ANTI-INFLAMMATORY PROPERTIES OF CERIUM OXIDE NANOPARTICLES ENCAPSULATED WITHIN POLY (E-CAPROLACTONE) (PCL) ELECTROSPUN FIBERS

by

UMMAY MOWSHOME JAHAN

(Under the Direction of Vladimir Reukov)

ABSTRACT

Cerium oxide nanoparticles (CeO_2NPs) have the unique property of scavenging reactive oxygen species (ROS), expressed in elevated levels in numerous pathologies. Findings about cytotoxicity of CeO_2NPs in the existing literature are contradictory. Many studies found CeO_2NPs exhibit low systemic toxicity, whereas other findings suggest that CeO_2NPs's exposure to cells elevates ROS levels under certain conditions leads to cell apoptosis. Also, the phagocytosis of CeO_2NPs by cells can result in substantial cytotoxicity. However, CeO_2NPs encapsulated with biocompatible polymers shows improved antioxidant and antimicrobial properties with reduced cytotoxicity, which can be utilized in various drug delivery and tissue engineering applications. This study aims to assess the cytotoxic effect and evaluate the anti-inflammatory properties of CeO_2NPs delivered directly and in poly(ε-caprolactone)- CeO_2NPs encapsulated state.

INDEX WORDS: Cerium Oxide, Nanoceria, Oxidative Stress, Encapsulation, PCL, ROS.

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Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
August 2021

DEDICATION

To,

My Father, A.K.M. Shahjahan Sharker

Abbu, you are not a superhero, but you own more superpowers than any fictional superhero.

ACKNOWLEDGEMENTS

I would like to express my sincere and heartfelt gratitude to my advisor, Dr. Vladimir Reukov, to whom I am thankful for many things, especially for giving me the opportunity to research under his supervision. Dr. Reukov inspired me to develop this research topic, gave me unwavering support and guidance during my research process, and criticized me constructively to achieve the best outcome possible. His strong mentorship helped me improve my academic knowledge, research ability, and professional skills needed for my future academic career.

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of the University of Georgia for their help and kindness. Special thanks to Md Mazbah Uddin for helping with my graduate studies and research works for the past two years.

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In my bleakest time, I found you by serendipity. You became my magic shop; believing in your galaxy comforted me, brought me out from the darkness, awakened the best in me, gave me strength to face reality. I purple you, BTS.; you are indeed the cause of my euphoria.

I am thankful to my mother, Monoara Jahan, for always believing the best in me and giving me the greatest support; my father, A.K.M. Shahjahan Sharker, for making my every dream possible and creating the strong person I am now; my sister Munmun Jahan, for having the utmost dreams for me and looking after me all the time; my youngest sister Ferdous Jahan for loving me and entertaining me continuously; my best friend and my other half Nasif Mahmood for holding my hand for the past ten years. The unconditional love, support, and dedication from all of you are only one thing that kept me going.

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CHAPTER 1

INTRODUCTION

Cerium is the first element in the lanthanide/rare-earth group, which can exist in both 3+ and 4+ states [1], producing a responsible redox couple catalytic activity [2]. Therefore, the cerium oxides can be found as CeO_2 and Ce_2O_3 in the bulk state [3]. However, while cerium combines with oxygen to form nanoparticles, cerium oxide produces a mixture of cerium in the 3+ and 4+ states on the nanoparticles' surface. The number of 3+ sites on the surface increases if the nanoparticle's diameter decreases and oxygen vacancies occur [4]. As the surface area-to-volume ratio increases in the nanoparticles, the cerium oxide nanoparticle has a higher concentration of Ce3+ and greater redox activity concerning larger particles [5].

As the CeO_2NPs has exceptional redox property, high oxygen storage capacity, and UV absorbing ability [6], [7], [8] it has numerous applications in engineering and biological sectors, including high-temperature oxidation protection materials [9], solar cells [10], solid oxide fuel cells [11], gas sensors [12], UV screens [13], catalytic materials [14], and potential pharmacological agents [15].

Although CeO_2NPs has been used in different industrial fields for more than a decade, the biomedical prospects of CeO_2NPs mainly started from 2005, after some research presented CeO_2NPs as a potential antioxidant agent in cell culture models [16], [17], [18], [19]. Several studies proved that CeO_2NPs has many multi-enzyme-mimetic properties, including superoxide dismutase (SOD) [20], peroxidase [21], catalase [22], oxidase [23], and phosphatase [24]. Also, CeO2NPs can scavenge hydroxyl radicals [25], peroxynitrite [26], and nitric oxide radicals [27].

Hence, it has shown its potentiality to be used in bioanalysis [23], [28], [29], [30], drug delivery [31], [26], and biomedicine [15].

Reactive oxygen species (ROS) can be termed as oxygen-containing reactive species, include superoxide (O_2▪−), hydrogen peroxide (H_2O_2), hydroxyl radical (OH▪), among others [32]. Cells produce ROS as the by-products of their aerobic metabolism and essential for cellular growth, proliferation, and differentiation [33], cell signal transduction process [34], immune response, and proper cardiovascular system regulation [35]. ROS react with lipids, proteins, and DNA [36], and a variety of cytosolic antioxidant enzymes counteract the production of ROS, maintaining an oxidative redox balance. However, if the ROS surpasses the average level or the antioxidant level reduces in our body, a phenomenon called "oxidative stress" arises. Oxidative stress has detrimental effects on DNA, proteins, and lipids of cells, causing damage to cell health and contributing to developing various physical and mental conditions [37]. On the other hand, if the level is too low, reductive stresses occur and can also cause pathologies ranging from cancer to cardiomyopathy [38].

The exchangeable ionic state for the redox properties of CeO_2NPs enable them to provide the cells with antioxidant protection against reactive oxygen species ROS [34], [26], [39], [40], [41]. Therefore, CeO_2NPs has been considered as a potential material for use in biomedical applications such as in radiation therapy; chemotherapy; treatment of sepsis, neurodegenerative diseases, cardiovascular diseases, and Alzheimer’s disease [25], [34], [42], [43], [44], [45].

In nanomedicine, CeO_2NPs is now considered as one of the most potent therapeutic metallic oxides for many pathogenies related to oxidative stress. The possibility to counteract the ROS-associated diseases using antioxidants made CeO_2NPs one of the most promising materials in nanomedicine, and a wide range of studies have been conducted exploring the potential use of

CeO_2NPs in regenerative medicine and tissue engineering [46], [47], [48], [49]. CeO_2NPs has shown promising results for wound therapy [50], [51] by promoting cell proliferation in vitro [48], [52], [53], and in the in vivo animal model [54]. CeO_2NPs's ability of ulcer healing in diabetes have been studied by Kobyliak and co-workers in an animal model [55], whereas Lushchak et al. investigated that CeO_2NPs could enhance the wound healing of type 2 diabetic patients [56].

Although numerous studies show that CeO_2NPs can reduce oxidative stress and associated inflammation, many studies reported the cytotoxic effects of CeO_2NPs [57]. CeO_2NPs can produce oxidative stress under certain conditions, and cause cytotoxicity, leading to cell apoptosis. Additionally, the phagocytosis of CeO_2NPs by cells can cause substantial toxic effects to cells. However, Weaver et al. studied that embedding CeO_2NPs into a polymer could reduce its cytotoxicity [58]. The CeO_2NPs-embedded polymeric materials have been used for advanced biomedical application, as the core properties of both polymers and CeO_2NPs have enhanced in this nanoceria-based polymer composite [59], [60]. Several studies performed on various cell lines, like human neuroblastoma cells [61], human bronchial epithelial cells BEAS-2B [62], [63], Human lung adenocarcinoma A549 cells [64] , etc. shows that the regulation and metabolism of cells are harmfully interrupted by the CeO_2NPs.

Several publications suggested targeted delivery of nanoparticles instead of direct delivery to alleviate nanoparticles' damaging effects [65]. Many of them used biocompatible polymers to encapsulate the CeO_2NPs and developed an effective delivery method [66], [67], [68], [69].

This study plans to present evidence on CeO_2NPs's potentiality to use it as an antioxidant agent while providing comparative data of the cytotoxicity of CeO_2NPs with burst delivery and targeted delivery via biocompatible polymer polycaprolactam (PCL).

CHAPTER 2

LITERATURE REVIEW

2.1 Nanotechnology in medicine and biomedical applications

2.1.1 Nanoscience and nanotechnology

The study on structures, molecules, and matters on the nanoscale, ranging from 1-100 nm, is defined as nanoscience, and the practical application of this study is defined as nanotechnology [70], where manipulation occurs at the molecular or atomic level for new unique properties which differ from the materials bulk properties.

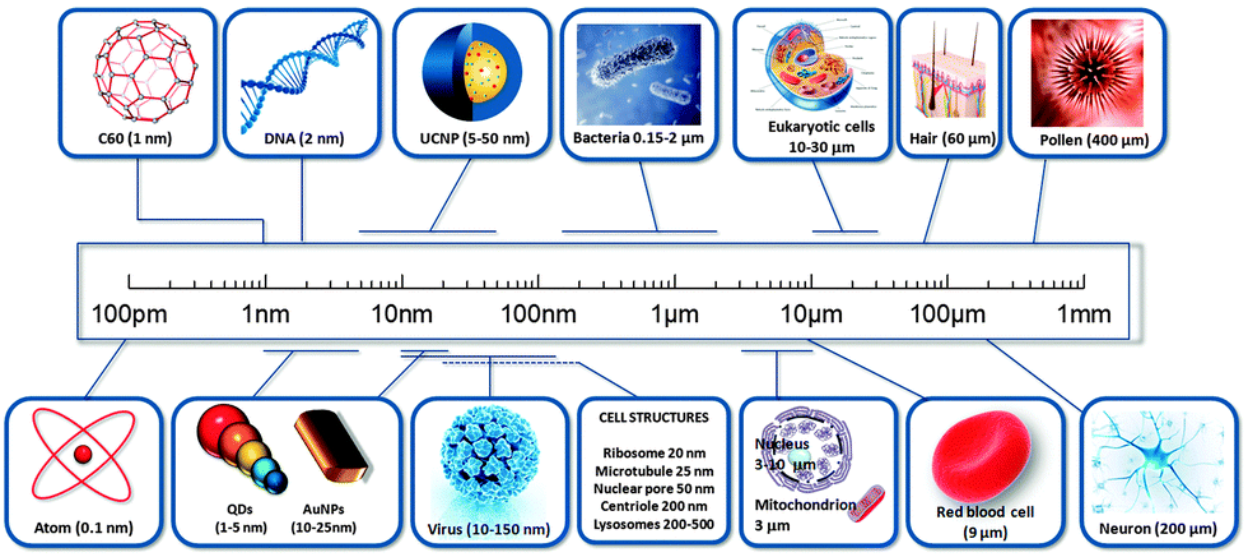

Figure: 2.1 The size of nanoparticles referenced various biologically relevant compounds, cells, and organisms. The image is adopted from [71]

2.1.2 Nanotechnology in biomedical applications

The advances in nanotechnology have created new opportunities in the biology and medicine fields, including tissue engineering, cancer treatment, cells, and biomolecules manipulation, detection of pathogens and proteins, drug, and gene delivery, etc.

