less attracted to similarly charged lysozymes at pH 4.3 than negatively-charged CeO_2 MNMs [103].

The objective of this research was to explore the relationship between surface coating and CeO_2 MNM toxicity to *C. elegans*. We used identical CeO_2 MNM cores with neutral (dextran [DEX]), positively-charged (diethylaminoethyl dextran [DEAE]), or negatively-charged (carboxymethyl dextran [CM]) surface coatings. Endpoints included mortality, reproduction and oxidative and nitrosative damage of proteins. We hypothesized that positively-charged CeO_2 MNMs would be more toxic, as measured by mortality and reproduction, to *C. elegans* than neutral or negatively-charged CeO_2 MNMs. We also hypothesized that exposure to CeO_2 MNMs would induce oxidative and nitrosative stress.

2.2 Materials and methods

CeO_2 MNMs synthesis and characterization

Dextran coated CeO_2 MNMs were synthesized using a modified method developed by Perez et al. [85]. 1.0 M $CeCl_3$ was combined with 1.9212 μM 10 kDa dextran (Sigma Aldrich catalog number 9004-54-0, St. Louis, MO, USA) in a 1:1 volume ratio and left to incubate overnight. The $CeCl_3$/dextran solution was combined with 3 M NH_4OH (2:3 volume ratio) at 323 K with constant stirring for 24 hours. The resulting suspension was transferred to a Teflon vessel and held at 353 K for 24 h to further crystallize DEX-CeO_2

MNMs. To purify the suspension, the MNMs were dialyzed in 18 MΩ deionized water with a 1000 Da cut off membrane for 72 hours changing the dialysis water every 24 hours. Removal of NH_4^+ from solution was verified by phenol hypochlorite assay [123]. Synthesis of CM- and DEAE-CeO_2 MNMs was accomplished by combining 10 mL DEX-CeO_2 MNM solution with 1.2 g of $C_2H_2ClNaO_2$ or $C_6H_{15}Cl_2N$, respectively. These solutions were combined with 2 N NaOH (1:1 volume ratio) and stirred overnight at room temperature. CM-CeO_2 MNMs and DEAE-CeO_2 MNMs were dialyzed using the same method as the DEX-CeO_2 MNMs.

CeO_2 MNMs were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), and phase analysis light scattering (PALS). Further characterization of the functionalization of the coatings was performed using Fourier transform infrared spectroscopy (FTIR). The FTIR results are reported by Collin et al. [29]. TEM was performed using a JOEL 2010 F microscope (Tokyo, Japan). To prepare grids for TEM analysis, a drop of CeO_2 MNM suspension was placed on glassine paper and spread into a thin layer. A lacey carbon copper coated Cu grid (400 mesh, Ted Pella, Inc., Redding, CA) was placed on the thinnest part of the solution and allowed to dry overnight before imaging. Both DLS, to determine hydrodynamic diameter, and PALS, to determine ζ potential, were completed using a Malvern zetasizer (NanoZS 90, Malvern, United Kingdom). ζ potential and hydrodynamic diameter were determined in *C. elegans* exposure media (moderately hard reconstituted water, MHRW: 96 mg $NaHCO_3$, 60 mg $CaSO_4$, 60 mg $MgSO_4$, 4.0 mg KCL per 1000 L DI H_2O [124]) at pH 7.4. The Hückel approximation was used to calculate ζ potential from electrophoretic mobility of the particles.

Ce concentrations in the CeO_2 MNM suspensions were analyzed with inductively couple plasma mass spectrometry (ICP-MS) (Agilent 7500cx, Santa Clara, CA). 50 μL of

CeO_2 MNM suspensions were dissolved in 6 mL trace metal grade HNO_3 and 3 mL H_2O_2 in Teflon vessels using a MARS Xpress microwave digestion system (CEM, Matthews, NC) following US EPA methods 3015 [125].

Mortality

Caenorhabditis elegans (Wild type, N2 Bristol Strain) were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). Nematode stocks and age synchronization were performed with previously established methods [1, 126].

Age synchronized *C. elegans* were exposed at either L3 or L2 larval stages for 24 or 48 hours, respectively, in MHRW. For each experiment, 9-11 *C. elegans* were exposed in 1 mL of CeO_2 MNM suspension for 48 or 24 h in a polycarbonate twenty-four well plate with three replicates per dose. Nematodes exposed from the L3 stage were kept in CeO_2 MNM suspensions for 24 hours either with or without 10 μL/mL *E. coli* (OP-50 strain). The inclusion of food can detract from stress the nematodes may encounter with CeO_2 MNM exposure. To establish safe doses for sublethal reproductive studies, nematodes were exposed from L2 stage in CeO_2 MNM suspensions for 48 hours and fed 10 μL/mL OP-50. The food was included to prevent *C. elegans* from entering into dauer stages. CeO_2 MNM suspensions were changed and the worms given an additional 10 μL/mL OP-50 every 24 hours. At the end of the exposure periods, *C. elegans* were determined dead if unresponsive to a gentle prodding [127]. Dose-response curves were generated from the collected data.

Reproduction

To observe CeO_2 MNM effects on nematode reproduction, L1 nematodes were exposed to 3 mL CeO_2 MNMs in MHRW in 6 cm polystyrene petri dishes with 10 μL *E. coli*

OP-50/mL exposure suspensions. The nematodes were allowed to hatch on K agar petri dishes seeded with OP-50 and were approximately 12 hours old when placed in CeO_2 MNM suspensions. Exposure suspensions were changed at 24 hours and an additional 10 μL/mL *E. coli* food was provided. Upon completion of the exposure period, one nematode was placed on a 6 cm K-agar filled polystyrene petri dish seeded with an *E. coli* lawn. The nematode was allowed to lay eggs for 48 hours and then moved to a new petri dish with K-agar. This was repeated for a total of three petri dishes. After moving the adult nematode, the newly hatched nematodes were stained with 0.5 mg/L rose Bengal stain (Acros Organics, New Jersey, USA) in DI water, heat killed for one hour at 50°C, and scored. Nematodes were counted as viable offspring if they were fully emerged from the egg cuticle. Each CeO_2 MNM dose included five to six replicates. Dose-response curves were generated from the collected data.

