Investigating Ceria Nanocrystals Uptake by Glioblastoma Multiforme Cells and its Related Effects: An Electron Microscopy Study

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ABSTRACT

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Cerium oxide nanoparticles have been utilized widely nowadays in cancer research. It has been suggested by many studies that these nanoparticles are capable of having dual antioxidant behavior in healthy and cancer microenvironment; where in physiological condition, they act as antioxidant and do not affect the healthy cells, while in tumor-like condition; they act as an oxidase, and result in a selective killing for the cancer cells. In this experiment, the interaction of nanoceria with glioblastoma and healthy astrocyte cells was examined, and further correlated with the in vitro cytotoxic effects of various nanoceria concentrations (100 and 300 µg/ml) and exposure times (12, 24, and 48 hours). Electron microscopes were used to investigate the cellular-NPs interactions, and to examine the related cytotoxic effects in combination with trypan blue and propidium iodide viability assays. Our data suggest the following results. First, the two cell lines demonstrated capability of taken up the ceria through endocytosis pathway, where the NPs were recognized engulfed by double membrane vesicles at various regions over the cellular cytoplasm. Secondly, cerium oxide nanoparticles were found to affect the glioblastoma cells, but not so severely the corresponding healthy astrocytes at the various concentrations and incubation times, as revealed by the viability assays and the electron microscopy analysis. Thirdly, the viability of the glioblastoma cells after the treatment displayed a declined trend when increasing the ceria concentrations, but did not
show such dependency with regard to the different time points. In all cases, the healthy astrocyte cells showed slight alterations in mitochondrial shape which did not influence their viability. Among the various nanoceria concentrations and exposure times, the most efficient dose of treatment was found to be with a concentration of 300 µg/ml at a time point of 24-hour, where higher reduction on the viability of glioblastoma cells was achieved, with minimal toxicity to the healthy astrocyte cells.
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LIST OF ABBREVIATIONS

BF  bright field
BMSCs  bone marrow stromal cells
BSE  backscattered electrons
CCD  charge-coupled device
CDN  cerium dioxide nanoparticles
Ce  cerium
CNS  central nervous system
CPD  critical point drying
CTRL  Control
DF  dark field
DMEM  Dulbecco’s Modified Eagle’s medium
DNA  Deoxyribonucleic acid
D-PBS  Dulbecco’s phosphate buffered saline
EDX  energy Dispersive X-Ray
EELS  electron energy loss spectroscopy
EM  electron microscopy
ER  endoplasmic reticulum
ETC  electron transport chain
FACS  fluorescence-activated cell sorting
FEG  field emission gun
GBM  glioblastoma multiforme
HMDS  hexamethyldisilazane
LaB₆  lanthanum hexaboride
LM  light microscopy
MEEETES  (6-[(2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy]-hexyl)triethoxysilane)
NADPH  nicotinamide adenine dinucleotide phosphate
NPs  nanoparticles
OXPHOS  oxidative phosphorylation
PDGF  platelet-derived growth factor
PI  propidium iodide
ROS  reactive oxygen species
RT  room temperature
SOD  superoxide dismutase
SPM  scanning probe microscopy
SEM  scanning electron microscopy
SEs  secondary electrons
TEM  Transmission electron microscopy
TMZ  temozolomide
UV  ultraviolet
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CHAPTER 1: INTRODUCTION

1.1 Cancer as a microevolutionary process

1.1.1 Cancer hallmarks

Cancer is defined as a group of diseases that arise from mutations in the DNA sequences of the cell genome, which lead to bypass the normal molecular networks that control cell proliferation, differentiation, and cell death, with the potential to colonize territories belong for other cells. [1] Despite the diversity of cancer types, which are reported to exceed a hundred distinct types, with many other subtypes in each organ, [2] all cancers share some features that distinguish them from other diseases, which are known as the cancer hallmarks. Such features define the physiological alterations that start with mutations in a single cell and develop to form tumors that behave autonomously, in deviation to the normal cellular regulatory circuits. The cancer hallmarks are six essential features, that were first postulated by D. Hanahan and R. Weinberg in 2000, as guidelines to dictate malignant growth, and still accepted, as revealed by a huge number of citations and as reviewed by many recently published studies. [3] These six features are: “self-sufficiency in growth signals, insensitivity to antigrowth signals, apoptosis resistance, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis”, [2] which will be discussed with some details in the next paragraphs.

The first of these features is the capability of self-sufficiency in growth signals, which allow cancer cells to proliferate by mimicking normal growth signals, even without exogenous growth stimulation from neighboring cells. For instance, glioblastoma, which is a highly invasive tumor that affects the brain, has the capability to
manufacture PDGF (platelet-derived growth factor) that influence a positive feedback proliferation loop, which obviates the dependence on external growth factors from other cells within the brain tissue. [2] [4] On the other hand, cancer cells are insensitive to antigrowth signals, the feature that also leads to alter the normal control of growth. [2] An example of this insensitivity is displayed by the loss of contact inhibition in cell culture, the mechanism that functions to keep cells growing into a monolayer, and stop proliferating whenever contacting each other, but, cancer cells lose this property and pile up on top of one another forming overlapping layers of cells. [5]

Another distinctive feature of cancer cells is the ability to resist apoptosis, mainly through the alteration of apoptotic signaling pathways. Mitochondria play an essential role in apoptosis, because in the normal situation most of the apoptotic signals converge on the mitochondria, which in turn respond by releasing the enzyme cytochrome c that catalyze the apoptosis mechanism. [2] However, cancer is known to affect mitochondria functions throughout the alteration of the glucose metabolism, by which normal cells rely mainly on the oxidative phosphorylation (OXPHOS) mechanism as a source of energy supply, which is performed in the mitochondria under aerobic conditions, and at the same time inhibit the cytosol glycolysis, while cancer cells exhibit an increase in glycolysis even in the presence of oxygen, [6] as shown in figure 1. This phenomenon is termed aerobic glycolysis or Warburg effect [7] from Otto Warburg, who first described the phenomena in 1956.

Aerobic glycolysis leads to many consequences not only for the mitochondria functions and shape, but also to the cellular microenvironment as a whole. First, with
regard to the mitochondria, such behavior decreases the rely on mitochondria as a main source of energy supply, which result in affecting the mitochondria shape, since mitochondrial morphology is altered according to cellular energy state, as illustrated in figure 2, where glioblastoma multiforme (GBM) is used as an example of malignant tumor. [6] In the OXPHOS mechanism the mitochondrial network seems interconnected with enlarged cristae compartment, and therefore the mitochondria preserve an elliptical shape, whereas in aerobic glycolysis mechanism the mitochondria matrix is expanded and the intra-cristae space is reduced, and thus the mitochondria appear in spherical shape. Such impairment also leads to defects in the electron transport chain (ETC), which results in insufficient production of nicotinamide adenine dinucleotide phosphate (NADPH), and thus excessive production of reactive oxygen species (ROS), the reactive chemical molecules that increases the cellular oxidative stress.

Figure 1: Warburg effects (aerobic glycolysis) in tumor cells glucose metabolism. A: shows the normal mechanism where most of the sugar converted into energy in the
mitochondria. B: shows the effect of tumorigenesis and proliferation on sugar metabolism, where most of the sugar (85%) is converted in the cytosol even in the present of $O_2$. [1]

Secondly, with regard to the cellular microenvironment, aerobic glycolysis leads to the conversion of cytosolic glucose into lactic acid, which in turn lead to the acidification of the extracellular environment. [6] As a result of this mechanism, cancer microenvironment exhibits more acidity than the corresponding normal tissue. Such postulation is proved in a number of studies; by which it is nowadays confirmed that cancer tissues are 0.3-0.5 pH units lower than healthy tissues. [8] For instance, in soft tissue sarcoma, a malignant tumor that affects connective tissue, the pH is about 0.45 unit lesser than the corresponding healthy tissue. Besides, other studies confirmed that in breast cancer the pH is 0.35 unit lower than the corresponding breast tissue and in glioblastoma the pH is 0.2 unit lower than the pH of the corresponding brain tissue. [8]

![Figure 2: Mitochondrial shape alterations according to energy state in cancer and healthy cell. A: Schematic representation of the mitochondria shape alteration in cancer cell comparing to that of healthy cell. B: Typical structure of a normal and glioblastoma mitochondria (GBM). See text for details. [6] [9]]
The concomitant above-mentioned features give rise to the fourth hallmark of cancer, which is the capability for limitless replicative potential. All healthy cells have an intrinsic program that limits their replication, such a limit is known as Hayflick limit, which illustrates that most mammalian cells undergo between 40-60 divisions before slowing down and eventually stop dividing. [10] In contrast, cancer cells disrupt such a program and continue the process of replication bypassing the mentioned restriction. [2] For example, glioblastoma cells exhibit increased mitosis activity and elevated cell density, which refers to the ability to produce large number of cells away beyond Hayflick limit in a short time. [11] The fifth hallmark of cancer cells is the ability to sustain angiogenesis, by developing new mechanisms to form new blood vessels to get nutrition and oxygen. [2]

The last hallmark of cancer cells is the capability to invade tissues and produce metastases. This property relies on the other five hallmarks, and reported to cause 90% of cancer deaths in human. [12] This feature of the cancer cells consists in their capability to migrate from the host tissue to invade and form colonies in other body tissues. In cultured cells, many cancer cells exhibit increased membrane ruffling, which is indicated by the formation of a motile cell surface that contains a meshwork of newly polymerized actin filaments and filopodia formation. This ruffling is a characteristic feature of migrating cells, and has been shown to be related to metastatic status and the invasion capability of tumor cells. [2] [13] [14]

1.1.2 Cancer classification

However, although cancers share the above-mentioned hallmarks, it is important to highlight that cancer exists in many diverse types, and is not considered as a single
disease, but rather a complex array of many diseases. Different cancer forms exhibit different degree of capability to more or less display the mentioned hallmarks. Moreover, this diversity does not only create a state of inter-tumor heterogeneity between different tumors, but also gives rise to intra-tumor heterogeneity, which consists in the presence of different cells within a given tumor. For instance, gliomas, which are subtypes of brain cancer, have higher tendency toward malignancy than basal cell carcinoma, which rarely metastasizes, [15], and furthermore cells within glioblastoma tumor exhibit diversity in shape, size, and cellular structure. [16]

Cancers are classified according to the tissue type and cell line; from which they are initiated, and sub-classified according to the ability of spreading into benign tumors, which lack the ability to invade adjacent cells, and malignant tumor, which destroy the integrity of the tissue and spread over neighboring cells. For example, adenoma, [17] is a benign epithelial tumor with a glandular structure, whereas the corresponding malignant form of this tumor is adenocarcinoma. [1] Similarly, the term glioma refers to the tumor initiated in the glial cells of the brain or the spinal cord, and sub-classified based on the cell lines or the pathologic evaluation grades according to the World Health Organization Classification of Tumors. [18]

Nevertheless, in the next section gliomas, and more specifically glioblastoma multiforme (GBM), would be discussed in details, with regard to the classification, characteristics, behavior, and pathophysiology, by which this specific form of cancer would be the focus of this study.
1.1.3 Gliomas: The cancer of glial cells

Tumors of the central nervous system (CNS) have many classifications, due to the complexity of the genetic basis of oncogenicity and the capability toward malignancy, beside the diversity of cell types, from which the tumor is first initiated. For example, in the 2016 World Health Organization Classification of Tumors of the CNS, tumors were classified into 46 distinct types, with 155 subtypes. [18]

Overall, CNS tumors are classified into primary brain tumors, which start within the brain tissue and tend to stay in the brain, and secondary brain tumors, also called metastatic tumors, which refer to tumors that migrate from somewhere else, mainly breast or lung tumor, to invade the brain tissue. The incidence and prevalence of the metastatic tumors are by far more frequent than the primary tumors, which outweigh about 4:1 ratio. [19] Primary brain tumor, on the other hand, is classified into many subtypes, according to the affected cell type, with gliomas being the most common intracranial malignant tumors, [20] which account for 80% of all brain malignant tumors, and the second most common primary brain tumor, with around 27% of all brain tumors, after meningiomas, which represent 36.4%. [21]

Gliomas, as introduced above, refer to the tumors that affect glial cells, which are non-neuronal cells that provide support and protection for neurons in the nervous system. Glial cells outnumber neurons in the nervous tissue, and exit in three different type of cells, astrocytes, oligodendrocytes, and ependymal cells. [22] Astrocytes, which are the cell of focus in this study, are more numerous than the other two types of glial cells. They are star-shaped cells, with no axon or dendrites, but rather characterized by multiple end-feet that connect the cells to the capillary walls, to assist the maintenance of the blood-
brain-barrier, or to connect to the synapses, to encapsulate the neurotransmitters release. Moreover, astrocytes have many other functions that relate to the structural support of the brain, maintaining homeostasis, and repairing injured nerve cells. [23]

Each of the three mentioned glial cells give rise to a distinctive type of tumor, which corresponds to the three classes of gliomas: astrocytomas, oligodendrogliomas, and ependymomas, as illustrated in figure 3. [24] Astrocytomas are the most common tumors of glioma, and represent approximately 75% of all gliomas. [21] They are classified, according to their invasion’s potential and the tendency toward malignancy, into four different grades, by which grade IV astrocytomas, or commonly known as glioblastoma multiforme (GBM), is the most aggressive primary brain tumor. [25]

![Glioma classification diagram](image)

Figure 3: Glioma classification. As described in text, glioma accounts for 80% of all brain malignant, and astrocytomas represent 75% of all glioma. GBMs constitute about 15% of brain tumor and 55% of all glioma [21].
1.1.4 Glioblastoma as a fatal disease

GBM corresponds to 55.1% of all gliomas, and 15.1% of all primary brain tumor. [21] It is the most quickly growing astrocytomias, and thus, described in the literature as a fatal disease. [25] The rate of survival with this tumor is less than a year, with only 6% survival rate after five years of diagnosis, [24] and a final mortality rate of nearly 100%. [25] Up to the present time, only rare curative outcome and enduring survival of glioblastoma cases have been reported in the literature and the specialized studies. [26] [27] [28] [29] The aggressive malignancy, rapid spreading, and heterogeneity in molecular profiles, along with the lack of effective treatments result in complicating the diagnosis process of glioblastoma, making this disease one of the most malign clinical prognoses.