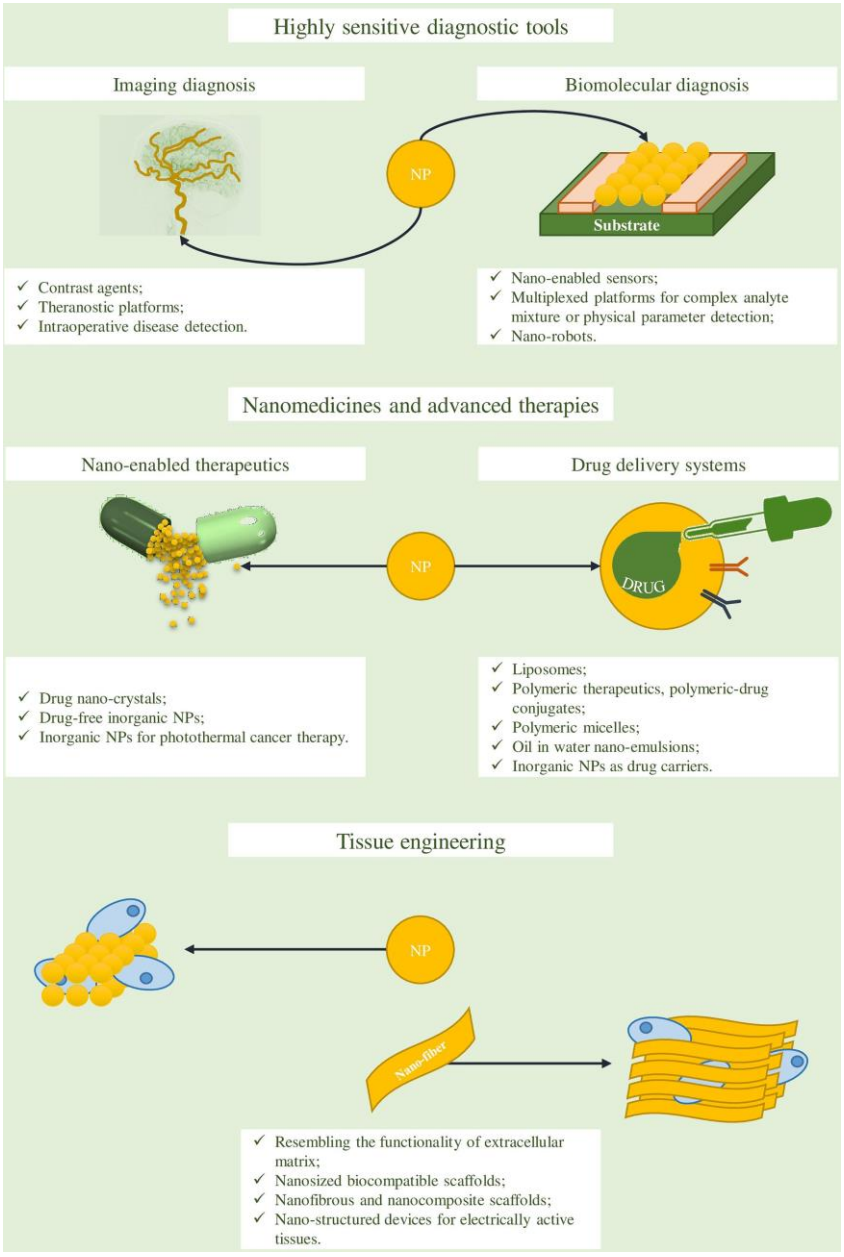

Figure: 2.2 Applications of nanotechnology in biomedical fields. The image is adopted from [72].

The targeted delivery of nanoparticles is used widely in medicine for its multifunctional purpose, including therapeutic and diagnostic use [73], [74].

2.1.3 Biocompatibility of nanomaterials

Biocompatibility is the main requirement for a novel nanomaterial intended for biomedical application. Careful evaluation of the physio-chemical properties of the nanoparticles on the biological response is mandatory before it becomes a clinical reality [75].

Our body's immune system defends against invaders, such as viruses, bacteria, and foreign bodies. In our body, macrophages, antigen-presenting dendritic cells, and other phagocytic cells are responsible for recognizing the foreign invaders, and they are equipped to respond against them. Before in vivo administration of engineered nanoparticles, the nanoparticles-immune interactions are the most critical aspect to consider [76], [77].

Depending on the physical and chemical properties of the nanoparticles, such as size, shape, surface charge, etc., the specificity of cellular uptake pathways is determined. Additionally, the cell type also impacts the selection of the path of cellular uptake; for example, macrophages in the lung utilize a different repertoire of recognition molecules than macrophages in the bone marrow. Therefore, the understanding of the mechanism of uptake, biodistribution is vital for determining potential adverse effects in vivo and for process and design optimization for future application of nanoparticles in biomedicine [78]. The properties of nanoparticles are size, shape, and structure dependence; therefore, these properties must remain controlled in vitro and in vivo. In an adequate media, the nanoparticles colloidal stability is an important issue to consider its safety [79].

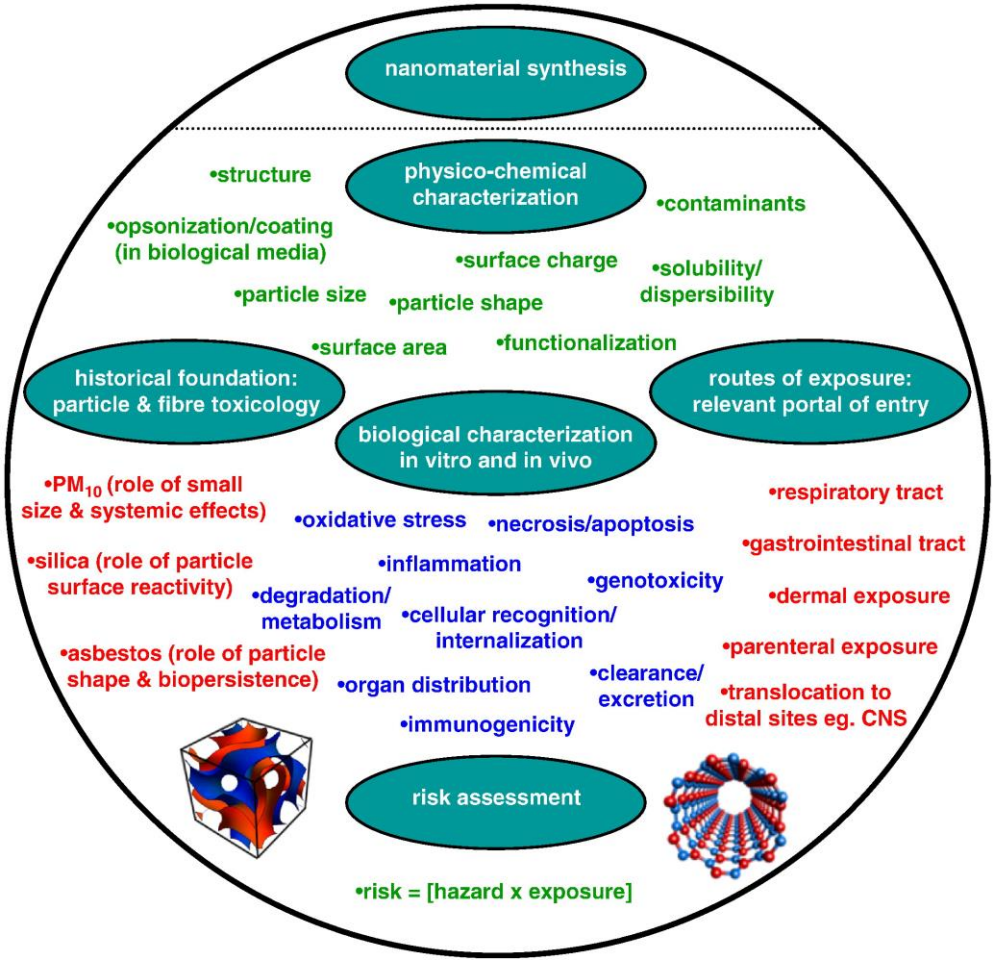

Figure 2.3 Aspects of the biocompatibility and toxicological properties to consider for nanoparticles used as biomedical applications. Figure is adopted from [80]

2.2 Cerium oxide nanoparticles (Nanoceria)

All the lanthanoids (La 57 - Lu 71), scandium (Sc 21), and yttrium (Y 39) are named rare earth (RE) elements, as they are naturally dispersed in minerals. They have similar ionic radii; they are strongly electropositive, and they have similar chemical properties [81].

Cerium is the first element in the lanthanide/rare-earth group, and it is the most abundant rare-earth element found in the earth's crust. It can exist in trivalent and tetravalent states like the

other rare earth elements. However, while the other lanthanide elements are stable only in the trivalent state, cerium is stable in trivalent and tetravalent states [82].

Cerium reacts readily with oxygen, forming Ce_2O_3 or CeO_2, preferably the dioxide structure in normal temperature and pressure in the bulk state [3]. The dioxide of cerium has a cubic fluorite structure where eight oxygen anions coordinate one cerium cation, and each oxygen atom is bonded to four cerium atoms (Figure 2.3).