Oxidative and nitrosative damage of proteins

L1 *C. elegans* were exposed to 6 mL CeO_2 MNMs with 10 μL/mL OP-50 in 15 mL polypropylene conical centrifuge tubes (Corning Life Sciences, Tewksbury, MA) for 48 hours. We exposed the nematodes to 500 mg/L DEX-CeO_2 MNMs, 750 mg/L CM-CeO_2 MNMs, and 3.25 mg/L DEAE-CeO_2 MNMs, which corresponded to the EC_{30} values for reproduction for each particle type. CeO_2 MNM suspensions were changed and 20 μL/mL *E. coli* added after 24 hours. For an additional 24 hours, nematodes were allowed to grow on a 10 cm K agar filled vented polystyrene petri dish seeded with an *E. coli* lawn. Nematodes were then washed off the petri dish with K-Media (2.36 mg KCl and 3 mg NaCl/1000 mL DI H_2O). The nematodes were washed twice more with K-media to remove *E. coli* and suspended in 0.5 mL sucrose isolation buffer (0.32 M sucrose, 2 mM EDTA, 2 mM EGTA,

20 mM HEPES) with a protease inhibitor cocktail (104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leipeptin, 1.5 mM pepstatin A). The nematode pellet was sonicated in an ice bath with a Misonix Ultrasonic Liquid Processor (Misonix, Inc., New York, USA) using the microtip for 20 seconds at 30% amplitude, with a 10 second pause after the first 10 seconds to avoid excessive heating. Protein concentration was determined using a BCA Protein Assay Reagent kit (Pierce Protein Biology Products, Rockford, IL). Extracted proteins were stored at -80°C before analysis.

The nematode protein samples were first derivatized to determine levels of protein carbonyls (PC) [128]. This step conjugates a 2,4-nitrophenol group to the protein carbonyl to form the hydrazine, which is then recognized subsequently by an antibody. Protein solutions (5 μL) were combined with 5 μL of 12% sodium dodecyl sulfate (SDS) and vortexed. 10 μL of 2,4-dinitrophenylhydrazine (DNPH) solution were added and the solution allowed to incubate at room temperature for 20 min. Neutralization solution (7.5 μL)(EMD Millipore, Billerica, MA) was added, and the solution was vortexed. Lastly, samples were diluted with 72.5 μL phosphate buffered saline (PBS) to a final concentration of 250 ng protein /mL.

Proteins set aside for 3-nitrotyrosine (3-NT) and 4-hydroxy-2-nonenal (HNE) slot blots were not derivatized since primary antibodies can bind directly to protein resident 3-NT or protein-bound HNE. Samples were prepared as follows: 5 μL of protein solution, 5 μL of 12% SDS, and 10 μL of Lamelli buffer (0.125 M Trizma (pH 6.8), 4% SDS, and 20% glycerol) were vortexed and allowed to rest at room temperature for 20 minutes. The solution was then diluted with 80 μL PBS to a final volume of 250 ng/mL.

Slot blot analysis was then performed with a Bio-Dot SF apparatus (Bio-Rad Laboratories, Eugene, OR). The apparatus contained three filter papers and one nitrocellulose membrane (0.2 μL, Bio-Rad) that were previously soaked in PBS. 250 μL PBS

were washed through the membrane. Then 250 μL of sample in three replicates each were placed in the slots and allowed to incubate for five minutes. After washing the samples through the membrane, a final wash of 250 μL PBS was performed to elute unbound protein. The nitrocellulose membrane was placed in blocking solution (3% w/v bovine serum albumin in 20 mL washblot (0.2% (w/v)) Tween 20 and 0.01% (w/v) sodium azide dissolved in PBS stored at room temperature) was rocked gently overnight at 5°C.

The following day, the membranes were developed with the following primary antibodies: Rb x dinitrophenol (DNP) for protein carbonyls (EMD Millipore, Billerica, MA), anti-nitrotyrosine for 3-NT (Sigma Aldrich, St. Louis. MO), or anti-4-hydroxy-2-nonenol polyclonal antibody for HNE (Alpha Diagnostics International, San Antonio, TX). 2.5 μL primary antibodies were added directly to the 20 mL blocking solution and rocked gently for two hours. Following three washes with washblot to remove excess antibody, 2.5 μL of anti-rabbit IgG alkaline phosphatase produced in goat was added to 20 mL washblot. This solution was rocked gently over the membrane for one hour. Upon completion, the membrane was washed thrice with washblot. Lastly, the membrane was developed for colorimetric analysis with 30 mL alkaline phosphatase, 99 μL 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 198 μL nitro blue tetrazolium (NBT). The membrane was allowed to dry overnight. Membranes were scanned into a TIFF data file form using a ChemiDoc MP System with ImageLab software (Bio-Rad).

Statistical analyses

For the mortality data, we calculated the percent mortality and normalized the data to controls. Two-tailed Bonferonni corrected student's T-tests compared each treatment and dose to controls. Lastly, significance was evaluated at α=0.05 and α=0.01 levels.

Reproduction was quantified by counting the total offspring per nematode. The data were then analyzed using the same statistical methods at mortality. Normalized data from the immunochemical slot blots were analyzed with analysis of variance (ANOVA), followed by Dunnett's post hoc comparisons between treatments and control when ANOVA was significant at α=0.05. For all statistical tests the normality and homoscedasticity assumptions were tested using Shapiro-Wilk's and Bartlett's tests respectively. For the slot-blot analyses, the technical replicates (individual blots) were averaged for each biological replicate and therefore the n corresponded to the number of biological replicates, which were independent exposures.