Glioblastoma multiforme shows high capabilities toward the six cancer hallmarks described above. Beside that, it is characterized microscopically by the elevated cell density, the resistance of the normal programmed death (apoptosis) but rather undergoing necrosis, due to genetic mutations on the apoptotic factors, [30] and the microvascular proliferation with increased tumor angiogenesis, as demonstrated in figure 4. Additionally, glioblastoma preserves increased mitotic activity, which along with the other mentioned histological features display higher rate of the limitless replicative potential, the fourth cancer hallmarks, and results in an infiltrative tumor mass. [31]
Furthermore, GBM displays nuclear and cellular anaplasia, which is the reason behind describing glioblastomas as "multiforme": it refers to its poor differentiation capability, and losing some of the morphological features of mature cells with regard to each other. [20] This property leads, in many cases, to a state of de-differentiation, or backward differentiation, which brought to hypothesize that glioblastoma keeps the characteristics of cancer stem cells. [34] [35] As a result of anaplasia, glioblastomas display distinct nuclear pleomorphism, i.e. the variability of nucleus size and shape, by which cells may either have expanded nuclei that reach 1:1 nucleus-cytoplasmic ratio, or have multiple nuclei in a single cell. [16]

However, the above-mentioned diversity gives rise to a state of intra-heterogeneity between cells within the glioblastoma. This diversity does not only complicate the process of diagnosing the tumor, but also challenges the task of designing
effective therapies. Tumor sampling is a common practice in diagnosing tumors, by which a biopsy of a tumor is analyzed in order to design the effective therapy, but, in case of heterogeneous tumors, this analysis would be misleading since a single sample would lack important information about other regions of the tumor. [16]

1.1.5 Efficacy of current treatment options of GBM

The ultimate goal of cancer therapy is to target a tumor specific molecular abnormality with accuracy, efficacy, and potentially less toxicity to surrounding healthy tissue. [36] However, to date, none of the available treatment options of GBM satisfies these requirements, being that supported by the low survival rate and the low improvement of the quality of life of patients suffering from this disease. These options, which are similar to those to treat any other malignant tumor, are resection surgery, chemotherapy, and radiotherapy, through which each possess particular challenges that limit its efficacy, as will be explained below.

First, the resection therapy is the first stage for treating GBM, which aim to reduce the GBM tumor mass that has an average of $10^{11}$ cells, to an average of $10^{9}$ cells. [37] However, brain is a difficult organ with regard to managing the side effects of surgical therapies, and the removal of portion of one hemisphere would not only lead to bad consequences to the patient’s quality of life, but it is also useless due to the infiltrative nature of GBM, since the tumor would likely occur again on the other hemisphere of the brain. [38] Also, statistics shows that in the best-case scenario, and after a successful surgical removal of 90% of GBM tumor, patients have around 66% of survival at 2 years. [24]
Secondly, radiotherapy and chemotherapy can also be used to treat GBM, by which they are combined by the administration of temozolomide (TMZ) followed by a radiation treatment. Nevertheless, this treatment results only in improving the survival rate from 12 up to 15 months. [39] Furthermore, both techniques have other limitations, by which chemotherapy is obstacle by the selectivity of the blood brain barrier, which prevents chemical and toxins from passing into the brain tissue, and radiotherapy results in inflammation around the radiated area, and may affect blood vessels in the brain tissue, and thus, cause neurological late side effects on the brain functions including cognitive disabilities. [38] [40]

Therefore, from the discussion reported above it can be concluded that GBM challenges the treatment process for the several limiting factors just listed. Hence, this highlights the need of designing a new approach that could overcome these limitations and better satisfy the declared requirements of a successful cancer therapy. One of these approaches, which has been investigated widely in cancer research nowadays, is the use of nanoparticles to selectively target tumor as will be discussed in the next section.

1.2 Cerium oxide nanoparticles as agents for cancer therapy
1.2.1 Nanoparticles in medicine

Nanoparticles have been used widely nowadays in medicine for multiple purposes. They are used as components of targeted drug delivery systems, for contrast enhancement in bioimaging, as luminescent and magnetic agents, and more importantly for cancer diagnosis and therapy. For example, gold, silver, and zinc NPs have been used as photosensitizers in photo-thermal therapy, [41] and similarly, iron oxide and cobalt ferrite NPs have been studied exclusively in magnetic hyperthermia treatments. [42] On
the other hand, cerium oxide NPs have shown very promising results, as agents for redox-directed cancer therapy, due to their unique characteristics toward the redox selectivity and the relative low cellular toxicity. [8]

1.2.2 Redox activity of nanoceria

Redox directed approach for cancer therapy relies on using agents that either selectively induce or protect from oxidative stress, under certain condition. This selectivity should be determined by the agent capability to distinguish between healthy and cancer cells. [43] As discussed before, one of the main characteristics that distinguishes between healthy and cancer cells is the microenvironments’ pH value, by which in GBM is reported to be 0.2 pH unit lower than healthy astrocytes, due to the process of aerobic glycolysis mechanism of malignant tumor. [8] Therefore, the selectivity of the redox directed approach can be achieved by using substrates that respond to variation in pH value of the medium. Cerium oxide nanoparticles, also known as nanoceria, is very promising in this regard, since one of the main characteristics of these nanoparticles is their ability to change their antioxidant properties with pH through the switch between Ce$^{3+}$ and Ce$^{4+}$ state. [8] [44] as will be explained below.

Cerium atom (Ce) preserves electron configuration with two partially filled orbitals, 4$f$ and 5$d$, which allow it to exist in either a fully reduced state Ce$^{3+}$ or in a fully oxidized state Ce$^{4+}$. [45] Cerium oxide is formed when a cerium atom in the oxidized state (Ce$^{4+}$) is combined with oxygen at the nanoscale, which results in a fluorine-like crystalline structure. Nevertheless, this structure is accompanied with intrinsic defects that arise from the presence of some Ce$^{3+}$ on the lattice structure, which facilitate redox reactions. The cerium atom, in this regard, preserves a unique property, through which it
can easily switch between Ce³⁺ and Ce⁴⁺ to adjust its electron configuration to better fit its environment, which suggests a dual role of cerium oxide NPs as an oxidation catalyst and a reduction catalyst, based on the reaction conditions. [46]

Recently, Korsvik and colleagues [47] have demonstrated the ability of cerium oxide NPs to mimic the activity of two enzymes that play an essential role in protecting cells from free radicals through the elimination of reaction oxygen species (ROS), which are superoxide dismutase (SOD) and catalase. SOD is an enzyme that protects cells through the dismutation of superoxide radical (O₂⁻) into O₂ and H₂O₂, while catalase is the enzyme responsible for the conversion of hydrogen peroxide H₂O₂ into O₂ and H₂O. The chemical mechanisms of the nanoceria mimicking SOD and catalase were proposed by Celardo and co-workers [48] as illustrated in figure 5A and B respectively.

![Figure 5: Nanoceria mechanism in mimicking SOD and catalase activities. A: Nanoceria mechanism for the dismutation of superoxide into oxygen and peroxide (mimicking SOD activity). B: Nanoceria mechanism for the dismutation of peroxide into oxygen and water (mimicking catalase activity). Modified from [48]](image)

According to many recent studies, the pH value influences the catalytic properties of nanoceria. [44] [49] [50] Under physiological pH, the SOD and catalase-mimetic activity of nanoceria lead to the conversion of superoxide into peroxide followed by the
conversion of peroxide into water and oxygen, and thus, nanoceria provides the protection for healthy tissue through the removal of ROS, as described above. However, under acidic pH (tumor-like environment) the SOD-mimetic activity of nanoceria is not affected, but the catalase-mimetic activity is significantly diminished, which lead to the accumulation of peroxide that lead to the selective killing of tumor cells through the oxidative stress. [46]

The cellular mechanisms underlying these reactions were demonstrated in another study, [51] which describes the effect of nanoceria on mouse bone marrow stromal cells (BMSCs) as represented in figure 6. The mechanism shows that with healthy cells nanoceria it prevents the formation of \( \text{H}_2\text{O}_2 \) by the switching from \( \text{Ce}^{4+} \) to \( \text{Ce}^{3+} \) oxidative state, which leads to maintain the normal shape and function of mitochondria, and thus, protects the cell. Conversely, with cancer cells the opposite result of nanoceria oxidative switch is achieved, \( \text{Ce}^{3+} \) to \( \text{Ce}^{4+} \), which results in inducing ROS formation, leading to two main consequences: causing DNA damage in the nucleus and altering mitochondria shape and function, through affecting the mitochondria membrane potential that regulates matrix configuration and cytochrome c release. The two effects finally result in inducing the selective killing of the cancer cells by apoptosis.
Figure 6: The mechanism of cerium oxide NPs protecting BMSCs from apoptosis induced ROS formation. See text for details. [51]

The assumption of the selective protection of nanoceria toward healthy cells under oxidative stress has been validated by Zholobak and colleagues in a pioneer experiment, [52] as illustrated in figure 7. The goal of this experiment was to investigate the protective effects of nanoceria on two types of cells, healthy swine testicular cells (ST) versus laryngeal carcinoma cells (HEp 2), under oxidative stress conditions caused by UV radiation or the introduction of H₂O₂ solution. First, both cell lines were treated with cerium dioxide NPs (CDN) and exposed to UV radiation to induce oxidative stress, and the result showed high protection by nanoceria toward both the healthy and the malignant cells, as demonstrated in the histograms reported in the bottom part of Figure 7. Then, cells were exposed to a hydrogen peroxide H₂O₂ solution, which severely affect the viability in both cell types. Lastly, healthy and malignant cells were injected with nanoceria and exposed to a hydrogen peroxide solution, and the result was almost full protection toward healthy cells, while vast effects on the cell viability of the malignant cells caused by oxidative stress, as displayed in the last column. [8]
1.2.3 Cellular uptake of nanoceria

There are many factors that influence the cellular uptake of cerium oxide NPs, such as NPs size and shape, surface charge, degree of crystallinity, oxygen stoichiometry stability, along with other factors. [8] These factors have been studied and modified in a number of works, in order to enhance the uptake mechanism of nanoceria by cells. For instance, a study shows that nanoceria smaller than 5 nm results in better cellular uptake and lower cytotoxicity toward healthy cells. [53] Also, another study illustrates that the coating of nanoceria with positively charged particles result in better affinity toward the cell membrane, which is negatively charged, similar to other NPs, as shown in figure 8. Also, the more positive zeta potential results in better absorption, which demonstrates that electrostatic interactions are crucial in cellular uptake of NPs. [54]
On the other hand, concerning the mechanism of the cellular uptake of nanoceria, many studies suggest an uptake by endocytosis as underlying mechanism, as reported in figure 8. [8] [55] Zhang et al. illustrated that cerium oxide NPs are taken up by multiple endocytosis mechanisms in bone marrow stromal cells, followed by the even distribution in the cytoplasm without entering the nucleus. Another significant result of that study is the illustration that nanoceria, while on the cytoplasm, maintain the normal shape and function of mitochondria to reduce the amount of ROS. [51] Another study, which utilized fluorophore-conjugated-nanoceria, elucidates the widespread of nanoceria in mitochondria, lysosomes, endoplasmic reticulum (ER), beside the abundant spread in the cytoplasm and the nucleus of keratinocyte model [56]. This study also supports the uptake of the nanoceria through clathrin-dependent endocytosis from lipid rafts, and reveals the wide spread of nanoceria as an indicator for the efficiency of the redox-directed cancer therapy. [56]