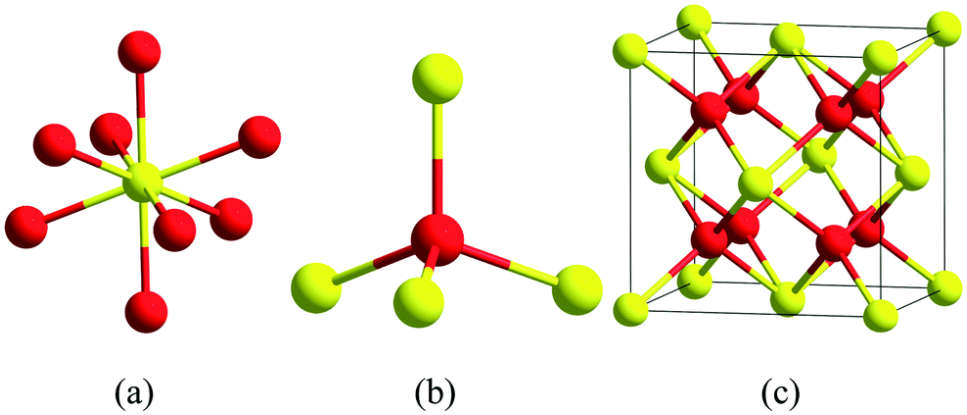

Figure 2.4 Structural analysis of ceria crystals and unit cells. Eight-fold coordinated cerium atoms (yellow) with four-fold coordinated oxygen atoms (red) in ceria crystals (a and b) and the primitive unit cell (c) (Figure is adapted from [83])

The vacant oxygen is found in the cerium oxide structure, making the cerium oxide compounds having stoichiometric values. The cerium oxide has two types of oxidation states, +3 and +4, and cerium oxide can switch between these two states. Ce^{+4} is more stable and produces a redox couple responsible for catalytic activity. The corresponding oxygen vacancy balances the reduced positive charge of Ce^{+4}. However, while cerium combines with oxygen to form nanoparticles, cerium oxide produces a mixture of cerium in the +3 and +4 states on the nanoparticles' surface. The number of +3 sites on the surface increases if the nanoparticle's

diameter decreases and oxygen vacancies occur [4]. As the surface area-to-volume ratio increases in the nanoparticles, the cerium oxide nanoparticle has a higher concentration of Ce^{3+} and greater redox and catalytic activity concerning larger particles [5] (figure 2.5).

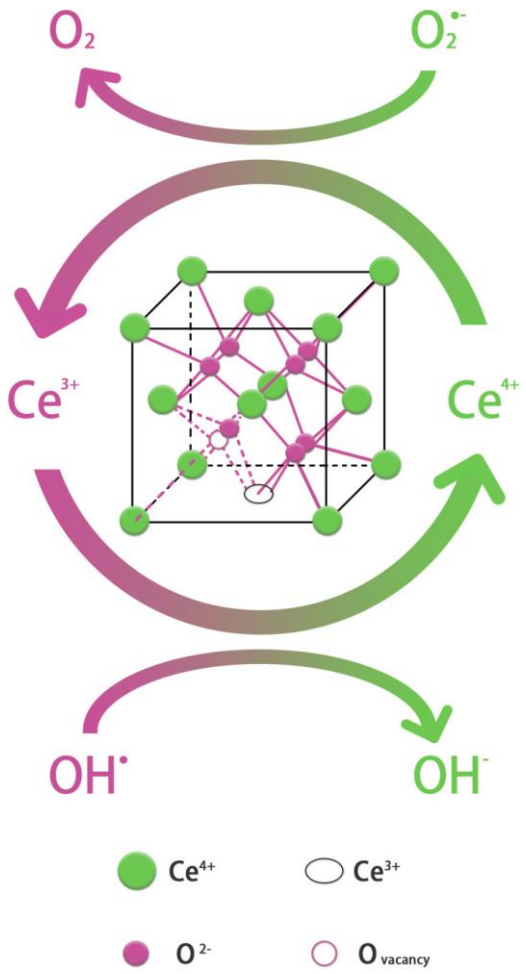

Figure 2.5 The structural representation of CeO_2NPs, and its self-storage stability and self-regeneration capacity exerting antioxidant chemical reaction (Figure is adopted from [84])

Additionally, cerium oxide nanoparticles have high oxygen storage capacity and UV absorbing ability [6], [7], [8]. Therefore, the production, application, and advancement of the cerium oxide nanoparticle have increased significantly during the last few years. The

applications of cerium oxide nanoparticles have spread in engineering and biological sectors substantially. Some most important applications of cerium oxide nanoparticles including high-temperature oxidation protection materials [9], solar cells [10], solid oxide fuel cells [11], gas sensors [12], UV screens [13], catalytic materials [14], and potential pharmacological agents [15].

2.3 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) can be termed as oxygen-containing reactive species, include superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), among others. Cells produce ROS as the by-products of their aerobic metabolism and essential for cellular growth, proliferation, differentiation, cell signal transduction, immune response, and proper cardiovascular system regulation. There are exogenous sources (UV light, radiation, environmental agents, the impact of pharmaceuticals, etc.) and endogenous sources (e.g., electron transport chain in mitochondria, NADPH (nicotinamide adenine dinucleotide phosphate) oxidases, peroxisomes, endoplasmic reticulum, etc.) responsible for ROS formation. ROS react with lipids, proteins, and DNA, and a variety of cytosolic antioxidant enzymes counteract the production of ROS, including glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), etc, maintaining an oxidative redox balance [36].

However, if the ROS surpasses the average level or the antioxidant level reduces in our body, a phenomenon called "oxidative stress" arises. Oxidative stress has detrimental effects on DNA, proteins, and lipids of cells, causing damage to cell health and contributing to developing various physical and mental conditions [37].

ROS control various signaling pathways depending on the amounts present, affecting all cellular processes, from proliferation to differentiation and apoptosis.

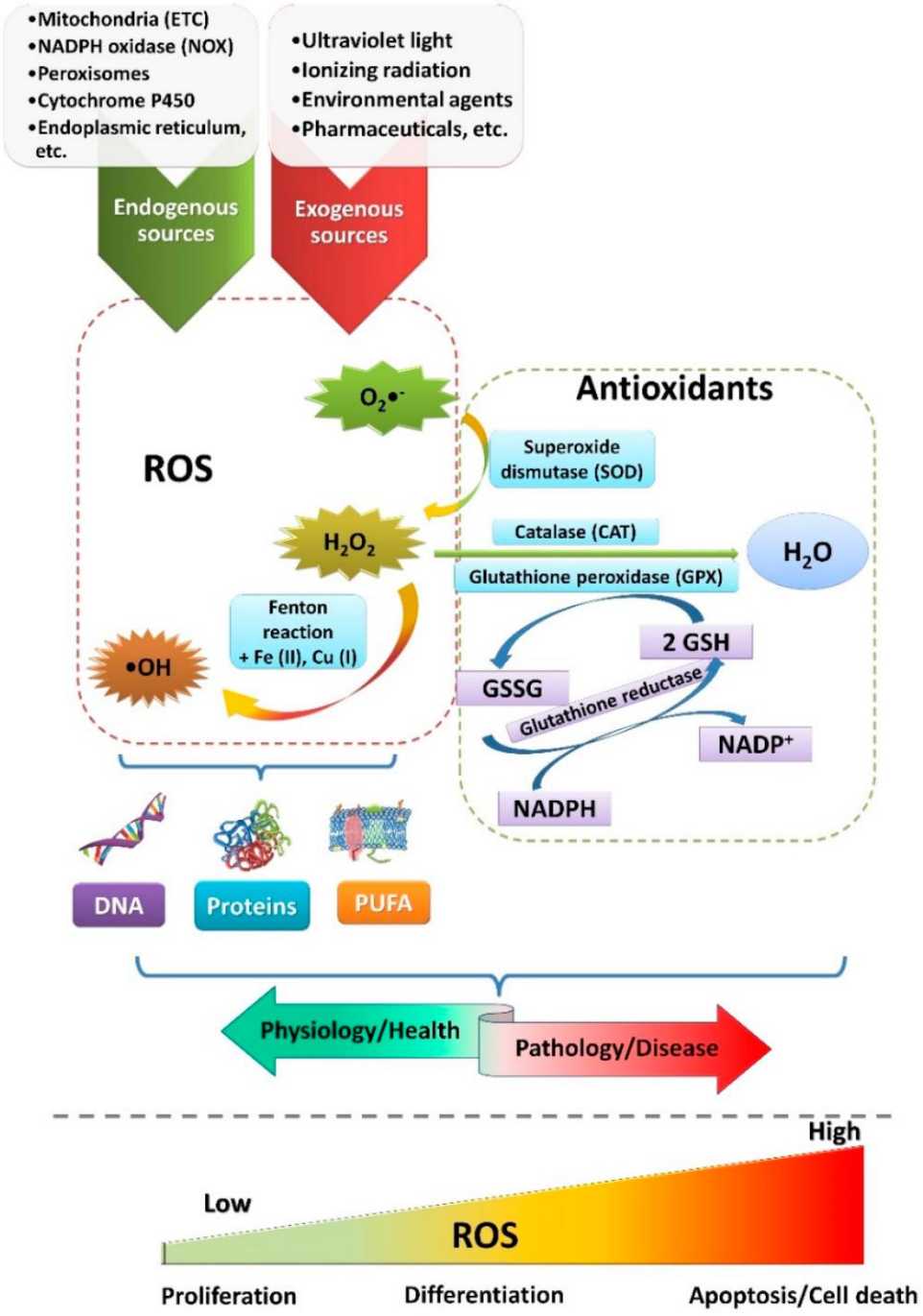

Figure 2.6 Schematic figure of generation of reactive oxygen species (ROS) and their impact on cells. Figure is adopted from [33].

The average amount of ROS can support the epidermal cells' proliferation and can promote wound healing. However, ROS production produces superoxide, damaging the tissue by decreasing antioxidant manufacture and activity [85]. An elevated ROS level in the wound environment slows down the effects on angiogenesis, causing a stagnant inflammatory stage, which is responsible for additional tissue damages by producing extreme ROS or by activating the intermediates of ROS, inflammatory cytokines, proteases, and proteases pro-apoptotic proteins. Moreover, this phenomenon increased the damage and death of the cell, which lead to wounds with decreased healing. On the other hand, if the level is too low, reductive stresses occur and can also cause pathologies ranging from cancer to cardiomyopathy [38].