2.3 Results

CeO_2 MNM characterization

TEM analysis showed the diameter of the DEX-CeO_2 MNMs to be 3.99 nm ±0.71 (mean ± standard deviation), CM-CeO_2 MNM 3.36 nm ± 0.80, and DEAE-CeO_2 MNM 3.88 nm ± 0.90 (**Fig 2.1***)*. The Z-average (intensity weighted) hydrodynamic diameters, as measured by DLS analysis were15.69, 8.72 and 13.54 nm for DEX-CeO_2 MNM, CM-CeO_2 MNM and DEAE-CeO_2 MNM. The mean ζ potentials were 2.27 mV, -22.2 mV, and +27.0 mV for DEX-CeO_2 MNM, CM-CeO_2 MNM and DEAE-CeO_2 MNM respectively.

C. elegans mortality

When exposing L3 *C. elegans* to CeO_2 MNM without food, no surface coating treatment induced greater than 50% mortality. The control mortality rate was 3.2 ± 4.7 %.

The highest mortality rate observed for CM-CeO_2 MNM was 16.4 ± 8.1 % at a concentration of 1000 mg Ce/L. A concentration that induced comparable mortality in

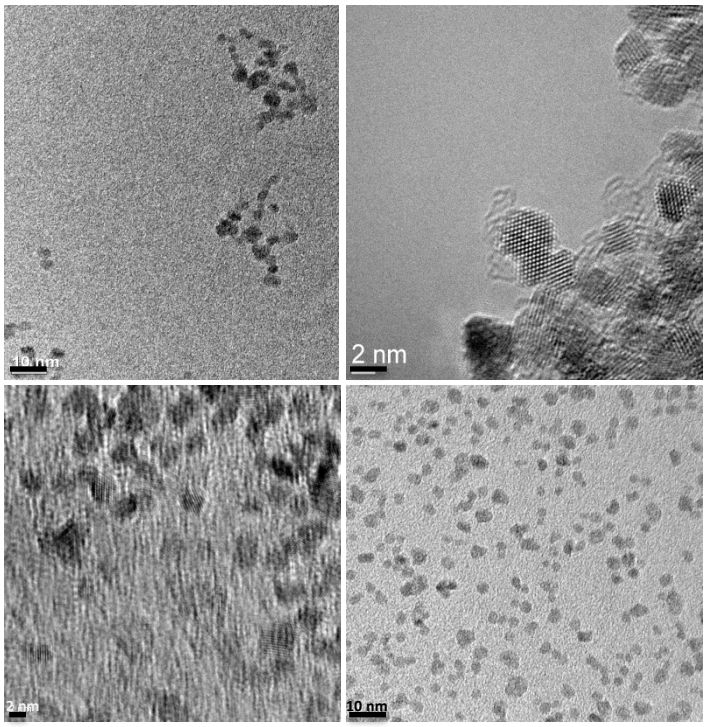

Figure 2.1. Transmission electron microscopy images of synthesized CeO_2 MNMs. Clockwise from top left: dextran (DEX), dextran (DEX) [higher resolution], diethylaminoethyl-dextran (DEAE), carboxymethyl-dextran (CM) coated CeO_2 manufactured nanomaterials.

DEAE-CeO_2 MNM (16.3 ± 6.1%) was 100 mg/L, which was a full order of magnitude lower. The mortality decreased steadily as the dose increased to 5000 mg/L DEX-CeO_2 MNMs. Mortality in DEX-CeO_2 MNMs exposures were significantly different from control (p<0.05) at 10, 100, and 2500 mg/L. Mortality in response to CM-CeO_2 MNMs exposure was significantly greater than control at 100 and 1000 mg/L (p<0.05). All but one concentration of DEAE-CeO_2 MNMs showed a significantly greater mortality than control (p<0.05). (**Fig 2.2***)*

In L3 exposures with feeding, CM-CeO_2 MNM did not induce any significant mortality below 5000 mg/L, where it was 14 ± 7%. Similar mortality was observed for DEAE-CeO_2 MNMs and DEX-CeO_2 MNMs at 10-fold lower concentration of 500 mg/L with mortality of 16 ± 10% and 10 ± 1%, respectively. As concentration increased, DEAE-CeO_2 MNMs elicited greater mortality than DEX-CeO_2 MNMs. Ultimately, DEAE-CeO_2 MNMs induced 80 ± 10% mortality at 5000 mg/L compared to DEX-CeO_2 MNM's 25 ± 6% and 14.1 ± 7.1% for CM-CeO_2 MNM's at the same concentration. At all tested concentrations of DEX-CeO_2 MNMs and DEAE-CeO_2 MNMs and the highest CM-CeO_2 MNM concentration mortality was significantly greater than in control (0 ± 0%) (p<0.05). Additionally, mortality in all DEX-CeO_2 MNM concentrations and the highest concentration for CM-CeO_2 MNM and DEAE-CeO_2 MNM were significantly greater than control (p<0.01) (**Fig 2.3**).

When the L2 nematodes were exposed for 48 hours with food, CeO_2 MNM induced higher mortality then in the L3 exposures. CM-CeO_2 MNM cause 10 ± 6% mortality at 750 mg/L. DEX-CeO_2 MNM induced a similar mortality (8 ± 7%) at 300 mg/L. DEAE-CeO_2 MNM, however, vastly increased in toxicity. At 50 mg/L, 45 ± 19 % mortality was induced. All DEAE-CeO_2 MNM concentrations were significantly greater than control (p<0.01).