Figure 8: Scheme of interaction between ceria NPs and cell membrane with different surface charge. a: low affinity of uncharged and negatively charged NPs. b: high affinity
of positively charged NPs. Also, the scheme illustrates the endocytosis mechanism of the cellular uptake of NPs. [8]

Moreover, a third study indicated that the localization and internalization of nanoceria in lung carcinoma cells plays a critical role in defining the nanoceria cytotoxicity profile. It illustrated that uncharged nanoceria internalize and localize frequently into the cytoplasm of cells showing no cytotoxicity, while the positively or negatively charged coated nanoceria can localize either in the cytoplasm or the lysosomes, based on the cell type. The localization of charged nanoceria in the cytoplasm still shows no cytotoxicity, while the localization in the lysosome exhibits toxicity, by which the low pH of lysosome activates the nanoparticle oxidase activity, as illustrated in figure 9. [8] [57] [58] A further study also indicated that the localization of nanoceria in the nucleus is also toxic, and leads to genomic effects: it applied functional genomic analysis to investigate the effects of naked nanoceria on mouse neuronal cells, and demonstrated that these nanoparticles contributed more than 83% of gene alterations associated with neurological diseases and cell cycle regulations. [59]
Figure 9: The localization and internalization of differently charged cerium oxide NPs define their cytotoxicity profile. Neutral nanoceria tend to reside in the cytoplasm showing no toxicity, while charged nanoceria localize, based on the cell type, either in the cytoplasm, where no cytotoxicity is observed, or in the lysosome, where their acidity activates the oxidase exhibiting toxicity. Coating agents used: dextran (neutral), PAA(−): Poly(acrylic acid); APAA(+) : Aminated PAA [8]

1.2.4 Cerium oxide NPs applications in glioma treatment

Due to the above-described selectivity of nanoceria to differentiate between tumor and healthy cells, these nanoparticles have been investigated widely, in the last few years, as agents for the treatment of brain tumors. Schubert et al. [60] demonstrate that cerium oxide NPs aid in protecting healthy nerve cells (HT22 cell line) from oxidative stress through the significant reduction of the ROS formation. A similar result was also reported by Das and colleagues, [61] where the uptake of CeO₂ NPs suggest neuro-protection to adult rat spinal cord neurons. Contrarily, Minchenko and co-workers. [62] show that nanoceria cause toxic effects on human astrocytes (NHA/TS cell line) through the alteration of genes responsible for controlling cell proliferation.
On the other hand, Park et al. [63] analyzed the effect of nanoceria on a human glioblastoma cell line (T98G), with a nanoceria concentration of 5 μg/ml, at various incubation time of (24-48-72-96 h) and NPs size (15, 25, 30, 45 nm), in correlation with GBM cells viability. In this study, it was observed that the cell viability was slightly decreased in a time dependent manner, but also that different NPs size cause no effects on the viability tests. The slightly decrease in viability suggests that higher concentration is required for more efficient treatment. When tracking the NPs internalization, it was observed that NPs were taken up into the cytoplasm of GBM cells in about 1.5 h, and within 3-12h the NPs were located and accumulated around the nuclear envelope, where they might induce direct interaction with cellular molecule to cause cellular response.

As clarified above, cerium oxide NPs and cellular interaction is influenced by many factors, but more importantly the exposure concentration and time that allow better internalization of the NPs to induce cellular responses. Still, several studies refer to the fact that cells response to nanoceria changes from one cell line to another, [44] [64] [65] and for that reason a literature review was performed to identify the ranges of cerium oxide NPs concentrations and incubation times that were used specifically with nerve and glial cells, along with their corresponding malignant tumors, on published studies [61-77]. Table 1 below shows the result of the most five relevant studies, which demonstrate variable concentrations that range from 5-500 μg/ml, with incubation times of 20h-4days. These findings allow the estimation of the most promising concentrations for astrocytes and GBM cells to be within 100-300 μg/ml, with incubation times of 12-48 hours.
However, the cellular-nanoceria interactions still need further investigations, and then more efforts should be done to investigate the cellular-NPs uptake. [66] This fact highlights the demand to examine such interactions, using tools that are capable of tracking the uptake mechanisms at the cellular scale. Electron microscopy, in this regard, is an efficient technique for performing such an analysis, due to its high resolution, up to the nanoscale, along with the concomitant capability of performing chemical analysis even in biological specimens through technique like Energy Dispersive X-Ray Spectroscopy (EDX), as will be explained in the next section.

Table 1: The most relevant published studies of glioma-nanoceria interaction.

<table>
<thead>
<tr>
<th>Cell Model</th>
<th>[CeO₂]</th>
<th>CeO₂ Incubation time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human glioblastoma (T98G)</td>
<td>5 μg/ml</td>
<td>24-48-72-96 h</td>
<td>[63]</td>
</tr>
<tr>
<td>Human astrocytes (NHA/TS)</td>
<td>170 and 340 μg/ml</td>
<td>20 h</td>
<td>[62]</td>
</tr>
<tr>
<td>Nuroblastoma (SH-SY5Y)</td>
<td>100 μg/ml</td>
<td>24 h</td>
<td>[67]</td>
</tr>
<tr>
<td>Nerve cell line (HT22)</td>
<td>100 μg/ml</td>
<td>20 h</td>
<td>[60]</td>
</tr>
<tr>
<td>Rat brain (in vivo)</td>
<td>30-100-500 μg/ml</td>
<td>Perfusions</td>
<td>[68]</td>
</tr>
</tbody>
</table>

Other studies involved in the literature review for the most suitable nanoceria concentration include: [69] [70] [71] [72] [73] [74] [75] [76] [77]
1.3 Electron microscopy as a tool for examining cellular-NPs interactions

1.3.1 Basics of electron microscopy

Microscopy are classified into three main types: light microscopy (LM), scanning probe microscopy (SPM), and electron microscopy (EM). Light and electron microscopy are widely used in biological applications nowadays. They are similar in their working principle, but differ in their capabilities of magnifying objects and their resolving power, as demonstrated in figure 10. Light microscopy uses light as a source of illumination and transparent glass lenses that are capable of focusing light and magnifying objects up to 1000 times. LM is a very powerful tool in investigating biological specimen, also because it allows observing live specimens in their native environment. However, the resolving power of LM is restricted by a physical limitation related to the light wavelength used, which ranges from 380-750 nm, as seen in figure 11-A, and according to Rayleigh criterion, shown in figure 11-B, the best resolution that can be achieved out of this range of wavelengths is about 100 nm, when using a high refractive index medium. Therefore, the details of most of the cellular organelles cannot be clearly observed using light microscopy, these details requiring higher magnification and much higher resolving power. [78]
Figure 10: Resolving power of microscopies. Human eyes cannot see objects smaller than 100 µm, while LM provides up to 1kX enhancement in the resolving power and allows observing objects with size down to 100 nm. EM provides up to 1MX improvement in resolving power if compared to the human eye, allowing visualizing objects of few angstroms. [79]

Figure 11: Elements that define the LM resolution. A: The electromagnetic wavelengths of visible light. [80] B: Rayleigh criterion. [81] C: Refractive index values of selected media. [82]
Electron microscopy is similar to LM, as shown in figure 12, but instead of using light as a source of illumination and glass lenses for magnifying objects, it uses a beam of electrons and electromagnetic lenses, to overcome the physical limitation encountered when visible light is used. According to De Broglie law, electrons can travel in straight lines with wave-like properties, and can be then directed and focused using electromagnetic field, similar to the action performed by glass lenses in LM. Besides, De Broglie equation shows that the main advantage of using electrons is that their wavelength is about $10^5$ times shorter than that of light, and therefore, they enable a magnification that is $10^3$ times higher than LM, with an ultimate resolution that could be less than 0.1 nm. [78] [83]

Figure 12: Schematic representation of LM and EM major components. TEM is very similar, with regard to its working principles, to LM, except that LM uses light as a source of illumination and glass lenses, while TEM makes use of a parallel beam of electrons and electromagnetic lenses. SEM slightly differ with regard to the configuration of the lenses and the sample holder, and contains scanning coil for deflecting the beam into a raster fashion. [84]
Electrons interact with matter in different ways, as shown in figure 13, which can be classified into two different types, elastic scattering, when the incident electrons energy is completely conserved, and inelastic scattering, when some energy is transferred from incident electrons to the specimen to give rise to the production of several phenomena and related signals, as displayed in figure 13. Transmission electron microscopy (TEM) relies on the detection of the electrons transmitted by the sample they passed through, while scanning electron microscopy (SEM) exploits secondary and backscattered electrons emission by the irradiated sample to look at its external morphology. Both TEM and SEM, if equipped with EDX detectors, can use the X-Ray signal emitted by the sample for determining its chemical composition. [85]

Figure 13: Schematic representation of the electron interactions with matter. These interactions define the basics of the different electron microscopies; where SEM relies mainly on SE, TEM relies on TE, and EDX on characteristics x-ray. [86]
Despite the differences between the electron microscope types, they all share three main components: the electron source (also called electron gun), a vacuum system, and the same kind of lenses. The electron gun that is responsible for producing electrons, where three main types are used; tungsten, lanthanum hexaboride \((\text{LaB}_6)\), and field emission gun (FEG). Secondly, both TEM and SEM contain electromagnetic lenses made of multiple coils and responsible for condensing or spreading the electrons into a convergent or parallel beam. Another common feature is the vacuum system. The evacuation of the whole column is necessary to avoid the collision of electrons with gas molecules that cause both scattering and absorption of the electrons, in order to provide them with a mean free path with meter’s magnitude. Lastly, modern electron microscopes are equipped with digitally controlled electron detectors to form an image of the sample looked at. [87]

1.3.2 Types of Electron microscopy

Transmission electron microscopy

Transmission electron microscopy (TEM) has basically two imaging modes, which are called bright field (BF) and dark field (DF), respectively. BR imaging relies on signals created by electrons with no change in their original trajectory. The DF imaging is constituted by the electron diffracted by crystalline samples, and then is not of particular interest for observing biological matters [88] After having passed through the sample, the electrons are two-dimensionally projected into a fluorescence screen, for real-time imaging, as illustrated in the schematic diagram reported in figure 12, while by a CCD (charge-coupled device) camera is possible recording images.
**Scanning electron microscopy**

Scanning electron microscopy (SEM) relies mainly on secondary (SEs) and backscattered (BSE) electrons emitted from the sample. Due to the low energies of SE (~2 to 50 eV) they are ejected only from near-surface layers, being suitable for recording topographical information. They are collected using dedicated detectors which can be located in the specimen chamber in close proximity to the sample and/or inside the SEM lens. BSEs are characterized by a higher energy than SEs, usually ranging between 60 and 80% of the energy of the primary electron beam. They are able to provide topographical and chemical information about the sample. These electrons are usually collected by an annular detector placed at the very end of the pole-piece. After condensing the electron beam, it is passed through a scanning coil, which is responsible for scanning it onto a rectangular area over the sample surface. [78]

**Analytical electron microscopy**

Analytical electron microscopy refers to the use either SEM or TEM concomitantly with an ancillary spectroscopic technique, *i.e.* EDX spectroscopy and/or electron energy loss spectroscopy (EELS), the latter being possible just on TEM. These techniques are used to analyze the elemental composition of samples, even in a spatially resolved fashion. [89] EDX, which is the analytical tool that has been used in this study, relies on characteristics X-ray signals created by the interaction of the electron beam with the sample, as displayed in figure 13. The X-Ray photons are created when incident electrons excite core electrons of the atom and eject them from their own shells creating vacancies; then, electrons of higher energy shell fill the electron vacancies, by means of
the possible shell transitions as schematized in 14-A, and the difference in energy between the two shells involved could be released in form of an X-ray. Thus, counting X-rays as function of their energy allow to plot an EDX spectrum with different peaks, as shown in figure 14-B. Their intensity, via an appropriate treatment, allows to determine both which elements are contained in the sample and their amount. [90]

![Figure 14: EDX working principle. A: Atomic electron energy levels (K, L, M, and N). B: Representation of a typical EDX spectrum produced from a multi-element sample. [89]](image)

### 1.3.3 EM imaging of biological specimens

Biological sample features, such as physiological wet environments and considerable body volumes, make these specimens problematic to be imaged by electron microscopy. The high vacuum present in any EM, together with the intrinsic low contrast of any biological specimen’s thin slice in view of its TEM imaging, represent the main obstacles for ultrastructural analysis as seen in figure 15.