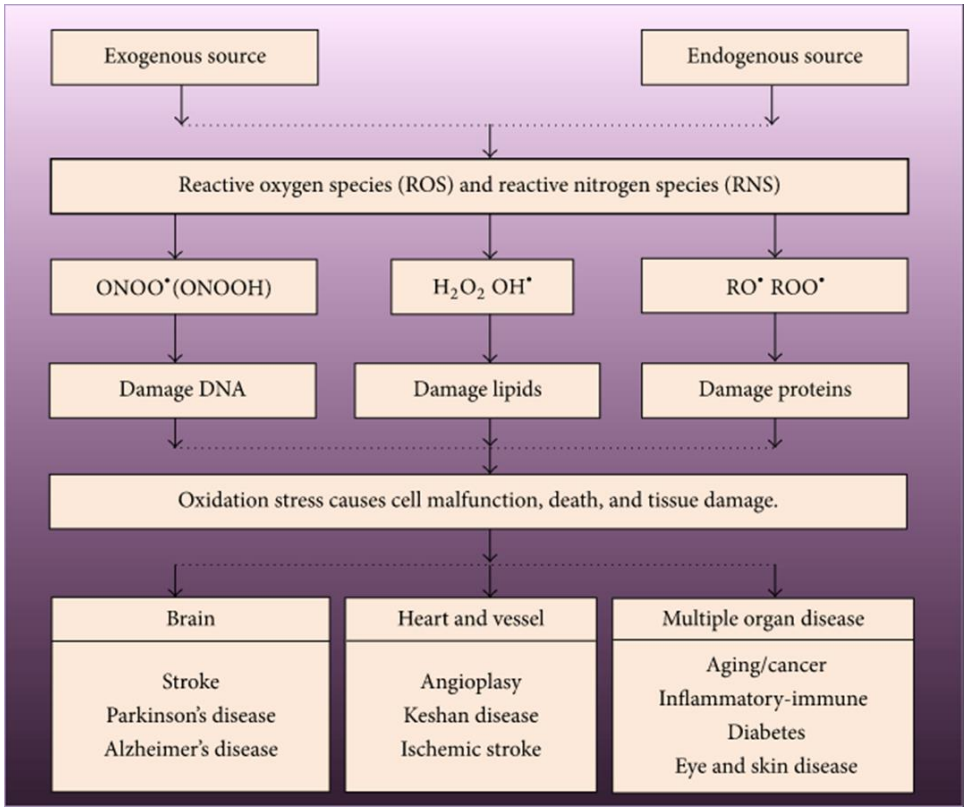

Figure 2.7 Reactive oxygen species induced oxidation stress damage to human health. Figure is adopted from [86].

2.4 Cerium oxide nanoparticles as an antioxidant

The exchangeable ionic state for the redox properties of CeO_2NPs enable them to provide the cells with antioxidant protection against reactive oxygen species ROS [34], [26], [39], [40], [41]. Therefore, CeO_2NPs has been considered as a potential material for use in biomedical applications such as in radiation therapy; chemotherapy; treatment of sepsis, neurodegenerative diseases, cardiovascular diseases, and Alzheimer's disease [25], [34], [42], [43], [44], [45].

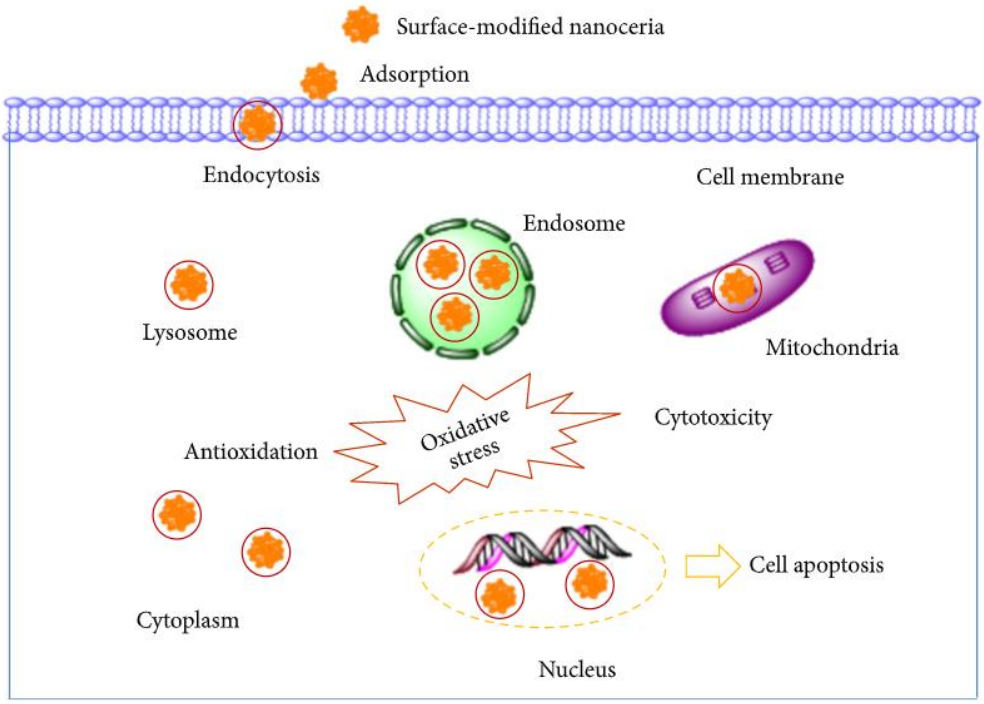

Figure 2.8 Mechanism of nanoceria in oxidative stress between cells and nanoceria receptor-mediated cellular uptake and translocation. Figure is adopted from [86].

In nanomedicine, CeO_2NPs is now considered as one of the most potent therapeutic metallic oxides for many pathogenies related to oxidative stress. The possibility to counteract the ROS-associated diseases using antioxidants made CeO_2NPs one of the most promising materials in nanomedicine, and a wide range of studies have been conducted exploring the potential use of

CeO_2NPs in regenerative medicine and tissue engineering [46], [47], [48], [49]. CeO_2NPs has shown promising results for wound therapy [50], [51] by promoting cell proliferation in vitro [48], [52], [53], and in the in vivo animal model [54]. CeO_2NPs's ability of ulcer healing in diabetes has been studied by Kobyliak and co-workers in an animal model [55], whereas Lushchak et al. investigated that CeO_2NPs could enhance the wound healing of type 2 diabetic patients [56].

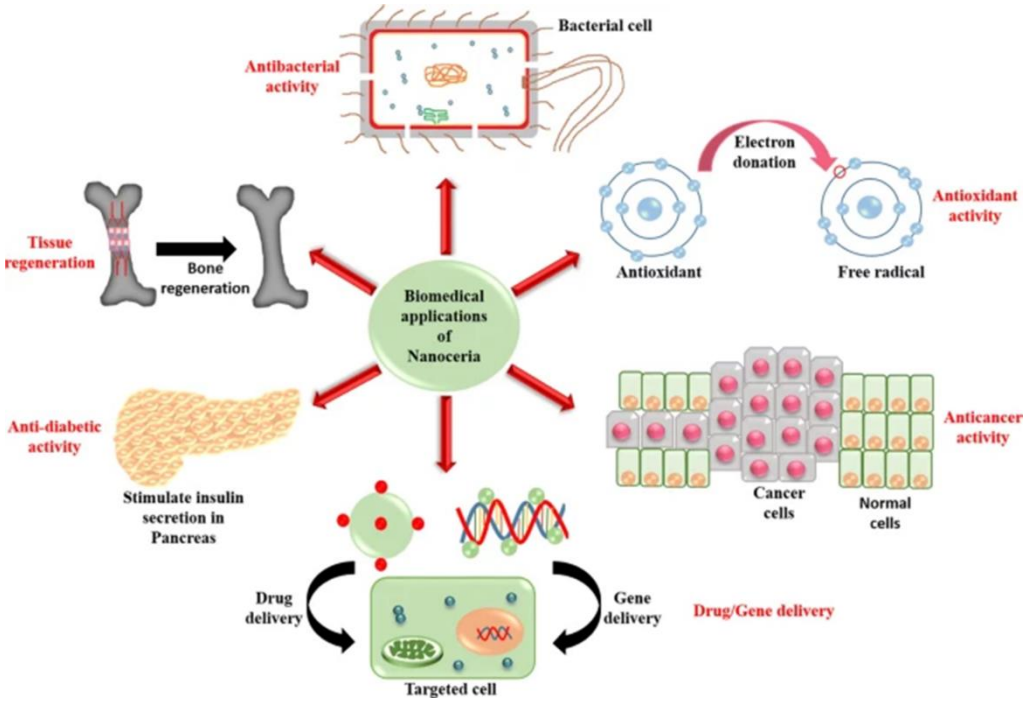

Figure 2.9 Schematic figure of biomedical applications of cerium oxide nanoparticles. Image is adapted from [87]

2.5 Research gap and overall objective

While the use of CeO_2NPs in biomedicine shows many perspectives, many research questions have remained unsolved, mainly the cytotoxicity of CeO_2NPs. Several publications show evidence of reduced toxicity of polymer encapsulated CeO_2NPs, and the potential use of PCL as the carrier fiber has also been explored. In this research, a simple approach of

encapsulating CeO_2NPs using a touch spinning system will be used. Therefore, it is essential to explore the process variables, prospects, and efficiency of this method. This research strategy, being comparatively simple and cost-effective, will help improve and accelerate the successful use of CeO_2NPs-containing polymers in the biomedical field, which will work as a basis for future innovative regenerative medicine.

The overall objective of this research is to synthesize biocompatible CeO_2NPs and to characterize the CeO_2NPs in terms of size, morphology, and concentration. After that, this research aimed to conduct a study to determine the cytotoxicity of CeO_2NPs and test nanoceria's ability to decrease inflammatory mediator production in stimulated cells. Further study also involved encapsulation of CeO_2NPs in PCL fiber mesh, characterization of the CeO_2NPs-embedded fiber mesh, analyzing CeO_2NPs-embedded fiber mesh's biocompatibility, and observing its encapsulation efficiency and antioxidant activity.

2.6 Hypothesis

In this study, we proposed the following hypothesis:

1. CeO_2NPs will reduce cellular oxidative stress.

2. The optimum amount of CeO_2NPs, which will work as an antioxidant, will not be toxic to cells.

3. The CeO_2NPs incorporated PCL fiber will reduce cellular oxidative stress.

4. The optimum amount of CeO_2NPs, which will act as an antioxidant while incorporating into PCL fiber, will not be cytotoxic.

CHAPTER 3

ANTI-INFLAMMATORY PROPERTIES OF CERIUM OXIDE NANOPARTICLES ENCAPSULATED WITHIN POLY (E-CAPROLACTONE) (PCL) ELECTROSPUN FIBERS

3.1 Introduction

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This study plans to present evidence on CeO_2NPs's potentiality to use it as an antioxidant agent while providing comparative data of the cytotoxicity of CeO_2NPs with burst delivery and targeted delivery via biocompatible polymer polycaprolactam (PCL).