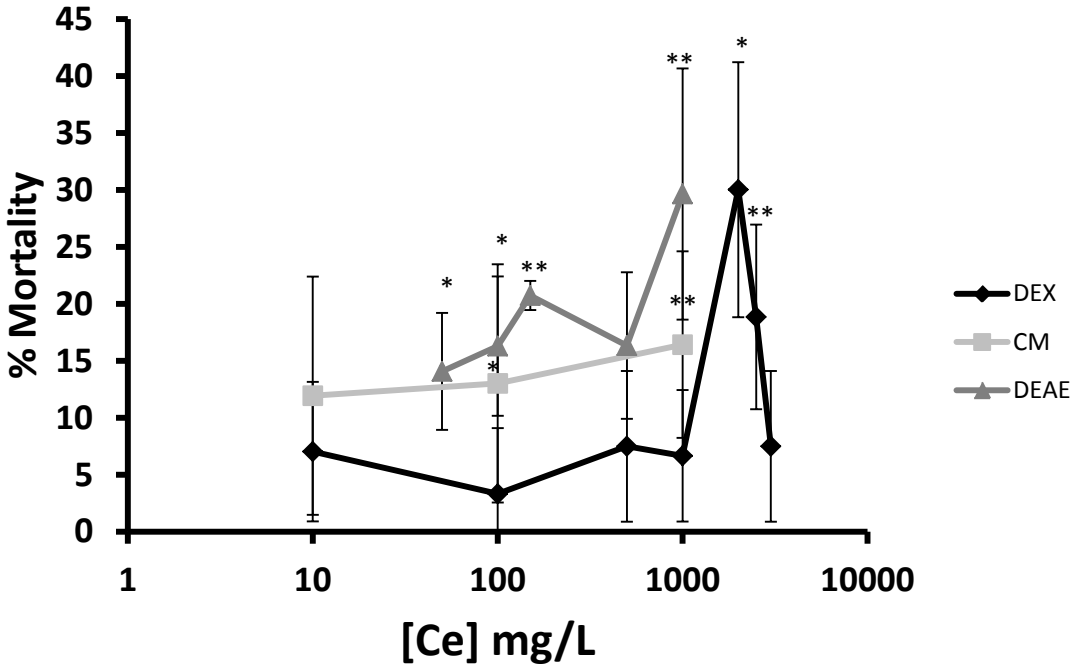

Figure 2.2. Mortality in L3 *Caenorhabditis elegans* exposed to dextran (DEX), carboxymethyl-dextran (CM), or diethylaminoethyl-dextran (DEAE) coated CeO_2 manufactured nanomaterials without feeding. Asterisks indicate that mortality is significantly greater than controls (*p<0.05, **p<0.01, error bars indicate standard deviation).

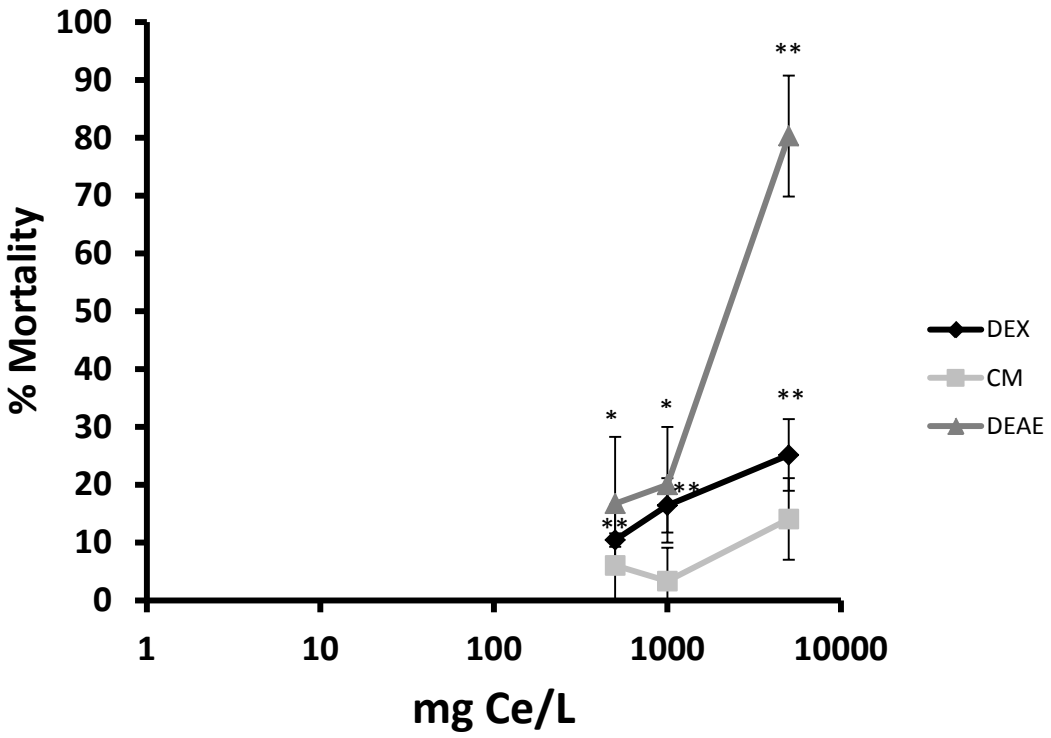

Figure 2.3. 24 Hour mortality in L3 *Caenorhabditis elegans* exposed to dextran (DEX), carboxymethyl-dextran (CM), or diethylaminoethyl-dextran (DEAE) coated CeO_2 manufactured nanomaterials with feeding. Asterisks indicate that mortality is significantly greater than controls (*p<0.05, **p<0.01, error bars from standard deviation).

Mortality at 750 mg/L CM-CeO_2 MNM = also significantly greater than control = mortality (9.8 ± 6.3%) (p<0.01). Lastly DEX-CeO_2 MNM 10, 100, 1000, 2500, and 5000 mg/L doses were significantly different from controls (1.2 ± 3.2%) (p<0.05) (**Fig 2.4***).*

Reproduction

The concentrations used for studying sublethal effects of CeO_2 MNM exposure were derived from the 48 hour mortality experiments in L2 larvae with feeding. We found that DEAE-CeO_2 MNMs caused decreases in reproduction at the lowest concentrations. The control treatment nematodes produced 168 ± 32 offspring, while nematodes exposed to 600, 700 and 1000 mg/L DEX-CeO_2 MNMs produced 82 ± 33, 82 ± 26, and 90 ± 64 offspring, respectively. Reproduction at all three concentrations were significantly lower than controls (p<0.05). While reproduction was significantly lower than control only at higher concentrations (p<0.05), a concentration response relationship is still evident (**Fig 2.5**). DEAE-CeO_2 MNMs and CM-CeO_2 MNMs were only significantly lower than controls at 3.0 mg/L (DEAE-CeO_2 MNMs) and 1000 mg/L (CM-CeO_2 MNMs) (p<0.05) (87.09 ± 49.46 and 86.67 ± 77.13, respectively) (**Fig 2.5**). As with the 48 hour mortality exposures, positively-charged DEAE-CeO_2 MNMs required much lower concentrations than the other treatments to take effect.