Therefore, they must be prepared to be placed in a high vacuum environment, usually by a process of chemical fixation and dehydration, which is the technique that has been used in this study, prior to loading them into the EM. The chemical fixation has three main goals that aim to overcome the mentioned drawbacks. First, it aims to fix and
dehydrate specimen in a controlled manner to avoid its external and internal structure damage, in view of both TEM and SEM imaging. The second aim is to make the sample mechanically stable to be cut into thin sections of less than 500 nm in case of need of TEM imaging, in order to allow electrons passing through them. Thirdly, it aims to stain the samples with heavy metal to enhance their contrast, being this again actually important for TEM imaging. On the other side and to improve the surface conductivity for the SEM, samples are coated by depositing a thin layer of heavy metal, such as gold or platinum. [91]

Figure 15: EM limitations with biological samples. A: Biological sample are wet and upon exposing them into the EM vacuum they will be dehydrated in uncontrolled manner. B: They are thick; while TEM require samples to be thin enough in order for electrons to pass through. C: they are composed of light elements that exhibit intrinsic low contrast in TEM. [92]

1.3.4 EM capability in analyzing NPs distribution in biological specimen

Engineered nanoparticles have been the heart of many recent researches in biology. Such a research field requires a complete understanding of the cellular nanoparticles interactions, which aims to define the cytotoxicity profile, through correlating the distribution and localization of nanoparticles within cells with the cellular responses to their exposure. [93] [94] Electron microscopy, mainly TEM, is considered
the standard technique for characterizing nanoparticles, [95] and has been widely used to perform their quantitative and qualitative analysis in terms of mean size and size distribution, morphology, even in case of their in biological applications, [96] [97] as recommended by the US Food and Drug Administration. [98] For instance, TEM was used to localize mesoporous silica NPs, [99] titanium dioxide NPs, [100] silver NPs, [101] and iron oxide NPs [102] in biological samples. Similarly, SEM were used to study the interaction between the external membrane of biological specimen with gold NPs [103] and silver NPs. [104]

Many other microscopy techniques have been utilized to study the localization of nanoparticles in biological samples, such as light microscopy, fluorescence microscopy, and Raman spectroscopy. [105] Even though these techniques provide an ease of use and high selectivity, as above-mentioned their main restriction is their limited resolution. Electron microscopy, on the other hand, also exhibits other limitations. First, EM is mainly utilized for the visualization of electron dense inorganic NPs, but it exhibits some challenges with organic NPs since these NPs show low contrast in cells and tissue. [106] Secondly, TEM requires staining biological samples with heavy metals, such as uranyl acetate and lead citrate to enhance the specimens intrinsic low contrast. However, possible agglomerates of these metals can be misinterpreted as NPs. [105] However, in this study, the latter problem was addressed by using spatially resolved EDX-based chemical analysis, while the former does not apply to cerium oxide NPs, which are electron-dense inorganic NPs.
Therefore, and due to the explained spatial resolution of electron microscopy, along with the capability to analyze the dispersion of nanoparticles in a given sample, this tool has been utilized in this study to examine the *in-vitro* cerium oxide nanoparticles internalization within GBM and astrocyte cells, along with evaluating the cellular responses to different doses of nanoparticles, and correlate that with the possible cytotoxicity in the examined cells.

1.4 Research objectives

1.4.1 Clinical need

Due to the aggressive tendency of GBM toward malignancy and heterogeneity, the high rate of tumor spread, beside the lack of effective therapy that leads to a very low survival rate, the experiment we report here have been designed first to image GBM cells and their relative healthy astrocytes from ultra-structural point of view. Second, they have been performed treating the cancer cells with the aim to use the selectivity of the redox property of ceria nanoparticles. Since they were expected to respond to changes in pH of GBM environment, which is reported in the literature to be 0.2 pH unit lower than the corresponding healthy astrocytes environment, it has been investigated if through which these NPs could selectively kill cancer cells, while providing radical scavenging function toward healthy cells.

1.4.2 Research goals

The ultimate goal of this experiments was to examine and characterize the interactions between rat GBM cell line (F98 ATCC® CRL-2397TM), along with the corresponding control, the healthy astrocyte cells (DI TNC1 ATCC® CRL-2005TM), and cerium oxide NPs. With this aim, viability assays and electron microscopy have been
used, to determine the efficacy of the cellular responses to two different doses of nanoceria (100 and 300 µg/ml) at 24h incubation time. These responses have been correlated with the internalization and localization of the nanoceria, and the possible ultra-structural alterations that might occur as a result of the cellular cytotoxicity in the two cell lines have been investigated. Then, the most effective concentration of nanoceria have been be used at different exposure times of 12, 24, and 48 hours. These are preliminary experiments that have aimed to determine a specific nanoceria concentration and exposure time that would be suitable for further studies.

1.4.3 Research design

In order to achieve the main aim of this research, the experiments were performed in two phases. The goal of the first phase was to select a proper ceria NPs concentration that could affect the glioblastoma cell line (F98), without harming the healthy astrocyte cell line (CRL 2005). This task has been established first by consulting the literature, already mentioned above, in order to identify a range of nanoceria concentrations that is believed to induce the desired effects on F98 and CRL 2005 cell lines. Based on that, the nanoceria concentration was found to be most effective in the range of 100-300 µg/ml. Thereafter, and in order to select the most appropriate concentration within this range, the two cell lines have been cultured and exposed to two different concentrations, 100 and 300 µg/ml for 24 hours. Then, the cellular viability of each group has been tested using a trypan blue assay facilitated by Burker chamber and inverted light microscope, to assess the effects on the two cell lines. Simultaneously, each group has been imaged using TEM and SEM to examine the cells-nanoparticles interactions, and to correlate that with the cellular viability of the two cell lines. Lastly data have been gathered from the viability
assay and the electron microscope imaging to identify one concentration to be used in the second phase.

After assessing the most appropriate nanoceria dose, the same experiments have been repeated in the second phase, which aims to check the effects of the chosen nanoceria dose when the cells are incubated for different times. First, a literature search indicated that the most effective time range for nanoceria to cause effects on similar cell lines is 12-48 hours. Therefore, the two cell lines, F98 and CRL2005, have been cultured and incubated with nanoceria for 12h, 24h, and 48h. After that, the cellular viability of each group has been tested using a trypan blue assay, followed by validating the cellular viability using propidium iodide assay aided by fluorescence-activated cell sorting (FACS). Then, each group has been imaged using TEM and SEM to correlate the NPs internalization into the cells with the cellular cytotoxicity of each cell lines, and also to correlate the findings with the cellular viability.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

In this experiment, two cell lines were used, which were purchased from ATCC®. The first one is an astrocyte cell line DI TNC1 (ATCC® CRL-2005™), which was extracted from a neonate rat brain tissue. [107] The other one is a glioblastoma cell line F98 (ATCC® CRL-2397™), which was isolated from a brain tumor of a fetus rat. [108]

The two cell lines, CRL2005 and F98, were cultured in Dulbecco’s Modified Eagle’s medium-high glucose (DMEM) (SIGMA, D6546), supplemented with 10% fetal bovine serum (Gibco, 10500-064), 200 mM L-glutamine (Sigma, G7513), and penicillin-streptomycin 100 IU (Gibco 15140-122) in a 75 cm² cell culture flask. Both of the cell lines were incubated at 37°C in air supplemented with 5% CO₂ (Thermo, Heracell 150i) until 70% confluence was achieved. Cells were washed with Dulbecco’s phosphate buffered saline (D-PBS), (Corning, 21-030-CMR), enzymatically detached from the surface of the cell culture flask using 0.25% trypsin (Sigma, 59418C-100ml) for CRL2005 and 0.05% trypsin for F98, as recommended by ATCC, and collected by centrifugation (Eppendorf, centrifuge 5810 R) at 1000 rpm for 5 min.

Cells then were counted using trypan blue (Sigma, T8154) assay, facilitated by a Burker chamber (BRAND, 718920), and an inverted light microscope (Nikon, Eclipse TS100), and diluted by DMEM to maintain a density of 20,000 cells/mL, where the following two formulas were used, with a starting cell suspension volume of 3000 ml, and a final cell suspension volume of 1 ml.
Next, the diluted cell suspensions were seeded into three 24-multiwell plates for each cell line as illustrated in figure 16, which shows the experimental setting for the first experiment with varying the nanoceria concentration. For the TEM and SEM imaging experiments, cells were seeded on 13 mm glass coverslips (0.5 mL), while for the trypan blue assay, cells were spread directly over the wells (1 mL). Then, cells were incubated at 37°C overnight.

The second experiment was designed for varying the nanoceria exposure time, with time points of 12, 24, and 48 hours. Cells were cultured in a similar way, as described above, then seeded in one 24-multiwell plate for the TEM and SEM protocol, and two 12-multiwell plate for the trypan blue assay, for each cell line, as displayed on figure 17. Also, for the propidium iodide assay, cells were cultured in the same way, but were seeded in triplicated petri-dishes for each time point.
Figure 16: Cell seeding multiwells for the first experiment, for CRL 2005 and F 98. Panel A shows the chosen wells for TEM and SEM protocol. Panel B shows the used wells for the trypan blue assay. Well area: 1.8 cm$^2$, cell density: 20,000 cells/ml.

Figure 17: Cell seeding multiwells for the second experiment, for CRL 2005 and F 98. Panel A shows the chosen wells for TEM and SEM protocol. Panel B shows the chosen wells for the trypan blue assay. Well area: 1.8 cm$^2$, cell density: 20,000 cells/ml.
2.2 Cerium oxide NP exposure

Cerium oxide NPs were synthesized in our lab using a precipitation method of ethylene glycol in water. The cerium oxide nanoparticle surface was modified by the functionalization with MEEETES (6-[2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy]-hexyl)triethoxysilane) to enhance the solubility and improve the dispersion of the molecules in water. The nanoparticles were characterized by TEM, which reveals spherical shape and monodispersion of the NPs size, with mean diameter of 7.4 nm and 1.6 SD. [109]

Two experiments were performed, one for CRL2005 and another for F98. In each, three multiwells were prepared as described above and shown in figure 16; one multiwell for TEM protocol, another one for SEM protocol, as shown in panel A, and a third one for the trypan blue assay, as shown in panel B. After seeding the cells for 24 hours, cerium oxide nanopowder was redispersed in sterile MilliQ water by ultrasonication in water bath (BRANSON, 5800) for 45 minutes. Afterward, three different CeO₂ concentrations (100, 200 and 300 μg/mL), were administrated to cells, which were subsequently incubated at 37°C in air supplemented with 5% CO₂ for additional 24 hours. Then, the same processes were repeated for the other experiments that involve varying the incubation time, and for the propidium iodide assay.

2.3 Cellular Viability Assays

Two viability assays were used in this study, as described in the research design, which are trypan blue assay and propidium iodide assay. These two assays belong to the same category, which is the cytolysis, or membrane leakage. The similarity between the
two assays comes from the fact that both work based on the principle that live cells preserve intact membrane that exclude the dye, while dead cells exhibit rupture membrane that allow the dye to pass through and therefore get detected. However, the difference between these two assays is that the trypan blue assay is performed by loading the cells-dye mixture into a chamber, followed by a manual counting of dead cell that appears bluish under an inverted light microscope, while for the propidium iodide assay the mixture is loaded into a FACS machine, where cells are counted automatically, through the detection of the fluorescent propidium iodide in dead cells, as will be described next.

2.3.1 Trypan blue assay facilitated by Burker chamber

Upon the completion of the 24h incubation time, the medium containing cells of each well was transferred into a 15 mL centrifuge tube, in order to not lose floating cells. Then, wells were washed with D-PBS, followed by the trypsinization with 0.25% trypsin for CRL2005 and 0.05% trypsin for F98, to detach the remaining cells from the wells. After that, the trypsin in each well was deactivated by adding a cell growth medium (DMEM), then the wells were further washed with D-PBS, and all the contents were transferred to the same tubes. Next, 10 µL of each cell suspension was mixed with 10 µL of trypan blue and loaded into a Burker chamber, as displayed in figure 18-A. The chamber was visualized using an inverted light microscope, and the cells put in three diagonal squares of the chamber (blue shaded area in figure 18-B) were counted. Dead cells appeared bluish under the microscope, due to taking up the dye through the rupture membrane, while live cells, which excluded the dye, appeared bright and colorless. The preliminary experiment, with varying the nanoceria concentration, was repeated in two
independent experiments, each in quadruplicate for each sample, while the second experiment, with varying the exposure time points, was repeated three times, again each in quadruplicate. Also, each counting was repeated two times by two observers. Then, the average of the data was calculated using Microsoft Excel, as displayed in the provided datasheet such as that shown Figure 18-C, where the viability was calculated using the following formula:

\[
\% \text{ Cell Viability} = \frac{\text{Average # of live cells}}{\text{Average # of live cells + Average # of dead cells}} \times 100
\]

Figure 18: Cell counting procedures using Trypan blue assay. A: Counting chamber with spring clips. B: Burker slide, with 9 large squares of 1 mm², each subdivided into 16 squares with 0.2 mm sides. C: Excel datasheet for averaging the data.