3.2 Materials and methods

3.2.1 Materials

Cerium (III) nitrate hexahydrate ($Ce((NO_3).6H_2O)$) (99.99% trace metal basis), citric acid, Resazurin sodium salt (Alamar Blue), H_2DCFDA (2,7-dichlorofluorescein diacetate), RAW 264.7 cell line, Hanks' Balanced Salt solution (HBSS), Phosphate buffered saline (PBS), Lipopolysaccharide (LPS from E. coli O8:K27), Calcein-AM, Ethidium Homodimer-1, Polycaprolactone (Mn 80,000 g mol^{-1}) were purchased from Sigma Aldrich (USA). Dulbecco's modified eagle's media-high glucose (DMEM), antibiotics and antimycotics, fetal bovine serum (FBS), Trypsin EDTA 0.25% were obtained from VWR International LLC (USA). Chloroform (ACS Grade) was purchased from Fisher Scientific. For cell culture, standard T-75 treated flasks, cell culture treated 6-well plates, flat-bottom cell culture treated 96-well plates were used, which were purchased from VWR International LLC (USA). All the reagents were used as received without any further purification.

3.2.2 Nanoceria synthesis

A one-stage method of synthesis of non-toxic and stable cerium oxide nanoparticles has been followed for this study. A 0.05 M aqueous solution of Cerium (III) nitrate hexahydrate ($Ce((NO_3)_3.6H2O)$) was prepared by dissolving it into D.I. water. Then, 0.24 g citric acid was mixed with this solution. After that, this solution was immediately poured into a 3M ammonia

solution (100mL) drop by drop under continuous stirring (400 rpm). White CeO_2 sol was formed in this stage. Then it was allowed to stand for oxidization overnight, under continuous stirring at 400 rpm. Then the sols were purified by washing with DI water several times following by centrifuging and sonication [88].

3.2.3 Characterization of CeO_2NPs

3.2.3.1 Dynamic light scattering

Dynamic light scattering (DLS) has been used to estimate the size of the synthesized nanoparticles. Cerium oxide nanoparticles were diluted with DI water, and a Malvern Zetasizer ZS DLS instrument was utilized to characterize the size.

3.2.3.2 Transmission Electron Microscopy (TEM)

The images of CeO_2NPs were acquired by using transmission electron microscope JEOL 1011 TEM, Japan. No additional coating was used during the TEM imaging.

3.2.3.3 Inductively coupled plasma mass spectrometry (ICP-MS)

The Inductively coupled plasma mass spectrometry (ICP-MS) analysis of CeO_2NPs was performed for determining the concentration of synthesized CeO_2NPs. For sample preparation, the nanoceria was digested into Nitric Acid and diluted 1000 times. The Plasma Chemistry Laboratory, UGA, performed the analysis.

3.2.4 In vitro cell studies

3.2.4.1 Cell culture

RAW 264.7 Macrophages were cultured with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 200 U/mL penicillin and 200 mg/mL streptomycin, and 10% fetal

bovine essence (FBE). The cells were incubated at 37 °C and 5% CO_2 and sub-cultured at about 80% confluency.

3.2.4.2 Cellular uptake efficiency of CeO_2NPs

The cells were grown to 80% confluence, and 2.5×10^4 cells were seeded into 96-well plates and cultured for 24h. Then the cells were exposed to 12 mM of CeO_2NPs and incubated for another 24h. Later the cells were fixed using 4% glutaraldehyde. The images of Cells with Cerium Oxide Nanoparticles were obtained by using transmission electron microscope JEOL 1011 TEM, Japan.

3.2.4.3 In vitro antioxidant study

The antioxidant efficiency of CeO_2NPs was evaluated by H_2DCFCA, a cell-permeable fluorogenic probe which can diffuse into cells and detect intercellular ROS level (72). For this study, RAW 264.7 Macrophages are culturing into cell culture standard up to 80% confluence. In a sterile flat bottom 96 well plates, 2.5×10^4 cells were seeded with 200µL of DMEM. After 24h of cell seeding, 3.02 mM, 6.04 mM, 9.06 mM, and 12.08 mM of CeO_2NPs were added into the wells. After another 24h, half of the plate was treated with Lipopolysaccharides (LPS) which will cause an acute inflammatory response by activating the release of inflammatory cytokines. Therefore, an abundant amount of reactive oxygen species (ROS) was produced. Then after another 24h, the old medium was removed from the wells and replaced with Hank's Balanced Salt Solution (HBSS) with 10µM of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFCA). After that, the plate was incubated in the dark for 45 minutes. Then, H_2DCFCA loaded HBSS was replaced by 100µL fresh HBSS. Finally, the fluorescence readings were measured at excitation at 495 nm and emission at 525 nm using Varioskan LUX Multimode Microplate Reader.

3.2.4.2 Cytotoxicity assay

RAW 264.7 Macrophages were seeded at a density of around 2.5×10^4 cells per well in a sterile 96-well plate with 200µL DMEM. After 24h of cell seeding, 3.02 mM, 6.04 mM, 9.06 mM, and 12.08 mM of CeO_2NPs were added into the wells. Then, the cells were treated with Alamar Blue solution (0.15 mg/mL in PBS, pH = 7.4) at 10% volume of cell culture medium and incubated for 3h. The active ingredient of Alamar Blue is a non-fluorescent compound, Resazurin, which is reduced to resorufin, a red and highly fluorescent compound after entering the living cells that is directly proportional to the number of living cells (73), (74). The fluorescence was measured at excitation at 540 nm and emission at 590 nm using Varioskan LUX Multimode Microplate Reader.

3.2.5 Fabrication of PCL- CeO_2NPs fibers

The polymer solution of PCL was prepared by mixing 10% w/w PCL in a Chloroform solvent system for 24h at room temperature. Two separate solutions were made, adding 0.05% and 0.1% w/w of dry CeO_2NPs to the polymer solution for spinning CeO_2NPs encapsulating fibers. All the samples were spun using an Electrospinning System situated at Nanostructured Materials Lab, University of Georgia. The fiber samples were prepared at room temperature with an electrospinning setup with a rotating drum collector (at drum rotational speeds of 2000 RPM). The nozzle (internal diameter of 0.9 mm) was connected to the electric power source of 20 kV. The flow rate of polymer solution through the nozzle was 20 $\mu L.min^{-1}$. The resulting electrospun fibers were collected on round acrylonitrile butadiene styrene (ABS) frames for characterization and cell culture studies.

3.2.6 Characterization of PCL- CeO2NPs fibers

3.2.6.1 Scanning electron microscopy

The morphological study of the PCL only and PCL-CeO_2NPs fibers was performed using scanning electron microscopy (SEM) using Thermo Fisher Teneo FE-SEM. After fabrication, the fibers were coated with 20 nm of Au/Pd. Then the samples were placed on a silicon wafer, and the imaging was done.

3.2.6.2 Fiber diameter

The average diameters of the PCL only and PCL-CeO_2NPs fibers were determined using ImageJ software (National Institute of Health, USA) from 50 randomly selected fibers from each sample.

3.2.6.3 Water contact angle

The Hydrophobicity of PCL only and PCL-CeO_2NPs fibers was measured by water contact angle measurement using the Sessile Drop Method. A water droplet of 10µL microliter was placed on the nanofibrous scaffold surface with a pipette. An image was taken with Abscam software, and the contact angle was calculated using ContAngle software. The test was performed for three different places of each sample, and an average value was estimated.

3.2.7 In vitro cell studies

3.2.7.1 Cell culture and treatment

RAW 264.7 Macrophages were cultured with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 200 U/mL penicillin and 200 mg/mL streptomycin, and 10% fetal bovine essence (FBE). The cells were incubated at 37 °C and 5% CO_2 and subcultured at about

80% confluency. For cell culture studies, fibers deposited on ABS frames were sterilized using UV light for 30 mins. 1×10^5 cells were seeded on each fibers sample with 6 mL of DMEM.

3.2.7.2 Cell viability assay

The viability of RAW 264.7 Macrophages on PCL-only fibers, PCL fibers with 0.05% CeO_2NPs, and PCL fibers with 0.1% CeO_2NPs was assessed by Alamar Blue assay over a period of 72 h. In a sterile 6 well plate, the fiber samples were placed, and 1×10^5 cells were seeded on each fiber sample with 6 mL of medium (DMEM). As a control, 1×10^5 cells were seeded in a well without any samples. Background fluorescence of phenol-red containing-medium, background fluorescence of Alamar Blue-containing medium, and background fluorescence of PCL fiber and ABS frame were considered during the assay. After every 24h, the wells were treated with Alamar Blue solution (0.15 mg/mL in PBS, pH = 7.4) at 10% volume of cell culture medium and incubated for 3h. The active ingredient of Alamar Blue is a non-fluorescent compound, Resazurin, which is reduced to resorufin, a red and highly fluorescent compound after entering the living cells that is directly proportional to the number of living cells. (73), (74). 200μL liquid was collected from each sample and triplicated in a 96-well plate. After that, the fluorescence was measured at excitation at 540 nm and emission at 590 nm using Varioskan LUX Multimode Microplate Reader. A graph of Florence versus the number of days was plotted.

3.2.7.3 Cytotoxicity assay

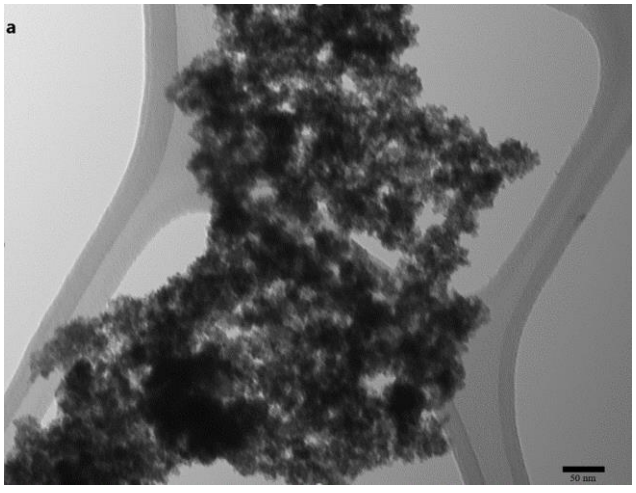
The cytotoxicity of RAW 264.7 Macrophages on PCL only and PCL-CeO_2NPs fibers was assessed by assay over a period of 72 h using green, fluorescent Calcein-AM, and red-fluorescent ethidium homodimer-1. Calcein-AM indicates intracellular esterase activity, which indicates the live cells, and Ethidium Homodimer-1 indicates the loss of plasma membrane

integrity, showing dead cells. For this assay, 1×10^5 cells were seeded on each fiber sample with 5 mL of medium and incubated. As a control, 1×10^5 were seeded without any samples. After every 24 h, the cells were treated with 1µM green-fluorescent Calcein-AM and 0.2µ red-fluorescent Ethidium Homodimer-1 in PBS. After 20 mins of incubation, the fluorescence images are taken using IVIS 100 (IVIS Spectrum imaging system).