Immunochemical slot blots

The concentrations used for slot blots were the estimated EC_{30} values for reproduction studies: 500 mg/L DEX-CeO_2 MNMs, 750 mg/L CM-CeO_2 MNMs, and 3.25 mg/L DEAE-CeO_2 MNMs. Protein carbonyl (PC) and 3-nitrotyrosine (3-NT) levels were not significantly greater than controls for all three treatments; however, mean PC levels were

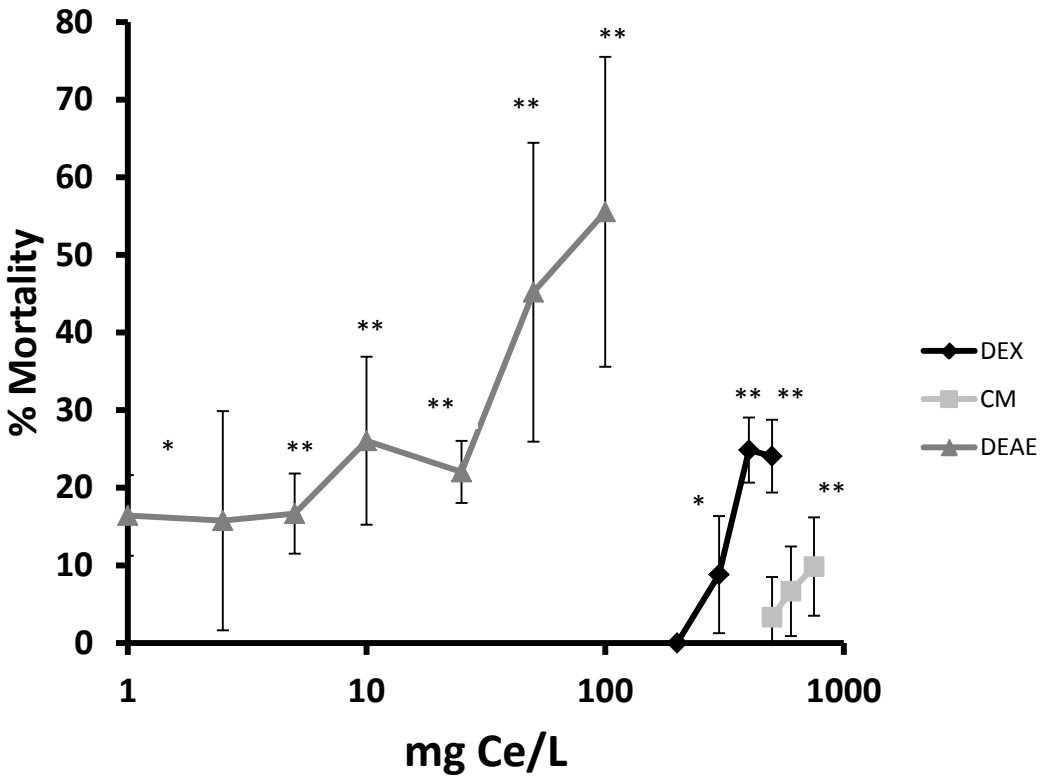

Figure 2.4. Mortality in juvenile C. elegans (L2) after 48 hr exposure to dextran (DEX), carboxymethyl-dextran (CM), or diethylaminoethyl-dextran (DEAE) coated CeO_2 manufactured nanomaterials with feeding. Asterisks indicate that mortality is significantly greater than controls (*p<0.05, **p<0.01, error bars from standard deviation).