2.3.2 Propidium iodide assay aided by FACS

For the propidium iodide (PI) assay cells were cultured in petri-dishes, as stated above, because more cells are required for this type of assay. Upon the completion of the
specified incubation time of cells with nanoceria, the same process described for the trypan blue assay was repeated, where the medium containing cells in each petri-dish was transferred into a 50-mL centrifuge tube, followed by washing the dishes with D-PBS, the trypsinization with 0.25% and 0.05 for CRL2005 and F98 respectively, the deactivation of trypsin using a cell growth medium, and lastly another wash with D-PBS, where all the contents were transferred into the same tube. Then, all the tubes were centrifuged at 1100 rpm for 5 minutes. Next, the supernatant of each tube was discarded and the pellet was dissolved using D-PBS. Afterwards, the aliquot of each tube was transferred into a FACS tube, which then were loaded into the FACS machine, to acquire data for unstained cells that constituted the positive controls. Lastly, PI staining solution was added to each sample, followed by loading the tubes into the FACS machine to run an automatic counting of viable cells. The experiment was performed in triplicate for each sample, and then, data were analyzed using Microsoft Excel.

2.4 TEM samples preparation and imaging with EDX analysis

The TEM specimens (along with the SEM specimen, which will be further discussed in 2.5) were taken out of the incubator after 24h, and then fixed for TEM (and SEM) imaging. First, four 35 mm glass petri-dishes were prepared for the TEM (and SEM) protocol for each cell line, by filling with fresh cell culture medium, DMEM, and labeling with the corresponding nanoceria concentration: CTRL, 100 and 300 µg/mL. Next, the glass coverslips were carried from the multiwells into the corresponding prepared petri dishes, and further prepared, as shown in figure 19-A and described in the next sections.
2.4.1 Chemical fixation and dehydration

The purpose of the chemical fixation is to preserve cell ultrastructure, and increase the cellular mechanical stability during EM ultrastructural analysis. To achieve this task, samples in the four petri-dishes were first incubated with 2% glutaraldehyde in 0.1M cacodylate buffer with pH=7.3 for 20 minutes at room temperature (RT), by which glutaraldehyde is known being able to crosslink with aminoacid residues, and thus to fix the cellular protein components, as shown in figure 19-B. Samples were then washed three times with 0.1 M cacodylate buffer, each for 10 minutes.

Next, samples were fixed with 1% osmium tetroxide in 0.1M cacodylate buffer for 20 minutes at RT, to stabilize the cellular lipid components, where osmium tetroxide has the ability to crosslink and preserve unsaturated lipids, as displayed in figure 19-B.
Then, samples were washed three times with distilled water, each for 3 minutes, and stained with uranyl acetate for 20 minutes at RT, followed by three washes with distilled water. After fixing the protein and lipid components, samples were dehydrated using graded ethanol series: 25%, 50%, 70%, 90%, 95%, each for 5 minutes, followed by two washes with 100% ethanol, and another two washes with propylene oxide, to remove the ethanol residues from the sample, for 10 minutes each.

2.4.2 Infiltration and embedding

This step aims to support the fixed ultrastructure, allow storing samples, and facilitate the ultrathin sectioning. Upon completing the process of the dehydration, samples were gradually infiltrate with Durcupan™ ACM epoxy resin; by the incubation with 25%:75%, 50%:50%, and 75%:25% (epoxy resin : propylene oxide), each for 90 minutes. Next, samples were incubated in 100% epoxy resin for 24 hours. In the next day, the process was repeated with new 100% epoxy resin for 60 minutes, followed by transferring the glass coverslips containing cells into glass slides and inverting Eppendorf centrifuge tubes, partially filled with epoxy resin, upside down at the center of the sample, as seen in figure 20-A. Lastly, samples were placed in an oven (Thermo Fisher Scientific, Precision™) at 60°C, for 48 hours to be polymerized, as shown in figure 20-A.

2.4.3 Sectioning

The purpose of the sectioning is to produce sections in thickness range of 50-70 nm to be transparent to electron beam in TEM. To achieve this task, the solidified resins were first trimmed, using thick and thin razors, into a trapezoid shaped pyramid with the section area being as small as possible around the part of interest. Then, the shaped resin was loaded into the vertical arm of an ultramicrotome (Leica, EM UC6), along with
loading a glass knife, which was prepared using a glass knife maker (Leica, EM KMR3), into the horizontal arm, as shown in figure 20-B.

The process of sectioning starts with aligning the sample to the glass knife boarder, followed by adjusting the speed of sectioning to 3 mm/s and selecting the thickness of the sections at 400 nm, which aims to produce semi-thin section in order to investigate the state of the cells under light microscope. Upon producing the semi-thin sections, they were collected on a glass slide, treated with sodium metoxide to remove resin traces, stained with crystal violet and basic fucsin, and lastly investigated under a light microscope (Leica, DM 2700 M) to check the state of cells and to localize the area of interest for the thin sectioning.

Then, the resins were further trimmed, to produce thin sections with an area of around 0.5 x 0.5 mm and thickness of 50-70 nm using a diamond knife. The same processes clarified with producing semi-thin sections were repeated, but with adjusting the speed to 1 mm/s and gradual adjustments of the thickness to 100, 90, 80, and lastly 70 nm, as illustrated in figure 20-C.

2.4.4 Mounting on grids

The projected colors of the sections provide indicators for the actual thickness, as shown in figure 20-C. Therefore, only silver to pale gold sections, which correspond to 50-70 nm, were collected. These sections were collected and usually placed on 300-mesh copper grids with diameter of 3 mm, as diaplayed in figure 20-D, and then, examined under a light microscope (Leica, S6D) to ensure a proper attachment of the sections to the
grid mesh. Finally, the grids were air dried on a filter paper, and then stored in a labeled grid box (Leica, Reichert 705525).

Figure 20: Illustration of the TEM sample preparation. A: sample embedded in epoxy resin. B: ultrathin-sectioning by ultramicrotomy. C: sections collecting. D: transferring sections on TEM grid.

2.4.5 Contrast enhancement

Biological samples are composed of light elements, mainly hydrogen, carbon, nitrogen, and oxygen, with low electron densities, which correspond to low intrinsic contrast for TEM imaging. Therefore, the contrast of these samples should be enhanced by utilizing high atomic number stains, such as uranyl acetate and lead citrate ($Z_U = 92, Z_{Pb} = 82$).

In this experiment lead citrate was used to stain the samples, by which the face of grids containing cells were placed into a droplet of lead citrate for about four minutes,
then drained with filter paper and washed plunging them in three distilled water beakers, as shown in figure 21. Finally, the grids were dried and stored in the grid box to be visualized by TEM.

![Diagram of contrast enhancement](image)

Figure 21: Contrast enhancement for TEM samples. A: configuration of the TEM grid on top of the lead citrate droplet. B: whole procedures of the contrast enhancement.

### 2.4.6 TEM Visualization and EDX analysis

After fixing, dehydrating, and staining the samples, they were taken to be imaged by a TEM (FEI TECNAI G2 Spirit), which operates using lanthanum hexaboride filament (LaB$_6$) as an electron source. The TEM operated with an acceleration voltage of 120kV and was equipped with EDX detector, bio-twin objective lens, and FEI eagle CCD camera.

The process started with loading the prepared grids, one by one, into the TEM sample holder, followed by inserting the holder into the TEM goniometer, as shown in
Images at different magnification were acquired for all samples, starting from about 1.2kX, which provides an overview of the cellular components, and up to about 40kX, with the aim to in-depth investigate the cells ultrastructure and image the eventual vesicles in which the ceria NPs could be contained. Then, EDX analysis was performed on the regions in which some electron dense aggregates were found, and a number of EDX spectra were acquired, as will be described in the results section.

Figure 22: Loading samples into the TEM. A: mounting a TEM grid into the sample holder. B: TEM. C: example of a typical TEM image.

2.5 SEM sample preparation and imaging with EDX analysis

2.5.1 Chemical fixation and dehydration

As described in the TEM sample preparation, samples were first fixed with 2% glutaraldehyde in 0.1M cacodylate and 1% osmium tetroxide in 0.1M cacodylate buffer each for 20 minutes at RT, to fix the protein and lipid components, respectively. Then, samples were dehydrated using 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95%
ethanol for 5 minutes each, followed by two immersions in 100% ethanol for 10 minutes each.

2.5.2 Samples drying

In this experiment, hexamethyldisilazane (HMDS) (Fluka, 52619) was used as a drying agent to prevent artifacts associated with air drying, such as the structural shrinkage and collapse. HMDS provides an alternative approach to critical point drying (CPD), which delivers minimal artifacts but requires specialized instrument. [110]

Using this chemical compound (HMDS), samples were dried gradually, by placing them into 25% : 75%, 50% : 50%, and 75% : 25% (HMDS : absolute ethanol) each for 15 minutes. After that, samples were placed in 100% HMDS for 20 minutes, followed by another change with fresh 100% HMDS for overnight, keeping the petri dishes open to allow complete evaporation.

2.5.3 Mounting on stubs

In the next day, the glass coverslips, were mounted onto Aluminum stubs previously covered with some conductive silver glue (TED PELLA, PELCO® 16034) to create a conductive path between the glass, containing cells and the stub, as shown in figure 23-A.

2.5.4 Gold sputter coating

The purpose of this step is to deposit a thin layer of conductive material, such as platinum or gold, on the samples surface to enhance the conductivity of the samples, as schematized in figure 23-C. To perform this task, the stubs were distributed into platform
supports of a sputter coater (TED PELLA, Cressington 208HR), where they were coated with 10 nm layer of gold (density of 19.30 gm/cm$^3$), using a current of 20 mA.

![SEM sample preparation procedures](image)

**Figure 23:** SEM sample preparation procedures. A: mounting samples on stub. B: sputter coater. C: thin layer coating’s illustration. D: Typical SEM image.

### 2.5.5 SEM Visualization and EDX analysis

Stubs, afterward, were placed into the sample holder of an SEM (Zeiss, Merlin), equipped with Schottky field emission gun and an Oxford EDX X-Max SDD detector with a detection area of 80 mm$^2$ and the AzTec Energy EDX analysis software. The SEM images of the samples’ surfaces/morphology were acquired, at different magnifications, collecting the secondary electron (SE) signal, with the microscope working at an acceleration voltage of 5kV and with a beam current of 200 pA. To collect the EDX elemental spectrum, the microscope operated at an acceleration voltage of 10kV and beam current of 3nA.
CHAPTER 3: RESULTS

3.1 Electron microscopy characterization of CRL2005 and F98 cell lines.

As a matter of convenience, from now on, we will refer to glioblastoma multiforme cells F98 (ATCC® CRL-2397™) only with the initials F98 and healthy astrocytes cell line DI TNC1 (ATCC® CRL-2005™) with the initials CRL 2005.

These cell lines were visualized first without nanoceria exposure. The aim of this initial analysis was to examine the structural and morphological alterations caused on the F98, as a result of tumorigenesis, comparing to the CRL 2005, and to define reference points for further analysis after the nanoceria exposure. However, little is known regarding these types of cells from ultrastructural point of view, and to the best of the author knowledge, these two cell lines have never been characterized by electron microscopy prior to this experiment.

3.1.1 Ultrastructural analysis of CRL2005 and F98 using TEM

First, overview images were acquired for the two cell lines, to observe the possible relevant ultrastructural differences of F98 in comparison with CRL 2005. Figure 24-A and B show the two types of cells at low magnification. The most significant feature to be noticed in these images are the diversity in shape and electron-density of the mitochondria between the two cells lines, along with a widespread of rough ER cisternae in the CRL2005 cell, beside the more remarkable presence of lipid droplets in the F98 cell.

Mitochondria are known to be altered as a result of tumorigenesis, as explained in the introduction. [111] Our findings support then this observation, where the CRL2005
cell show electron-dense mitochondria, as clearly shown in the image reported in Figure 24-A, with elongated shape and enlarged cristae compartments, called outer and inner membrane, respectively. [112] On the other hand, the F98 cell shows swelling mitochondria, with electron-lucent matrices (transparent to electrons), which cause them to be barely distinguished in Figure 24-B, but clearly recognized in the more magnified image reported in Figure 25-B. In addition, figure 26-B illustrates swelling F98 mitochondria, and cristae disarrangement with partial or total cristolysis and expanded matrices, which lead to a defective inner membrane. Moreover, the F98 cells display heterogeneous mitochondria, i.e. with variability in size, shape, and number, as well as the degree of severity of the internal structure. CRL2005 in figures 25A, 26A, and 27A display several rough ER cisternae that spread with different size over the cytoplasm.