3.3 Results and Discussion

3.3.1 Nanoceria Characterization

The shape of the CeO_2NPs was examined by TEM imaging, and the size of the synthesized CeO_2NPs was determined by DLS. The TEM imaging of figure 3.1 (a) showed that the synthesized nanoparticles were uniform in size. As demonstrated by the DLS particle size distribution graph, the average size of the particles was found to be ~64 nm, which is shown in figure 3.1 (b).



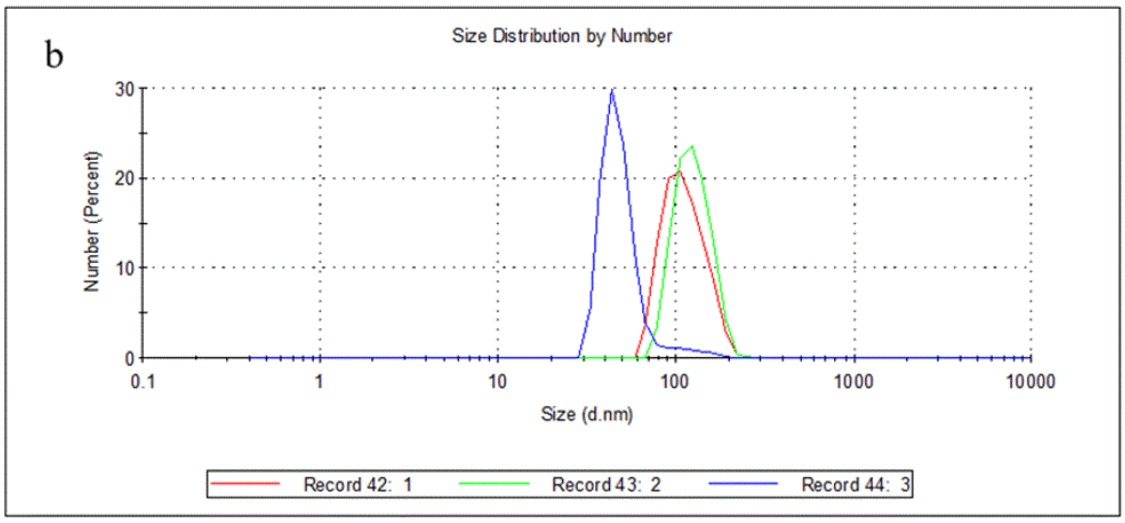


Figure 3.1 Characterization of nanoparticles: (a) TEM image of CeO_2NPs shows the homogenous distribution and size and shape of Nanoceria (b) Particle size distribution of CeO_2NPs by number using dynamic light scattering revealing the size range is in between 20 nm -110 nm.

3.3.2 Cellular uptake efficiency of CeO_2NPs

The RAW 264.7 cells were exposed to 12 mM of CeO_2NPs and incubated for 24 h. The intracellular localization of CeO_2NPs was investigated using TEM, which shows that the CeO_2NPs was taken up properly and distributed throughout the cells. The CeO_2NPs was seen to be localized in membrane-bound vesicles and free in the cytoplasm.

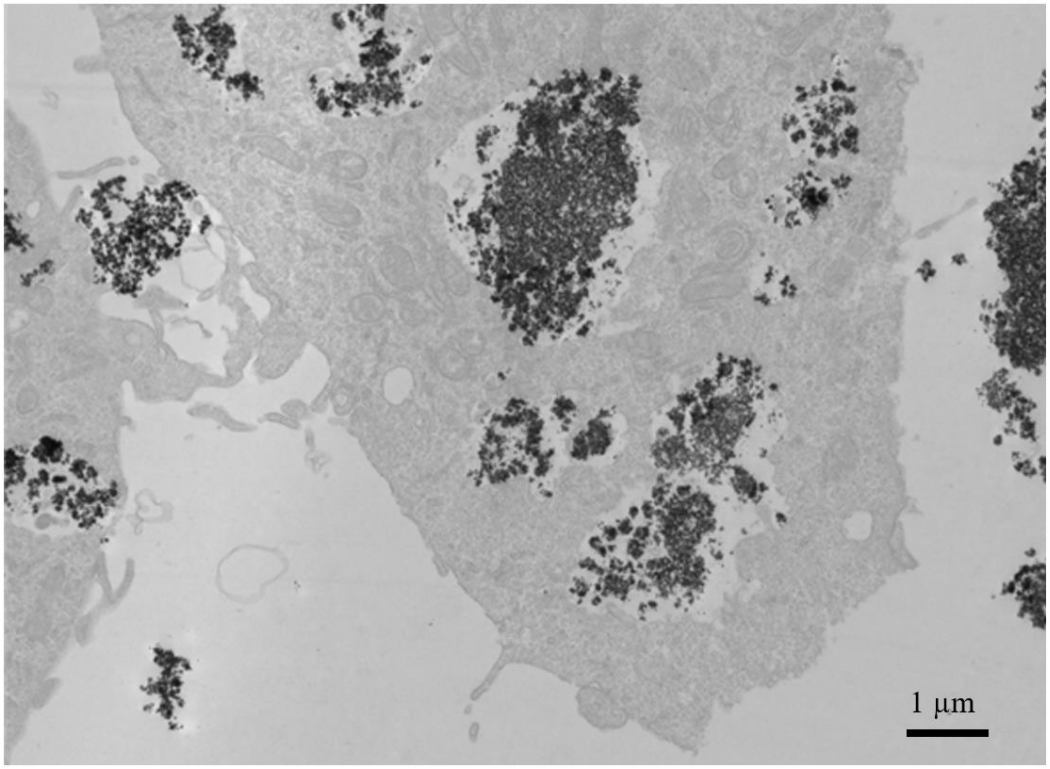


Figure 3.2 TEM image of Nanoceria treated RAW 264.7 cell showing the proper and even uptake of CeO_2NPs by cells, and localization of CeO_2NPs in membrane-bound vesicles and free in the cytoplasm.

3.3.3 In vitro antioxidant study

In order to explore the efficiency of nanoceria as an effective scavenger of ROS, an H_2DCFDA probe-based assay was carried out. The RAW 264.7 cells were treated with LPS to generate ROS into the cells. Afterward, the cells were incubated with different amounts of Cerium oxide nanoparticles (3.02 mM, 6.04 mM, 9.06 mM, and 12.08 mM). The results of the assay (Fig. 3.3) demonstrate that the LPS has successfully activated the macrophages. There is a ~67% decrease in fluorescence intensity of H_2DCFDA observed for cells treated with 12.08 mM

of nanoceria compared to the control cells i.e., untreated cells. The reduction in the fluorescent activity indicates a ROS scavenging potential of the cerium oxide nanoparticles.

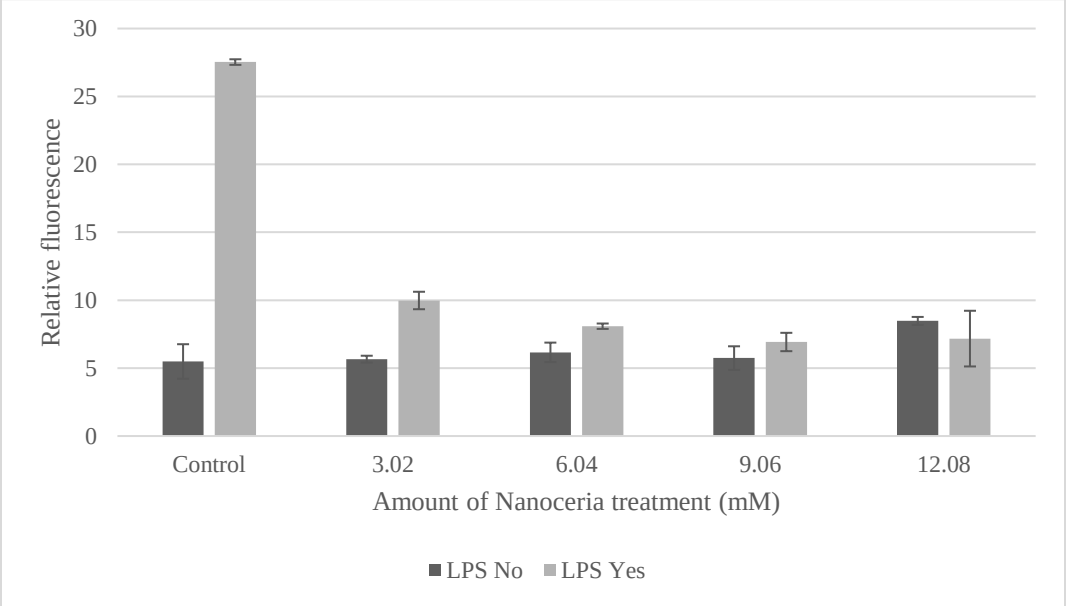


Figure 3.3 ROS level measurement in RAW 264.7 by H_2DCFDA fluorescence intensity.

3.3.4 Cytotoxicity assay

An Alamar Blue assay was performed to determine the effect of CeO_2NPs on the viability and proliferation of RAW 264.7 cells. The results (Fig. 3.4) illustrate a decrement of cell viability and proliferation incubated with the increment of treatment nanoceria for 24 h. There are ~14%, ~32%, ~48%, and ~56.26% decrements of cell viability observed in the cells treated with 3.02 mM, 6.04 mM, 9.06 mM, and 12.08 mM of cerium oxide nanoparticles compared to the control cells, i.e., untreated cells. This data indicates the cytotoxicity of cerium oxide nanoparticles in burst/direct delivery.