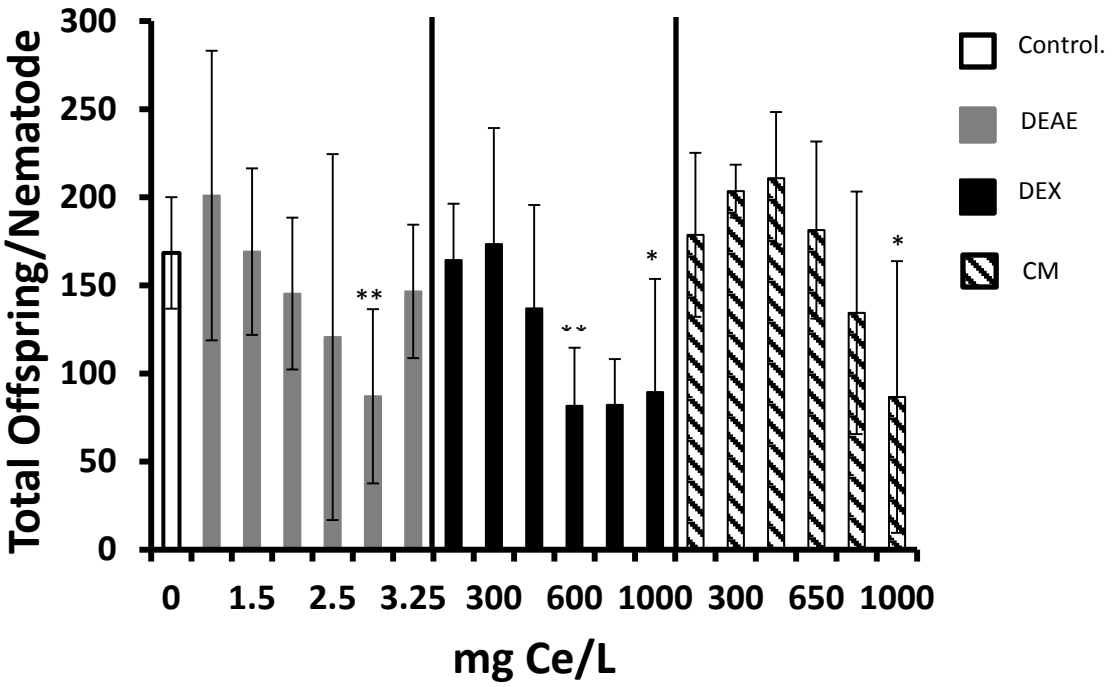

Figure 2.5. *Caenorhabditis elegans* reproduction based on total offspring per nematode exposed to dextran (DEX), carboxymethyl-dextran (CM), or diethylaminoethyl-dextran (DEAE) coated CeO_2 manufactured nanomaterials. Asterisks indicate that reproduction is significantly different from controls (*$p<0.05$, **$p<0.01$, error bars from standard deviation).

consistently higher than controls for all CeO_2 MNM treatments, although not significantly different (F=1.68 , p>0.2247). DEX-CeO_2 MNMs and CM-CeO_2 MNMs had no significant effect on 4-hydroxy-2-nonenol (HNE) levels (F=3.46, p>0.0512). There was a significant decrease in HNE levels for the DEAE-CeO_2 MNMs treatment as compared to controls as determined by Dunnett's test (p<0.05). (**Fig 2.6**)

2.4 Discussion

As outlined below, these results indicate that the surface coating of CeO_2 MNMs has a profound impact on their toxicity. Importantly, exposure to the surface coating materials used in this study alone does not explain the observed toxicity, as we demonstrated in a previous study [29]. But when present on the surface of CeO_2 MNMs, these compounds have a profound influence on CeO_2 MNMs's toxicity to *C. elegans*.

Increases in mortality occurred for DEX-, CM-, and DEAE-CeO_2 MNMs; however, this tended to occur at far lower concentrations for DEAE-CeO_2 MNMs than for DEX- or CM-CeO_2 MNMs, particularly in the experiments where L2 and L3 nematodes were exposed with feeding for 48 hr and 24 hr, respectively. L3 nematodes exposed without food generally experienced greater mortality for all particle types than those with food. This could be related to greater physiological vigor in the fed nematodes helping them resist CeO_2 MNM toxicity or it could relate to changes in the surface chemistry or bioavailability of the particles. If bound to the microbial cells, the bioavailability of the particles may have decreased versus water only exposures. Nematode absorption could have occurred either through the cuticle or by absorption from the gut [29]. The L2 nematodes experienced greater mortality than L3 nematodes. Both nematode's developmental stage and length of

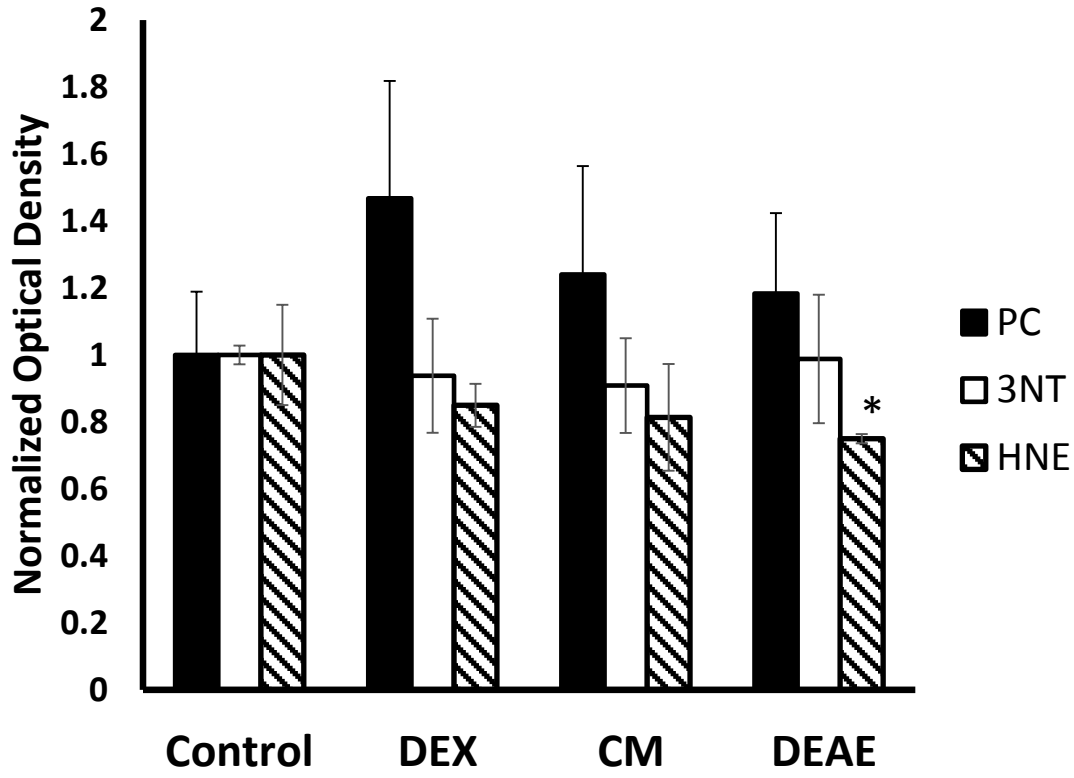

Figure 2.6. Immunochemical slot blots with *Caenorhabditis elegans* proteins probing for oxidative/nitrosative stress markers protein carbonyls (PC), 3-nitrotyrosine (3-NT), and 4-hydroxy-2-nonenol (HNE) after exposure to dextran (DEX), carboxymethyl-dextran (CM), or diethylaminoethyl-dextran (DEAE) coated CeO_2 manufactured nanomaterials. Asterisks indicate that reproduction is significantly different from controls (*p<0.05, error bars indicate standard deviations).

exposure could influence the increase in mortality rates in L2 nematodes, since they were exposed for 48 hours as opposed to 24 hours for the L3 nematodes.