The second principal difference observed in F98 cells in comparison with CRL2005 is the elevated formation of lipid droplets (LDs). Figure 27 shows again CRL2005 and F98 cells, where the healthy astrocytes show limited LDs, while the prevalent existence of LDs is seen in the glioblastoma cells. The lipid droplets are observed in different size and shapes, and they are, interestingly, accumulated between or in close proximity to the affected mitochondria. Some of the LDs were big enough to be observed even with the light microscope at low magnification, as shown in figure 28-A, where a number of CRL2005 cells are shown with no LD formation, while the corresponding F98 cells, displayed in Figure 28-B, exhibit different sizes of LDs all over the displayed cells.
3.1.2 Ultrastructural analysis of CRL2005 and F98 using SEM

An initial investigation was performed by acquiring scanning electron micrographs at low magnification for CRL2005 and F98, as reported in figure 29-A and B, respectively. F98 cells show different shapes, with the majority having spindle shapes (fibroblastic-like cells) and a smaller number of rounded and dendritic-like morphology. The rounded cells exhibit blebs and filopodia, and make many contacts with the flat and highly prolonged dendritic like cells, as shown in figure 29-D. The spindle shaped cells contribute to the majority of the illustrated cells, and they are characterized by the elevated amount of microvilli on their surfaces, as displayed in Figure 29-F. CRL2005 cells, on the contrary, are flatter, and with somehow uniform polygonal shape, as shown in Figure 29- C, and clear surface with no microvilli formation, as seen in Figure 29-E.

GBM cells are characterized by cellular anaplasia that give rise to the polymorphism conditions. The cellular polymorphism refers to the loss of morphological uniformity of the GBM cells, and with respect to other glial cells. This feature is illustrated in figure 30, where F98 cells display variation in morphology, as shown in Figure 30-B, where the same situation is not observed with the corresponding CRL 2005 cells, reported in Figure 30-A, which look very similar among each other.

Healthy cells in culture normally stop proliferating once they contact each other, or carpeted the dish; this phenomenon is well-known as contact inhibition. [1] Figure 31-A shows CRL2005 cells exhibiting this property, and the cells appear then to be distributed on a monolayer. In a different manner, F98 cells appear to disregard the contact inhibition restraints and to continue proliferating, in an uncontrolled way, even
when in contact with adjacent cells, forming overlapping layers of cells, as observed in figure 31-B, where round cells appear to pile up on top of a layer of dendritic-like cells.

**Figure 24:** Overview TEM of CRL2005 and F98. A: CRL2005 cell, which shows multiple dense mitochondria with elongated shape. The image also shows a widespread of rough ER cisternae. B: F98 cell, which shows affected mitochondria, with electron-lucent matrices, which cause them to be barley distinguished from the cytoplasmic background. It is also possible to detect lipids droplets inside the cytoplasm. N: nucleus, M: mitochondria, LD: lipid droplets, ER-C: ER cisternae.
Figure 25: Electron density of CRL2005 and F98 mitochondria. A: CRL2005 TEM, where the cell preserve electron-dense mitochondria, with an elongated shape and clearly distinguished cristae. B: F98 TEM, which shows electron lucent mitochondria, with cristae disarrangement. Mitochondria are indicated by black arrows.

Figure 26: Shape of CRL2005 and F98 mitochondria. A: CRL2005 TEM, which shows the normal shape of mitochondria in the healthy astrocyte cell. B: F98 TEM, where the mitochondria are swelling, and exhibit partial cristolysis and cristae disarrangement. Mitochondria are indicated by black arrows.
Figure 27: Lipid droplet formation at CRL2005 and F98 cells. A: CRL2005 TEM, which show the healthy astrocyte with no lipid formation. B: F98 TEM, through which the cells preserve elevated lipid droplets formations (black arrows) with multiple sizes.

Figure 28: Light microscope images of CRL2005 and F98 cells. A: CRL2005 cells that lack the appearance of lipid droplets (LDs). B: F98 cells, which show lipid droplets (LD) inside multiple cells with different sizes, as indicated by the black arrow.
Figure 29: Overview images of CRL2005 and F98 cell lines. A: CRL2005 SEM micrograph. B: F98 SEM micrograph, which illustrate the elevated amount of F98 density, comparing to the CRL2005 cells at A. C: CRL2005 SEM micrograph, which show the uniform shape of the astrocyte cells, with polygonal shape. (P) D: F98 SEM micrograph, which demonstrate a mixed population of spindle shapes (S), rounded (R), and dendritic-like (D) cells. The image also shows filopodia (F) contact between rounded and the dendritic like cells. E: CRL2005 SEM that show the lack of microvilli formation. F: F98 SEM that show the elevated formation of microvilli.
Figure 30: Cellular polymorphism. A: CRL2005, which shows uniformity of the cellular shape. B: F98, where cells display diversity in shape.

Figure 31: Contact inhibition property of CRL2005 and F98 cells. A: CRL2005 SEM micrograph, where cells appear to be distributed on a monolayer. B: F98 SEM micrograph, magnified at 3600X, which shows that glioblastoma cells forming overlapping layers of cells, where round cells pile up on top of a layer of dendritic-like cells.
3.2 Effects of various nanoceria concentration on CRL2005 and F98

The aim of this task was to investigate the *in vitro* effects of various nanoceria concentrations on the viability of CRL2005 and F98, and to correlate these effects with the internalization of the NPs into the two cell lines, and the eventual related ultrastructural alterations of the cellular organelles. This task was accomplished first by assessing the viability of CRL2005 and F98 after 24 hours of exposure to 100 and 300 µg/ml of cerium oxide NPs, using a trypan blue assay, followed by examining the nanoparticles internalization and localization inside the cells and investigating the cellular ultrastructure of each sample by TEM and SEM ultrastructure analysis.

3.2.1 Testing the cellular viability of CRL2005 and F98 at various nanoceria concentrations using trypan blue assay.

The viability of CRL2005 and F98 was assessed using trypan blue assay. The test was repeated in two independent experiments, each in quadruplicate for each sample. Figure 32 shows the results of the test for both CRL2005 and F98 at various nanoceria concentrations after normalizing the percent of each cell line to its control to compare the data. First, with regard to CRL2005 the graph shows that the cellular viability remained unaffected over the different nanoceria concentrations, but rather each concentration influences a slight increase (not significant) of the number of live cells by 2% and 10% over the control for 100 and 300 µg/ml, correspondingly, due to the cellular proliferation. F98, on the contrary, displays another trend, with the cells exhibiting a reduction in their viability by 6% after the treatment with 100 µg/ml. However, the viability was significantly reduced by 30% when F98 cells were treated with a nanoceria concentration of 300 µg/ml. Thus, the nanoceria concentration of 300 µg/ml has shown to induce the
most pronounced toxic effect, in fact a significant reduction in the F98 cells was achieved while no effects were seen for the CRL2005.

3.2.2 Structural analysis of CRL2005 and F98 at various nanoceria concentration using electron microscopy.

TEM was used first to verify the internalization and localization of the nanoceria inside CRL2005 and F98 cells at the different nanoceria concentration (100 and 300 µg/ml), and each examination was followed by an EDX chemical analysis, with the aim of confirming that the electron dense points observed were CNPs and not ascribable to staining agents’ agglomerates. The purpose of this analysis was also to ensure the effective internalization of the engineered cerium oxide NPs into the two cell lines, in order to link this event with the possible alterations in the ultrastructure of both CRL2005 and F98 cell lines. Figure 33 displays the localization of the nanoceria in the cytoplasm of the CRL2005 samples, each supplemented with an EDX spectrum to validate the presence of the nanoceria at X-ray energy of about 4.8, 5.2, and 5.6 keV, which correspond to the X-ray energy of $L_{a1}$, $L_{a2}$, and $L_{p1}$ of the cerium atoms.

The CNPs, in all samples, were observed to be encapsulated within double membrane vesicles, which suggest an endocytosis pathway, and localizes in various positions over the cytoplasm. While the majority of the vesicles that contain CNPs were localized in the cytosol in close proximity to the nuclear membrane, some cases were identified where vesicles containing nanoceria were localized inside a CRL2005 nucleus, as shown in figure 34-A. Also, figure 34-B displays various vesicles that are seen to spread over CRL2005 cellular cytoplasm, with one that seems internalizing the cellular nucleus. CNPs were observed at different stages of the endocytosis pathway. They were
recognized engulfed in early endosomes, late endosome (multi-vesicular bodies), and inside lysosomes, as highlighted in figure 35.

Afterwards the cellular ultrastructural of each sample was investigated, by acquiring TEM images at two different magnifications, as shown in figure 36, which displays overview images of the control cells, with no NPs, along with the samples exposed to CNPs at the various concentrations. The TEM images show no signs of alteration of the internal cellular organelles, except a slight variation in the mitochondria structure, as revealed in the magnified image of each treated sample. The mitochondria structures appear to exhibit a slender swelling and slim effects in the cristae arrangement, which increases with increasing the nanoceria concentration, and appear more clearly in the 300 µg/ml sample. Then, the CRL2005 samples were analyzed using SEM, where the process started with confirming the presence of nanoceria by EDX, and an example of the analysis is provided in figure 37, followed by investigating the possible alteration of the samples surfaces, as shown in figure 38. However, the SEM imaging also shows that in terms of cell external membrane no alterations for the three different concentrations are observed, even when compared with the control (not treated) cells.

Next, the same investigations were repeated with F98 samples. Figure 39 shows the nanoceria localization for the three different concentrations, where CNPs were observed to interact with the F98 cells in a similar way to what has been described happening with the CRL2005, where the NPs were recognized in various endocytosis pathway vesicles as demonstrated in figure 40. On the other hand, and concerning the ultrastructure of the F98 samples, the mitochondria indicate to be affected by extensive
alterations, and appear to undergo elevated levels of severity, as highlighted in figure 41. The mitochondrial shape exhibits swelling, and partial cristolysis with various degree of cristae disarrangement in both not treated and treated samples but, as shown in Figure 41-D, F, and H when compared with the not treated (control) cell reported in Figure 41-B, the treated samples appear to be more affected in terms of mitochondrial features. In particular, the sample containing 300 µg/ml nanoceria indicating very pronounced cristolysis and nearly diminished cristae with expanded matrices, as shown in figure 41-H. Golgi apparatus also seem to be affected by the treatment, as shown in figure 42, where the control cell, 42-A exhibits a slight flattened cisternae that are aligned into stacks, while after treatment, the samples undergo disorganization that ranges from moderate enlargement, as displayed in panels 42-B, to critical one, as shown in panel 42-C corresponding to the sample treated with 300 µg/ml nanoceria.

Then, F98 samples were analyzed by SEM, starting with investigating the interactions between nanoceria and F98 external membrane was analyzed by SEM, as reported in figure 43, which indicates a cluster of ceria in direct contact with the cellular plasma membrane. Thereafter, the surface of each F98 samples was examined to explore any possible alteration that might be caused by the treatment with the CNPs. The cells exhibit structural changes that suggest cellular injury, which include plasma membrane blebs cellular swelling, and microvilli distortions. Figure 44 shows some magnified images that display blebs formation, 44-A, at the outer membrane, and the cellular swelling, 44-B, of a sample treated with 100 µg/ml nanoceria, followed by overview images, in figure 45, of the control and the treated samples at different magnification, highlighting the cells that preserve blebs formation and those undergoing swelling
conditions. The surface of the sample treated with 300 μg/ml nanoceria appears to be the most affected, where the blebs and swelling cells were widely recognized, as highlighted in Figure 45-G and H.

Figure 32: Trypan blue viability assay result for CRL2005 and F98 at Different Nanoceria Concentrations of 100 and 300 μg/ml and incubation time of 24h. The samples treated with 300 μm/ml display the most reduction in the F98 cells without affecting CRL2005.
Figure 33: Nanoceria localization inside CRL2005 cells. A and B: Nanoceria concentration of 100 µg/ml, with TEM-EDX spectrum to confirm the presence of nanoceria (CNPs). C and D: For 300 µg/ml nanoceria. The cerium oxide NPs (CNPs) are encapsulated within double membrane vesicles, with a localization over the cellular cytoplasm, in close proximity to the nucleus.
Figure 34: Localization of nanoceria inside CRL2005 nucleus. A and B: Inside CRL2005 nucleus with an EDX spectrum to confirm the presence of nanoceria. C and D: Display various vesicles containing nanoceria that are seen to spread over CRL2005 cellular cytoplasm, with one that seems internalizing into the cellular nucleus.