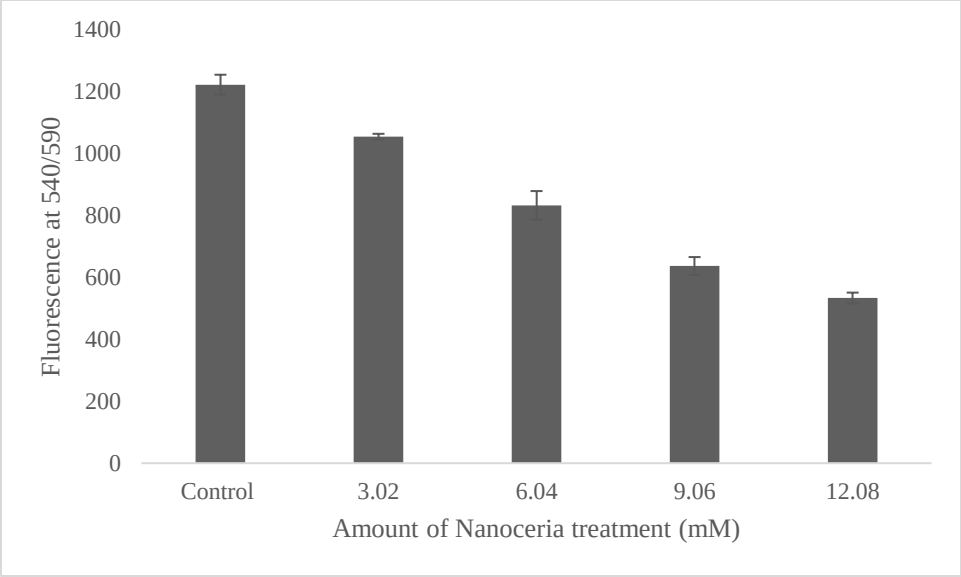


Figure 3.4 Cell viability of RAW 264.7 cells treated with nanoceria by Alamar blue assay.

3.3.5 Characterization of PCL- CeO_2NPs fibers

The Morphology analysis of PCL fibers with nanoparticles and without nanoparticles was carried out by observing the fibers using SEM. The SEM micrographs of PCL-only fibers, PCL with 0.05% cerium oxide nanoparticles, and PCL fibers with 0.1% nanoparticles shown in Fig. 3.4 (a, b, c) show isotropic and oriented electrospun fibers with an apparently smooth morphology. The average diameters of the PCL-only fibers were calculated to be in the range of 2.3µm-6.2µm with a mean fiber diameter of 4±1µm. Whereas the diameter of the PCL fibers with 0.05% CeO_2NPs was estimated to be in the range of 2µm-6µm with a mean fiber diameter of 3.8±0.9 µm, and the PCL fibers with 0.1% CeO_2NPs was estimated to be in the range of 2.4µm-6.33µm with a mean fiber diameter of 3.7±1.3µm.

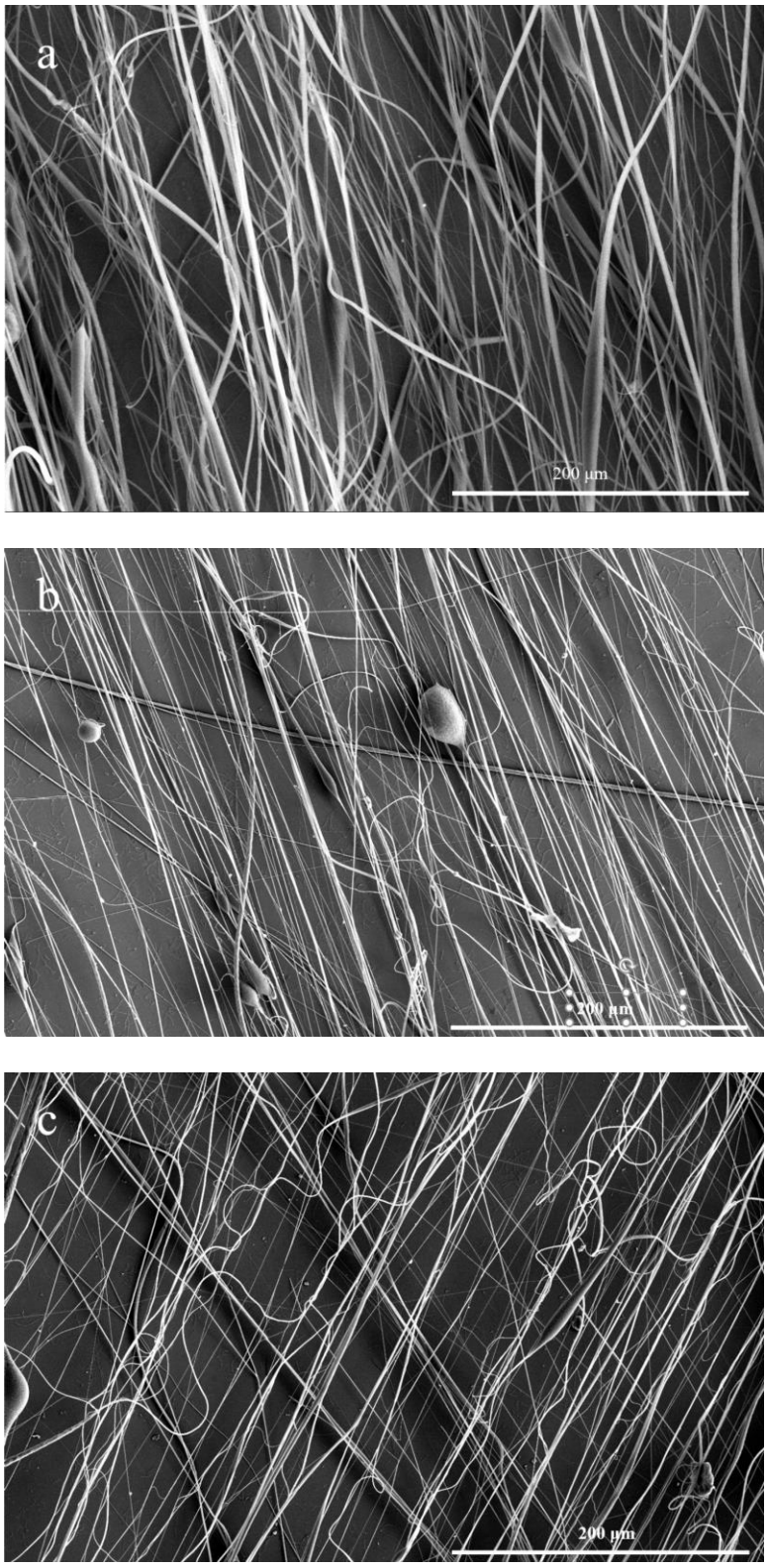


Figure 3.5 SEM images of oriented (a) PCL-only fibers, (b) PCL fibers with 0.05% CeO2NPs, (c) PCL fibers with 0.1% CeO_2NPs fabricated by electrospinning.

3.3.6 Water contact angle

The Hydrophobicity of PCL only and PCL-CeO_2NPs fibers was measured by water contact angle measurement by Sessile Drop Method, and the average contact angle value of PCL-only fiber was determined 104.2°±4; PCL fibers with 0.05% nanoceria were determined 91.1°±1, and the water contact angle PCL fibers with 0.1% CeO_2NPs was 85.6°±2. These measurements could assume that the hydrophobicity of PCL fibers decreases when cerium oxide nanoparticles are present. And the increased amount of cerium oxide nanoparticles increases the hydrophilicity of fibers. And the hydrophilic surface of fibers makes them more compatible for cell attachment and proliferation.

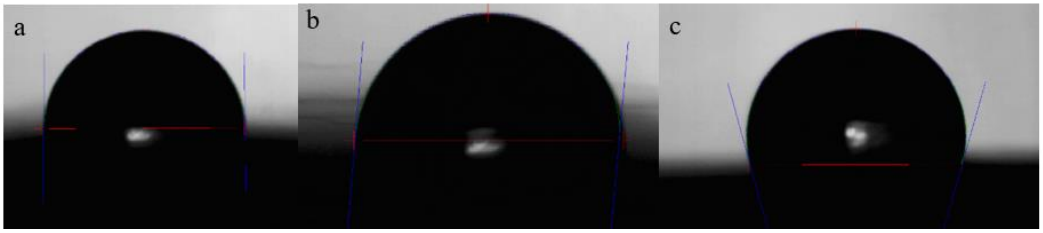


Figure 3.6 Water contact angle measurement of (a) PCL-only fibers, (b) PCL fibers with 0.05% CeO_2NPs, (c) PCL fibers with 0.1% CeO_2NPs fabricated by electrospinning.

3.3.7 Cell viability assay

The viability of RAW 264.7 Macrophages on PCL only and PCL-CeO_2NPs fibers was assessed by Alamar Blue assay throughout 72 h (Figure 3.7). It was observed that cells were viable and proliferating on the samples; however, the rate of cell proliferation was highest on the PCL fibers with 0.1% CeO_2NPs. The higher cell proliferation on PCL fibers with 0.1% CeO_2NPs continued till 72 h. The steady increase in cell proliferation on PCL fibers with 0.1% CeO_2NPs can be attributed to the encapsulated CeO_2NPs that would have released in the cell culture medium. The intracellular ROS, which is produced by cell metabolic activity, is responsible for

cellular damage, and it can reduce the viability and proliferation of cells. The released CeO_2NPs can be able to improve cell survival by decreasing the intracellular levels of ROS [52], [89].

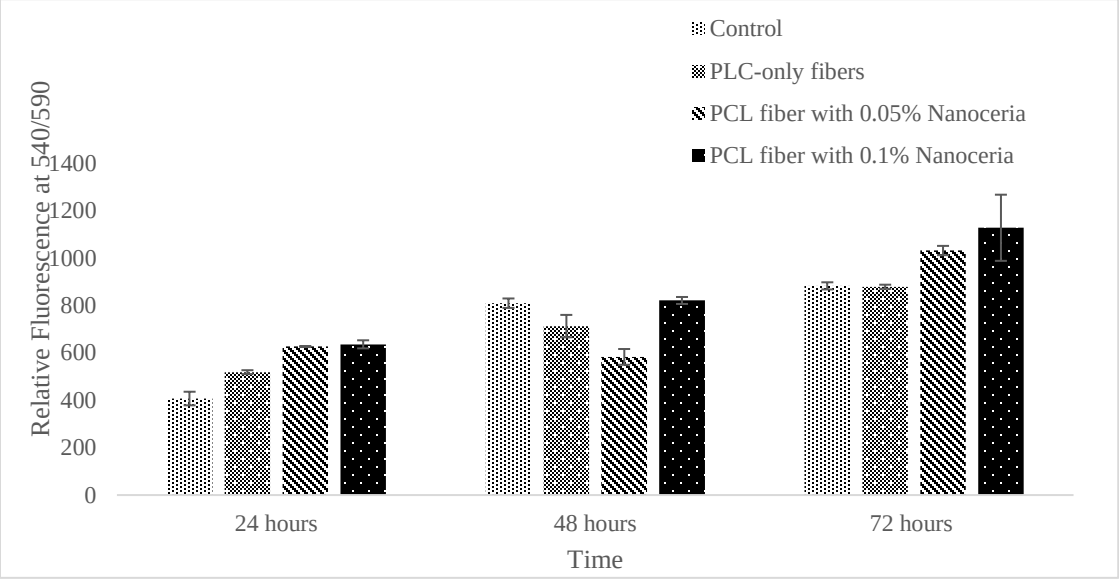


Figure 3.7 RAW 264.7 cell viability and proliferation study on PCL-only, PCL fibers with 0.05% CeO_2NPs and PCL fibers with 0.1% CeO_2NPs after 24, 48 and 72 h as measured by Alamar Blue assay.