All three CeO_2 MNM treatments affected *C. elegans* fecundity, but as with mortality DEAE-CeO_2 MNMs caused effects at more than 100 fold lower concentrations than the CM- or DEX-CeO_2 MNMs. However, the lowest observed effect concentration for the DEAE-CeO_2 MNMs was 3 mg/L, which is far greater than the concentrations (1 nM or 172 ng Ce/L) at which Zhang et al. observed decreased in lifespan of *C. elegans* [116]. Zhang did not control for toxicity of the molecule used to coat their particles, which was HMT. It is possible that either this coating molecule in the dissolved state is toxic or that the HMT coating greatly increases the toxicity of the CeO_2 MNMs. The exposure duration in their experiment was also longer and they looked at nematode lifespan, which is a different endpoint. Zhang exposed nematodes until death (up to 25 days), where as we exposed the nematodes for a set time period (2 days maximum). The HMT coated particles in this experiment were also positively-charged which may have contributed to their great toxicity as evidenced by our data.

Immunochemical slot blots were performed on proteins extracted from nematodes exposed to concentrations of CeO_2 MNMs that provided the same sublethal response rather than the same concentration (the EC_{30} for reproduction). DEAE-CeO_2 MNMs appeared to decrease the HNE concentrations *C. elegans*, suggesting that lipid peroxidation diminished. Additionally, the data suggested a trend that all three treatments produce greater concentrations of protein carbonyls as compared to controls; however, these were not significantly different from control at $\alpha = 0.05$. With more replicates, it is possible a significant difference could be realized. Given the difficulties in obtaining sufficient quantities of protein for this assay, we were limited to only 4 independent biological

replicates. Given previous results that show increases in protein carbonyls in rats exposed to CeO_2 MNMs [121], further investigation into oxidative stress as a possible contributor to the mode of action is warranted.

There are several explanations for the increased toxicity of positively-charged CeO_2 MNMs, but perhaps the most likely is that the particle uptake is greater for positively-charged particles. Collin et al. found that the coating of CeO_2 MNMs greatly influences bioaccumulation in *C. elegans* [29]. Positively-charged MNMs bioaccumulated in the nematode's tissue to a far greater extent than negative or neutral MNMs. Similarly, Asati et al. explored charged CeO_2 MNM internalization within normal and cancerous human cell lines. Positively-charged CeO_2 MNMs were generally taken up into the cells more so than negative or neutral CeO_2 MNMs [104]. Other explanations involve differences in the subcellular distribution of the particles. Our results add to this trend: positively-charged DEAE-CeO_2 MNM induced more toxicity to *C. elegans* in both the lethal and sublethal endpoints, sometimes with doses differing by one or more orders magnitude for the same effect; however, we found no differences in biomarkers for oxidative or nitrosative stress at the EC_{30} for each particle type. There was a distinct trend towards increased protein carbonyl content from all three treatments as well as an exception of a significant decrease in HNE only caused by the positively-charged DEAE CeO_2 MNMs.

Similar surface chemistry dependent effects have been seen on other MNMs as well [96]. Goodman et al. showed that 2 nm Au MNM coated with anionic side chains required an exposure concentration two to three times more concentrated than the same MNM with a cationic coating to see the similar responses from *E. coli* [101]. El Badawy et al. observed that toxicity of Ag MNMs with hydrodynamic diameters ranging between 10 and 18 nm depended on the charge of their coatings to impart toxicity to a Gram-positive *Bacillus*

species, measured with mortality and respiration [129]. Positively-charged Ag MNMs were the most toxic, negatively-charged Ag MNM the least toxic, and neutral Ag MNM were in between. Hoshino et al. synthesized ZnO coated CdSe 3.5 nm quantum dots. When they functionalized either a positively- or a negatively-charged group to the ZnO coating, they observed that positively-charged MNM were more toxic than negatively-charged. Interestingly, they noted that uncoated MNMs were not toxic to WTK-1 cells at concentrations as high as 400 mg/L [130].

In conclusion, we observed that the toxicity of CeO_2 MNM is highly dependent on the molecular charge of the surface coating. While all three treatments increased *C. elegans* mortality rates and lowered their reproduction rates, positively-charged DEAE-CeO_2 MNM affected the nematodes at much lower doses than negatively-charged CM-CeO_2 MNM or neutral DEX-CeO_2 MNM. While not statistically significant as compared to controls, there was a noticeable trend towards increased protein carbonyl content with all three CeO_2 MNM treatments. In future studies, we plan to further explore the role of oxidative stress in the mechanism of toxicity given that these results provided some indication that protein carbonyls may be elevated. Optimization of *C. elegans* exposures to provide greater replication for the slot-blot measurements will be necessary.

Chapter 3: Conclusions

The aims of this research were to better understand the role of CeO_2 MNM coating charge on toxicity to model soil organism *Caenorhabditis elegans*. By observing mortality, reproduction, and formation of biochemical oxidative stress indicators, we were able to add to the understanding of the properties of CeO_2 MNMs that contribute to toxicity. We hypothesized that positively-charged CeO_2 MNMs would more toxic to *C. elegans* and that CeO_2 MNM exposure will induce oxidative stress. To test these hypotheses, we first synthesized CeO_2 MNMs with either positively-, negatively-charged, or neutral dextran-based coatings. Toxicity was tested with both lethal and sublethal endpoints with exposure to a wide range of concentrations. We probed for oxidative stress indicators with a well-established oxidative stress assay. Overall we confirmed that coating charge plays a very important role in determining the toxicity of these materials. Furthermore, we also developed a set of model nanomaterials that have identical cores with polymer coatings that differ only in the nature of the functional groups present and the molecular charge. These materials were used in other concurrent studies [29] and will be a resource for future investigations on the role of coating charge on CeO_2 MNM toxicity.