Figure 35: Localization at principal components of endocytosis pathway of CRL2005. A: Early endosome. B: Late endosome, which is characterized by various internal vesicles. C: Lysosome, has distinctive feature, and characterized by internal membrane sheets that are filled with electron dense materials. These components were classified based on established morphological criteria, [113] [114]
Figure 36: Overview TEM images of CRL2005 control (A&B) and at different nanoceria concentration of 100 µg/ml (C&D), and 300 µg/ml (E and F). The images at different magnifications to highlight the general structures of each sample, with a focus on the mitochondria morphology. N: nucleus, M: mitochondria
Figure 37: Example of the SEM-EDX analysis of CRL2005. A: CRL2005 cell at low magnification, which shows the presence of the nanoceria of the cellular surface. B: CRL2005 cell at higher magnification, which show the strong attachment of the NPs to the cellular surface. C: EDX spectrum, which display peaks of the different element analyzed at point 16, where cerium can be seen at 4.8, 5.2, and 5.6 keV, which correspond to La1, La2, and Lβ1, respectively.
Figure 38: An overview SEM images of CRL2005 control (A, B) and at different nanoceria concentration of 100 µg/ml (C, D), and 300 µg/ml (E and F). The images at two different magnifications to highlight the general structures of each sample. The images show intact samples, with no sign of alteration as a result of treatment.
Figure 39: Nanoceria localization inside F98 cells. A and B: Nanoceria concentration of 100 µg/ml, with TEM-EDX spectrum to confirm the presence of nanoceria (CNPs). C and D: Localization of 200 µg/ml nanoceria, and E and F: For 300 µg/ml nanoceria. The cerium oxide NPs encapsulated within double membrane vesicles, with a localization over the cellular cytoplasm, in close proximity to the nucleus.

Figure 40: Localization at principal components of endocytosis pathway of F98. A: Early endosome. B: Late endosome, which is characterized by various internal vesicles. C: Lysosome, has distinctive feature, and characterized by internal membrane sheets that are filled with electron dense materials. These components were classified based on established morphological criteria, [113] [114]
Figure 41: Overview TEM images of F98 control (A and B) and at different nanoceria concentration of 100 µg/ml (C and D), and 300 µg/ml (E and F). The images highlight the general structures of each sample, with a focus on the mitochondria morphology at a higher magnification.
Figure 42: Golgi apparatus alteration in F98. A: Control, B: 100 µg/ml, C: 300 µg/ml nanoceria concentration. The image shows a developing swelling in the treated sample, with critical scattering of the Golgi stacks at 300 µg/ml treatment. Golgi apparatus are indicated by black arrows.
Figure 43: An example of the SEM-EDX analysis of F98. A: F98 cell at low magnification, which shows the presence of the nanoceria of the cellular surface. B: F98 cell at higher magnification, which show the strong attachment of the NPs to the cellular. C: EDX spectrum display peaks of the different element analyzed, where cerium can be seen at 4.8, 5.2, and 5.6 keV, which correspond to La1, La2, and Lβ1, respectively.

Figure 44: Examples of A: plasma membrane blebbs (Bl) and B: cellular swelling (S), at F98 cells treated with 100 µg/ml nanoceria. These indications are expected to be caused as a result of cellular injury.
Figure 45: Overview SEM images of F98 at different nanoceria concentration. A&B: CTRL, C&D: 100 µg/ml, E&F: 200 µg/ml, and G&H: 300 µg/ml. The images highlight the general structures of each sample, with a focus on the mitochondria morphology. The sample treated with 300 µg/ml nanoceria seems to be affected.
3.3 Effects of various nanoceria exposure time on CRL2005 and F98

After investigating the effects of the various nanoceria concentrations, both of the viability test results and EM analysis indicate that a nanoceria concentration of 300 µg/ml leads to the most pronounced effect on viability. Therefore, the next step was to examine the effects of varying the nanoceria exposure time on the CRL2005 and F98 cell lines, using a nanoceria concentration of 300 µg/ml. Three time points were selected for this task, as stated in the introduction: 12, 24 and 48 hours. The cellular viability was assessed by the trypan blue assay and the propidium iodide assay, and the results were validated using electron microscopy.

3.3.1 Testing the cellular viability of CRL2005 and F98 at various nanoceria exposure times using trypan blue assay.

The viability of CRL2005 and F98 was assessed first using a trypan blue assay. The test was repeated in three independent experiments, each in quadruplicate for each sample. Figure 46 shows the results of the viability test for both CRL2005 and F98 at various exposure time, after normalizing the percent of each sample to its control to compare the data. The figure indicates that the viability of CRL2005 at different time points results in a slight decreasing trend in the cellular proliferation, though the number of live cells in the three samples exceeded their corresponding control by 14%, 6%, and 4% when varying the exposure time for 12, 24, and 48 hours, respectively. Conversely, F98 cells display another behavior; in fact, the cellular viability exhibits a reduction of 9% when the cells were incubated with nanoceria for 12 hours, and as the incubation time lasted for longer time, 24 hours, the viability of the cells was significantly reduced by 14%. Surprisingly, when cells were administrated with nanoceria for longer time, 48
hours, the cellular viability raised again to a situation similar to that observed at 12 hours. In all cases, the trypan blue assay demonstrates similar results to those obtained when varying the nanoceria concentration, through which the 300 µg/ml treatment slightly alters the healthy cells, while significantly affects the corresponding cancer cells.

3.3.2 Testing the cellular viability of CRL2005 and F98 at various exposure times using Propidium Iodide assay.

The purpose of this test was to validate and better understand the data obtained by the trypan blue assay with regard to the cellular responses and trend, especially for F98 cells. By varying the nanoceria exposure times, such cells preserve different trends, as their viability was affected mainly at 24 hours, while it results less influenced at shorter (12 hours) and longer (48 hours) time.

Figure 47 shows examples of the fluorescence-activated cell sorting (FACS) graphs that illustrate a representative plot of the PI signals versus forward scatter (size of cells) for untreated F98 sample (A) and treated with 300 µg/ml nanoceria for 24 hours (C). These graphs demonstrate the distribution of live (red) and dead (green) cells, along with counting analysis of live (PI-) and dead (PI+) cells, where around 10,000 events were encountered. Also, the figure shows histograms distribution of live and dead cells for the untreated (B) and the treated (D) samples.

The results of the cellular viability obtained by the PI assay are displayed in figure 48, after normalizing the percent of each sample to its control. CRL2005 cells were not significantly affected by the treatment, as all samples show reductions of equal or less than 5%. On the other side, after an incubation time of 24 hours F98 cells encounter a
viability reduction of 16% comparing to the control, according to the trypan blue assay. However, at shorter time of 12 hours, the F98 cells preserve their viability, showing a slight reduction of 6%. Remarkably, cell viability seems to be recovered at the 48-hours, as observed with the trypan blue assay. Overall, the PI viability assay confirms the data obtained by the trypan blue assay with regard to the trend of the cellular viability of both cell lines.

3.3.3 Structural analysis of CRL2005 and F98 at various nanoceria exposure times using electron microscopy.

The first step was to verify the internalization of nanoceria by cells, using TEM. First of all, the internalization and the localization of CNPs do not seem to differ based on ceria concentrations. Next, CRL2005 cells were investigated by TEM, paying more attention to the mitochondria structure in the three different samples. As seen in figure 49, slight ultrastructural modifications in mitochondrial cristae appear in healthy cells after ceria treatment. On the contrary, SEM images do not show significant alterations in cell morphology, figure 50, and also verify the presence of the ceria on the cellular surfaces (EDX data are not shown).

The same process of investigation was repeated then with F98, where the morphological features of the three samples were compared to the control, as shown in figure 51. F98 treated for 12 hours (Figure 51-C and D) appear similar to untreated cells, preserving very comparable ultrastructure to that of the control (Figure 51-A and B). Conversely, when cells were exposed to 300 µg/ml nanoceria for 24 hours, their mitochondria were severely disrupted, with swelling and cristolysis, as shown in panel 51-E and F. However, when treated with nanoceria for 48 hours (Figure 51-G and H),
F98 cells also display altered mitochondria that undergo distinctive cristolysis and swelling, while the Golgi apparatus shows less alteration with just moderate enlargement (Figure 51-H).

On the other hand, SEM analysis shows that when cells F98 are exposed to nanoceria for 12 and 48 hours, they appear very similar to the control, because just few swelling cells are observed, with rare cases of membrane blebs alterations. Nevertheless, when cells are incubated with ceria for 24 hours, a large number of cells display swelling and blebs as can be seen in figure 52, suggesting cell injury.

Figure 46: Trypan blue viability assay result for CRL2005 and F98 at Different Nanoceria Exposure time of 12, 24, and 48h. The sample treated for 24h with 300 µg/ml display the significant reduction in the F98 cells and protection of CRL2005 cells. The sample treated with 300 µm/ml for 24h display the most significant reduction in the F98 cells without affecting CRL2005 cells.
Figure 47: Examples of the FACS graphs to analyze F98 viability, which include PI signals vs forward scatter for F98 Control (A) and untreated 300 µg/ml nanoceria for 24h (C), with histogram for live and dead cells distribution for the control (B) and the treated (D) samples.
Figure 48: PI viability test assay result of CRL2005 and F98 at Different Nanoceria Exposure Time of 12, 24 and 48 hours with a concentration of 300 µg/ml. The sample treated with 300 µm/ml for 24h display the most significant reduction in the F98 cells without affecting CRL2005 cells.
Figure 49: TEM analysis of CRL2005 control (A), and treated with nanoceria for 12 hours (B), 24 hours (C), and 48 hours (D). The images highlight the mitochondrial structure in the four samples. C and D demonstrate slight alterations on the mitochondria cristae arrangement.
Figure 50: SEM analysis of CRL2005 control (A), and treated with nanoceria for 12 hours (B), 24 hours (C), and 48 hours (D). The samples demonstrate no sign of alteration as a result of the nanoceria treatment.
Figure 51: Overview TEM images of F98 at different nanoceria time exposure. A and B: CTRL, C and D: 12 hours, E and F: 24 hours, and G and H: 48 hours. The images show the general structures of each sample, with a developing degree of severity in the mitochondria, with a total swelling and cristolysis. N: nucleus, M: mitochondria, G: Golgi apparatus.
Figure 52: An overview SEM images of F98 at different nanoceria exposure times. A: CTRL, B: 12h, C: 24h, D: 48h. S: Swelling, Bl: membrane blebs. The sample treated with nanoceria for 24h appear to be mostly affected.
CHAPTER 4: DISCUSSION

4.1 EM as a powerful tool to analyze the bioenergetics of cancer cells.

Mitochondria has gained an increased interest from the scientific community, mainly because this organelle plays important roles in many of the cellular regulation pathways, including cellular metabolism, energy supply, cell cycle control, development, and apoptosis. Current advanced techniques, such as EM, facilitate the characterization of this organelle in many diseases, including cancer, giving rise to a new field entitled bioenergetics of cancer. Cancer cells, and other highly proliferated cells, undergo different metabolism mechanism; they favor the glycolysis pathway, even under aerobic condition. This phenomenon results in changing mitochondria morphology, since the mitochondria shape is altered according to their energy state. Our findings here reported seem to support this observation, as we observed the F98 mitochondria were observed to undergo alteration in more than single aspects.

F98 cells exhibit mitochondrial swelling, with cristae disarrangement and partial cristolysis, along with electron lucent-matrices, as described in the result section. These observations agree with others works, in which similar alterations have been reported with gliomas in general and glioblastoma cells in particular. According to Morillo et al. in human astrocytoma the morphology of mitochondria, imaged by electron microscopy, provides an indication of their capability of producing energy, by which dense mitochondria demonstrate a capability of generating energy through oxidative phosphorylation (OXPHOS) pathway, while mitochondria with lucent-matrices lack the ability to do so. Ramirez et al. also demonstrated the same observation with human glioblastoma cells, where numerous swelling mitochondria were
reported, with different levels of cristae disarrangement and cristolysis, the observations that were linked with the deficiency of the human glioblastoma to produce ATP through the mitochondrial oxidative phosphorylation.

Therefore, these results suggest that F98 cells partially lack the ability to produce energy through the OXPHOS pathway, but rather, they produce energy through aerobic glycolysis, the mechanism that result in making the F98 cells microenvironment more acidic, as explained in the introduction, than the surrounding brain tissue, which is hypothesized to play a role in changing the nanoceria properties, as will be explained later.

The health of mitochondria can be recognized using electron micrographs by numerous indicators. The mitochondrial swelling with matrix lucent, cristae disarrangement, and cristolysis provide indication for defective mitochondria, which might associate with the lack of the cells ability to undergo OXPHOS pathway, but rather, to produce energy through aerobic glycolysis. These indicators will be utilized in the next sections to evaluate the condition of the mitochondria at the samples after the nanoceria treatment.