3.3.8 Cytotoxicity assay

The cytotoxicity of RAW 264.7 Macrophages on PCL only and PCL-CeO_2NPs fibers was assessed by staining the samples by1µM green fluorescent Calcein-AM and 0.2µ red-fluorescent Ethidium Homodimer-1 in PBS over 72 h, with 24 h interval.

The images show that cells' growth and proliferation, and viability are more significant in the PLC fibers containing nanoceria than the control cells and PLC fiber without nanoceria. The images of the 1st control samples, which are only RAW 264.7 cells without any nanoceria or PCL fibers, show some dead cells, where the number of dead cells is lowest in the images

containing PCL fibers with 0.1% nanoceria. This data supports the Alamar Blue results of increment of cell viability and proliferation with cerium oxide nanoparticles.

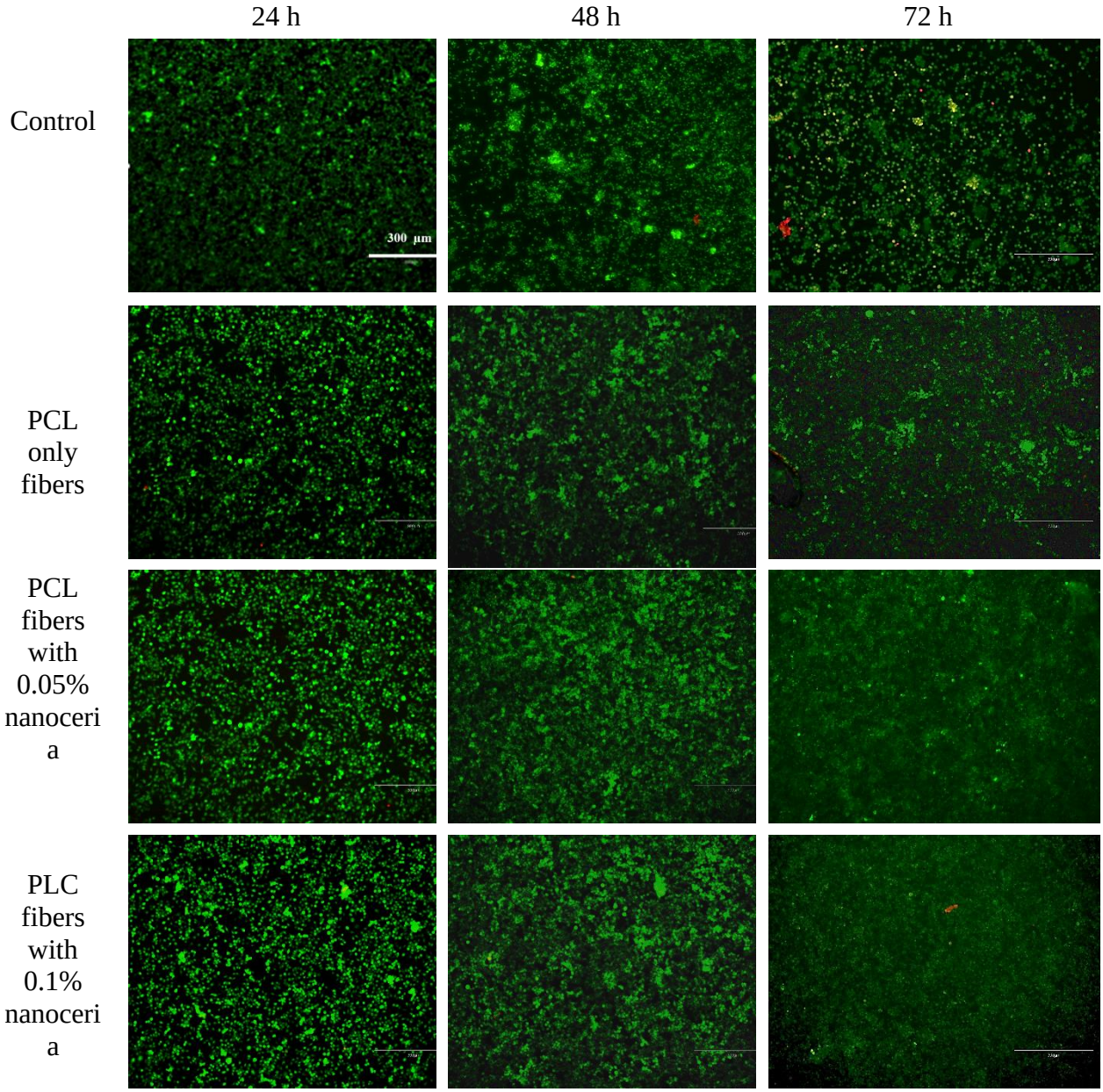


Figure 3.8 RAW 264.7 cell viability and proliferation study on PCL-only, PCL fibers with 0.05% CeO_2NPs and PCL fibers with 0.01% CeO_2NPs after 24, 48 and 72 h as measured by

fluorescence imaging. Cells were stained using 1µM green fluorescent Calcein-AM and 0.2µ red-fluorescent Ethidium Homodimer-1.

3.4 Conclusion

In conclusion, we presented a simple and effective technique for encapsulating cerium oxide nanoparticles via electrospinning. In this technique, a basic rotating-drum electrospinning device was used to fabricate the PCL fiber mesh where cerium oxide nanoparticles were encapsulated successfully, confirmed by EDS data. The TEM images show that the electrospun PCL fibers, with and without cerium oxide nanoparticles, maintained uniform morphology and consistent diameter. The water contact angle test confirmed the enhancement of hydrophilicity of the fiber surface due to the encapsulation of nanoceria. The proliferation and viability data of RAW 264.7 cells on the fibers demonstrated the biocompatibility of the PCL fibers, with and without nanoceria. The cell viability data demonstrated that the rate of cell proliferation on the PCL fibers with 0.1% CeO_2NPs was highest, and this trend was constant for 72 h. Hence, we could say that the encapsulated CeO_2NPs that would have released in the cell culture medium improved cell survival by decreasing the intracellular levels of Reactive Oxygen Species (ROS). Therefore, it can be concluded that, the anti-inflammatory properties of cerium oxide nanoparticles remain effective while encapsulated within the PCL electrospun fibers.

CHAPTER 4

APPLICATIONS AND FUTURE WORKS

4.1 Applications

The nanoceria-polymer composites enhance the properties of both nanoceria and polymers. Therefore, the application of nanoceria-polymer composites has been growing for the past few years. This study showed the successful encapsulation of cerium oxide nanoparticles in PCL fiber, proving its biocompatibility. Some potential applications using this study's model could be:

1. This system can be used to develop tissue engineering scaffolds (three-dimensional scaffolds, artificial-niche, or Electrospun fiber scaffolds) that can be applied for replacing, remodeling, or repairing whole tissues or their parts.
2. Another major application of this system could be in targeted and controlled delivery of therapeutic nanoceria.
3. Several studies show the antibacterial and antiviral effects of ceria-containing materials. Also, the encapsulation of cerium oxide nanoparticles can improve the rate of wound healing and decrease the severity of the treatment process [90], [91], [92], [93], [94]. Therefore, nanoceria-loaded electrospun fibers can be used to develop antibacterial and antiviral products. Additionally, it can be used to develop wound dressing materials that will promote wound healing.

4.2 Future works

Due to time limitations, some important studies have not been performed which need to be addressed in the future.

1. In our study, the degradation rate of the PCL fibers with and without cerium oxide nanoparticles was not studied. Additionally, the impact of the amount of cerium oxide nanoparticles on the degradation rate of the PCL fibers could be studied. As a more advanced study, the degradation rate of PCL fibers, with and nanoceria, within a biomimetic model will show a better understanding of the degradation behavior of PCL fibers in-vivo.
2. Determining the rate of release of cerium oxide nanoparticles into the cell medium is a critical study that could be well-addressed. Moreover, a long-duration study should be carried out to determine the effectiveness of cerium oxide nanoparticles as an active ROS scavenger over time, which could be a good indicator to determine if the nanoceria encapsulated polymers remain effective as implantation.
3. Another important study should be regarding the antioxidant activity of the PCL fiber encapsulated cerium oxide nanoparticle. In our study, the increased and steady viability of cells on the PCL fibers with 0.1% cerium oxide nanoparticles was observed and based on this trend; we concluded that the release of nanoparticles in the cell medium is acting as an antioxidant agent. for further verification, some other antioxidation detecting test could be carried out.

CHAPTER 5

CONCLUSION

method for encapsulating cerium oxide nanoparticles via electrospinning. In this method, a fundamental rotating-drum electrospinning device was used to produce the PCL fiber mesh, and cerium oxide nanoparticles were encapsulated in the mesh. The evidence of encapsulation was confirmed by EDS data. The electrospun PCL fibers, with and without cerium oxide nanoparticles, maintained uniform morphology and consistent diameter, which was confirmed by SEM imaging. After that, the water contact angle test proved the improvement of hydrophilicity of the fiber surface caused by the encapsulation of cerium oxide nanoparticles. The proliferation and viability data of RAW 264.7 cells on the fibers demonstrated the biocompatibility of the PCL fibers, with and without nanoceria. The cell viability data demonstrated that the rate of cell proliferation on the PCL fibers with 0.1% CeO2NPs was highest, and this trend was constant for 72 hours. Hence, we could say that the encapsulated CeO2NPs that would have released in the cell culture medium improved cell survival by decreasing the intracellular levels of Reactive Oxygen Species (ROS). Therefore, it can be concluded that, the anti-inflammatory properties of cerium oxide nanoparticles remain effective while encapsulated within the PCL electrospun fibers.

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