When MNM are manufactured, a surface coating is frequently employed to increase stability and decrease aggregation. Many of the studies previously published did not include surface coating as an experimental variable. But because the surface coating is the first part of MNMs to come into contact with biological membranes, it is vital to explore this aspect of MNM use and their toxicity [99]. Specifically of interest are coatings with different molecular charges. Previous research supports that MNMs with positively-charged coatings are more readily attracted to most biological membranes as compared to neutral or

negatively-charged MNMs [101, 102]. Furthermore, Wang et. al demonstrated that positively-charged CeO_2 MNM were repelled from positively-charged lysozymes [103]. Asati et. al also demonstrated that CeO_2 MNM surface charge is an important factor. By studying neutral, positively-, or negatively-charged CeO_2 MNM, they found that positively-charged and neutral CeO_2 MNMs internalized into the normal human cell lines studied. Negatively-charged CeO_2 MNMs did not interact with normal cells as much, but did internalize more into cancerous cells. Once inside, CeO_2 MNMs still relied on surface charge to impart toxic effects [104]. Our research lends strength to existing literature by showing that surface coating effects is a vital aspect of MNM synthesis to consider. On their own DEX, DEAE, and CM are not toxic to *C. elegans* [29]. But when bound to the CeO_2 MNM surface, they do influence the MNM's toxicity: positive DEAE-CeO_2 MNMs generated toxic effects at dramatically lower doses than both DEX-CeO_2 MNMs and CM-CeO_2 MNMs.

The differences between CeO_2 MNM's behavior observed in this research and previously published studies in *C. elegans* could be due to different sizes of MNM tested, exposure methods, or surface chemistry of the particles themselves. Arnold et al. published that CeO_2 MNMs were more toxic than bulk CeO_2 (>100 nm) [32]. Roh et al. studied the toxicity of 15 nm and 45 nm CeO_2 MNMs to *C. elegans*; the smaller particles were more toxic than the larger ones [92]. To provide context, our CeO_2 MNMs averaged 4 nm in diameter. DEAE-CeO_2 MNMs did induce similar toxic responses to previously published studies, but DEX- and CM-CeO_2 MNMs required far greater concentrations than previously studied particles. This suggests that the DEX and CM coatings greatly reduce the toxicity of CeO_2 MNMs; however, they did not appear to have antioxidant properties as measured HNE, 3-NT or PC at the concentrations tested. While we used established aquatic exposures [126], other researchers may have utilized other methods. Zhang et al. embedded CeO_2 MNMs

into solid nematode growth medium [116]. In comparison with liquid exposure, this could either limit the bioavailability of CeO_2 MNMs or even lead to agar-mediated surface modifications. Interestingly, both our research and Zhang's work show decreased fecundity in *C. elegans*; Zhang reported dramatically lower doses to see a significant effect. Bearing these differences in mind, our research adds to the general consensus that CeO_2 MNMs can impart toxicity and that the surface coating, particularly a positively-charged coating, is an incredibly important factor to consider.

The existing literature suggests that CeO_2 MNMs has potential to be both a prooxidant and an antioxidant [36, 87, 90, 116, 131]. At low doses, CeO_2 MNMs can act as an antioxidant [121, 132]. It can mimic catalase and superoxide dismutase [72, 88]. But at higher doses, CeO_2 MNMs instead could be a prooxidant [116]. In our study, exposure to positively-charged DEAE-CeO_2 MNMs decreased 4-HNE formation at 3.25 mg/L. However, a significant decrease in reproduction was observed at this concentration, so there was a net adverse effect on the nematodes. Furthermore there were also large, but not statistically significant increases in protein carbonyl levels. Regardless, if CeO_2 MNMs do not induce oxidative stress, they must impart toxicity through a different mechanism. We know from previous research that DEX, DEAE, and CM coatings by themselves are not toxic [29]. Several mechanisms have been described by which MNMs may cause toxicity independent of ROS. For example, Tsyusko et al. published evidence that Au MNMs causes endoplasmic reticulum stress and protein misfolding [58]. Another possibility is that our CeO_2 MNMs did not internalize into cells in exactly the same fashion as in previous research. Asati et al. report that the surface chemistry of CeO_2 MNMs dictate not only how much is internalized into the cell, but the final location as well. If CeO_2 MNMs are localized

in an acidic microenvironment, such as inside a lysosome, they are more likely to behave as a prooxidant [104].

In future research, we plan to probe further into *C. elegans* biochemical responses to CeO_2 MNM exposure. By identifying which proteins are modified either in expression or oxidative damage, we can outline CeO_2 MNM's mode of action more accurately and better understand where the MNMs localize within inside the nematode's cells. Additionally, we will explore other MNM surface modifications such as lower or higher molecular weight coatings. By using longer or shorter dextran molecules when synthesizing CeO_2 MNMs, observing physical changes in coating molecular weight and conformation rather than charge could lead to a deeper understanding of the role of surface coatings in CeO_2 MNM toxicity.

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VITA

Emily Oostveen was born in Houston, TX. She acquired a Certified Pharmacy Technician License from the National Pharmacy Technician Certification Board in 2007. In 2011, she received a Bachelor of Science in Biochemistry from Texas Tech University. While studying at Texas Tech University, Ms. Oostveen was accepted as a Research Fellow for Howard Hughes Medical Institute, where she researched nanotoxicology under Dr. Jaclyn Cañas-Carrell. In 2011, she was awarded a Research Fellowship from University of Kentucky's College of Agriculture, where she continued to pursue nanotoxicology with guidance from Dr. Jason Unrine. She was also awarded a Kentucky Opportunity Fellowship in 2012.

Ms. Oostveen's publications include:

Whitley, A.R.L., C.; Oostveen, E.; Bertsch, P. M.; Matocha, C. J.; von der Kammerd, F.; Unrine, J. M., *Behavior of Ag nanoparticles in soil: Effects of particle surface coating, aging andssewage sludge amendment.* Environmental Pollution, 2013. **182**: p. 141-149.

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