4.2 EM analysis of cerium oxide NPs interactions with CRL2005 and F98.

Cells are surrounded by a plasma membrane (PM), which is a selective permeable barrier that controls the process of communication with the biological environment. [121] PM not only regulates what enters the cell, but also regulates the amount of any given substrate that is allowed to enter. Small and non-polar molecules can diffuse freely through PM, but bigger particles, like NPs, require an uptake mechanism. [122] The
interaction between NPs and cellular PM is a key aspect that determines their efficient uptake. Such interactions are influenced by many factors: mainly the cell type, [123] and the NPs size, shape, and surface chemistry. [124] Yet, the cellular uptake of NPs can be viewed as a two-stages process; the binding of the NPs into the cell membrane and their following internalization. [125]

In this study, and as demonstrated in the results, SEM displays the presence of the cerium oxide NPs into the PM of CRL2005 and F98, which indicates that the physiochemical properties of the engineered cerium oxide NPs (CNPS) interact well with the PM of the two cell lines, which in turn lead to proper activation of the cellular uptake machinery. On the other hand, TEM confirms the nanoceria internalization, and provides further information about the localization of the NPs in the cytosol and in few cases inside the nucleus.

TEM also verifies that ceria nanoparticles were taken up through an endocytosis mechanism; as revealed by the localization of the NPs in the three principal components of the endocytosis pathway, which are early endosome, late endosome, and lysosome. These components were classified based on established morphological criteria, [113] [126] through which early endosomes are reported to refer to double membrane vesicles containing from 1 to 4 internal vesicles. Late endosomes, which are also known as multivesicular bodies, are vesicles with more than 4 internal vesicles. Lysosome, on the contrary, has distinctive feature, being characterized by a single membrane and internal membrane sheets that are filled with electron dense materials.
Cerium oxide NPs are electron dense inorganic NPs (CNPs), and display high contrast in cells. Therefore, they can be visualized easily by SEM and TEM. However, in order to avoid the misinterpretation that might be caused by the presence of possible staining agents’ agglomerates, SEM and TEM images of CNPs were followed by EDX chemical analysis, to be sure about the chemical identity of nanoceria. Cerium oxide X-ray energy can be detected at K, L, and M lines, as illustrated in table 2. However, the M-line characteristics X-ray peak lacks in accuracy due to the overlapping with light element peaks ($K_{\text{C,O,N}}$ and $L_{\text{Cu}}$) which have close values, as seen in the table and observed in the EDX spectra in the result section. Besides, the K-line is not measurable as the normal range of energy detection reaches values up to 20 keV. Therefore, the L line is the energy of choice, through which three detectable peaks can be detected at 4.8, 5.2, and 5.6 KeV, which correspond to $L_{a1}$, $L_{a2}$, and $L_{\beta1}$ respectively, [127] as shown in the EDX spectra. The peaks appear clearly in the TEM-EDX due to the use of high acceleration voltage of 120 kV, which increases the signal to noise ratio of X-ray peaks, while in the SEM-EDX a lower acceleration voltage of 10 kV was applied, resulting in a bit weaker X-Ray signal.
Table 2: X-ray energy of K, L, and M lines for elements in the samples. [127]

<table>
<thead>
<tr>
<th>Element</th>
<th>$K_a$ (keV)</th>
<th>$L_a$ (keV)</th>
<th>$M_a$ (keV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uranium (U)</td>
<td>98.4</td>
<td>13.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>74.9</td>
<td>10.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Osmium (Os)</td>
<td>62.9</td>
<td>8.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Cerium (Ce)</td>
<td>34.7</td>
<td>4.8</td>
<td>0.88</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>8.04</td>
<td>0.93</td>
<td>-</td>
</tr>
<tr>
<td>Carbon (C)</td>
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<td>-</td>
<td>-</td>
</tr>
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<td>Oxygen (O)</td>
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<td>-</td>
</tr>
<tr>
<td>Nitrogen (N)</td>
<td>0.39</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

4.3 EM ultrastructural analysis of F98 cellular injury caused by various nanoceria concentration

Cerium oxide NPs are redox directed agents that seem to selectively facilitate the formation of ROS, and thus induce oxidative stress, at acidic condition, while acting as a free radical scavenger at physiological pH. [44] Of particular significance, and due to the acidic environment of cancer, nanoceria have been utilized in this study for testing the selective killing of F98 cells, but not the corresponding healthy cells, CRL2005. Trypan blue assay provides a first hint, about the fact that different concentrations of nanoceria contribute to significant reduction of the F98 viability, without significant effect on CRL2005 viability. Indeed, these NPs seem to induce a slight, even though not significant, cellular proliferation in CRL2005 cells, as their viability seem to exceed the one of the control cells in a dose-dependent manner. This could hypothesize that this behavior might be related to a potential antioxidant effect of nanoceria; however, this
statement needs further investigations. The electron microscopy analysis also confirms intact and comparable ultrastructure compared to the control cells in CRL2005-treated cells, with no major alterations to the cellular organelles neither to the cells surfaces. However, the mitochondria, and especially the sample treated with the highest dose, undergo slender swellings, which is expected during proliferating.

These results coincide with the findings of recently published papers, where nanoceria were reported to stimulate the proliferation of mouse embryonic fibroblast [128] and human astrocytes. [129] It has been also declared that nanoceria provides neuro-protection toward healthy rat neurons, [130] and mouse hippocampal cells, [131] and protect against free radical species in various in vivo brain studies. [132] [133] [134]

On the contrary, and concerning the F98 cells, the trypan blue assay elucidates different cellular viability behaviors with the different nanoceria concentrations. Cells were observed to be affected when treated with 100 and 300 µg/ml of nanoceria, as shown in figure 32. The viability of the samples treated with 100 and 300 µg/ml of nanoceria display a decrease with increasing concentrations; in fact, live cells were observed to be reduced by 6% and 30%, when they are treated with 100 and 300 µg/ml of nanoceria, respectively. These results show that nanoceria exhibits a higher toxic effect on cancer cells compared to the healthy cells, which could be correlated to an excess in ROS formation, as demonstrated to other authors. [44]

The electron microscopy analysis also verifies severe damages for the F98 samples after treatment, morphologically and topographically, where the degree of severity was seen to vary in a dose-response relationship. First, TEM illustrates extensive
alterations of the mitochondria shape in all the treated samples, as seen in figure 41. These alterations vary with varying the nanoceria dose, with the most effects observed in the sample treated with the higher dose, 300 µg/ml, where mitochondria undergo total cristolysis and extreme swelling. In all cases, one of the mechanisms that could be taken into consideration is that defective mitochondria could less contribute to produce ATP via OXPHOS pathway, which possibly leads to a state of ATP depletion that severely affects the cellular survival. [135]

The mitochondria impairment is expected to be caused by the excess formation of ROS, [135] possibly initiated by nanoceria, which in turn induces a state of cellular oxidative stress. Another possible indicator of the cellular oxidative stress is the swelling in Golgi apparatus, through which this organelle is described as a “stress sensor” [136] playing an essential role in facing the effects of the oxidation stress, and getting affected by it. [137] Golgi apparatus were seen to range from moderate swelling, in the low dose (100µg/mL), to a critical enlargement in the highest dose of 300 µg/ml, which indicates a dose-response dependency, as seen in figure 42. SEM, on the other hand, provides potential indications of a state of reversible cell injury, [135] as shown in figure 45, which is initiated as a result of the cellular oxidative stress, that include, the cell membrane blebs, cellular swelling, and microvilli distortion. These alterations are reported in the literature to be reversible if the harming stimulus is removed, where cells move to the adaptation phase and recover themselves, otherwise, the injury transform into an irreversible stage, when cells cannot heal themselves and die. [135]
In conclusion, this part of the study demonstrates the following findings. First, the trypan blue assay provides a proof of concept for the selective role playing by nanoceria in different cell types, where the CNPs were not significantly harmful toward healthy cells, while selectively killing cancer cells. Secondly, EM analysis reveals that nanoceria possibly causes a state of reversible cell injury, through which most of the indications that are illustrated in the literature for identifying this form of injury were seen by TEM and SEM, which include: mitochondria and Golgi apparatus swelling, plasma membrane blebs, cellular swelling, and microvilli distortion. [135] Thirdly, both of the viability test and the EM analysis illustrate that the nanoceria concentration of 300 µg/ml could provide the most pronounced effects, in exerting severe effects toward the cancer cells.

4.4 EM ultrastructural analysis of F98 cellular injury caused by various nanoceria exposure times

Based on the results discussed in the last section, a nanoceria concentration of 300 µg/ml was used, and another set of experiments were designed to investigate the time dependency of the nanoceria treatment on CRL2005 and F98.

The viability assays, trypan blue and PI, that were used to test the effects of varying the nanoceria exposure time, figure 46 and 47, showed very similar trends in many aspects. First, they confirmed the overall assumption of the capability of cerium oxide NPs to affect the survival of cancer cells. Secondly, almost the same trend was observed in the two tests; in fact, CRL2005 viability were seen not to be strongly decreased by the treatment with varying the exposure time, while F98 cells were seen to suffer mainly at the 24-hours incubation time, but not at 12-hours, where the incubation time seemed not to be sufficient to induce damages to the cells, or 48-hours, where the
cells appear to adapt to the stress and escape injury. Thirdly, the viability of the cancer cells at the 24-hours exposure time was found to be relatively similar, with a reduction of 14% in the trypan blue assay, and 16% in the PI assay.

Electron microscopy analysis confirmed the viability assays observations for the two cell lines. Even though CRL2005 cells exposed to CNPs highlighted a slight alterations in mitochondria shape, as verified by TEM, they did not show any significant decrease in cell viability. F98 cells, on the other side, showed different behavior at the various nanoceria exposure times. The samples after 12-hours incubation time were observed to be relatively unaffected, comparing to the control, while the 24-hours exposure time was expected to lead to a state of reversible cell injury, as specified before, indicated by the extensive effects on the cellular structure, where cells displayed severe mitochondrial alterations and critical enlargement of the Golgi cisternae, and on the cellular surfaces, where cells appeared swelling and expressed a pronounced degree of membrane blebs.

However, after 48 hours of incubation with nanoceria, F98 cells looked to display recovering Golgi apparatus, where the Golgi cisternae displayed a parallel series and aligned stacks that resembled the structure of the healthy Golgi apparatus. Yet, cells of this sample preserved altered mitochondria, because damages on this organelle is reported in the literature to be constantly irreversible, even after the recovery from injury. [135] Moreover, the sample, as observed by SEM, showed recovered shapes, with less swelling and blebs which could confirm the result obtained by the viability assay, and
suggest that the cells within this time frame succeed to adapt to the oxidative stress and escape injury.

Overall, the results of the viability tests, along with the EM analysis, are hypothesized to lead to the following findings. First, the CRL2005 cells viability was seen not be significantly affected by the nanoceria treatment at various doses or exposure time. Secondly, the F98 cells viability exhibit a decrease with increasing concentrations, but did not show a clear trend with respect to the multiple time points. Thirdly, F98 cells were observed to be affected most at an incubation time of 24 hours, where cells might suffer from a state of oxidative stress that possibly led to a reversible cell injury. Lastly, F98 cells were observed to be subjected to recovery after 48 hours, when they were adapted to the oxidation stress, through modulating their structure and therefore escaping injury.
CHAPTER 5: CONCLUSION

The main objective of this study was to investigate the interactions of cerium oxide NPs with glioblastoma (F98) and healthy astrocyte (CRL2005) cells, and to correlate that with the *in vitro* cytotoxic effects of various nanoceria concentration (100 and 300 µg/ml) and exposure time (12, 24, and 48 hours) on the two cell lines. Electron microscopes (TEM and SEM) were utilized to assess the cellular uptake of the NPs, and to examine the related effects in combination with trypan blue and propidium iodide viability assays. Our data suggest that the two cell lines were capable of taken up the nanoceria through endocytosis pathway, where the NPs were recognized engulfed by double membrane vesicles inside the cellular cytoplasm. Cerium oxide NPs were found to affect the glioblastoma cells, but not so severely the corresponding healthy astrocytes at the various concentrations and incubation times, as revealed by the viability assays and the electron microscopy analysis.

Of particular interest, the viability of the samples treated with nanoceria display a decrease with increasing concentrations with regard to the effects on the glioblastoma cells, where higher ceria concentration of 300 µg/ml result in more reduction on the F98 viability. On the other hand, the treatment did not show such dependency with regard to the different time points, where the treatment for 24-hour result in better efficiency than 12 and 48-hours. In all cases, the healthy astrocyte cells showed slight alterations in mitochondrial shape which did not influence their viability. To conclude, among the various nanoceria concentrations and exposure times, the most efficient dose of treatment was with a concentration of 300 µg/ml at a time point of 24-hour, where higher reduction
on the glioblastoma cells was achieved, with minimal toxicity to the healthy astrocyte cells. These are preliminary experiments, and the above-mentioned results could be suitable for further *in vitro* or *in vivo* studies.
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