MECHANISM OF NANOPARTICLE AND NANOTUBE-INDUCED CELL DEATH

by

SYED KHAJA SOHAEBUDDIN

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ABSTRACT

NANOPARTICLE-INDUCED CELL DEATH MECHANISM

Syed Khaja Sohaebuddin, MS

The University of Texas at Arlington, 2008

Supervising Professor: Liping Tang

Recent advances in nanotechnology have lead to synthesis of high-quality nanoparticles which exhibit unique physical and chemical properties. The novel properties of these nanoparticles are widely being applied for diagnostic and therapeutic purposes and enable new means of biosensing and targeted drug delivery. However, increasing evidence has suggested that some nanoparticles may cause detrimental effects on cells and tissues. Using titanium oxide (TiO_2) nanoparticles (5-10 nm diameter), silicon oxide (SiO_2) nanoparticles (30 nm diameter), and multi-walled carbon nanotubes (MWCNTs) of different diameters (<8 nm, 20-30 nm, >50 nm), 0.5-2 µm in length as test subjects, we evaluated the in vitro interaction of these nanoparticles with 3T3 fibroblasts. Our goal was to unearth the underlying mechanism of nanoparticle-induced cell death. Our results have shown that cell viability following exposure to the nanoparticles was dependent on material composition, concentration, diameter, particle exposure time. The MWCNT 20-30 nm exhibited the strongest toxicity effects to 3T3 fibroblasts, 31% reduction in cell survival at 1 µg/ml compared to 20% reduction with MWCNT <8 nm, 14% reduction with SiO_2 nanoparticles at the same concentration. TiO_2 nanoparticles and MWCNT >50 nm were the least toxic to 3T3 fibroblasts causing approximately 10% reduction in viable cells at a concentration of 1 µg/ml. By assessing the reactive oxygen species (ROS) generation

and lysosomal membrane stability, it was observed that nanoparticles and nanotubes exposure resulted in increased generation of intracellular reactive oxygen species (ROS); this caused lysosomal membrane permeabilization (LMP) only in cells exposed to MWCNTs but not in cells exposed to TiO_2 and SiO_2 nanoparticles. TiO_2 and SiO_2 nanoparticles did cause an increase the activity of caspase-3/7, whereas cells exposed to MWCNTs did not show increased caspase-3/7 activity. These findings indicate that exposure to TiO_2 and SiO_2 nanoparticles caused cell death via DNA damage leading to apoptosis, whereas exposure to MWCNTs caused cell death via leakage of lysosomal hydrolases leading to necrosis.

We believe that a better understanding of nanoparticle and nanotube-induced cell death will help us in the development of safe and better nano-devices.

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LIST OF ABBREVIATIONS

TiO_2 – Titanium Oxide

SiO_2 – Silicon Oxide

MWCNT – Multi-walled carbon nanotube

SWCNT – Single-walled carbon nantoube

AO – Acridine Orange

ROS – Reactive Oxygen Species

LMP – Lysosomal Membrane Permeabilization

IL-8 – Interleukin-8

H_2DCFDA – Dichlorodihydrofluorescein diacetate

PBS – Phosphate Buffered Saline

DMEM – Dulbecco's Modified Eagle's Media

CS – Calf Serum

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay)

ATCC – American Type Cell Culture

CHAPTER 1

NANOMATERIALS FOR MEDICAL APPLICATIONS

It is anticipated that nanotechnology can have an enormous impact on human health. The application of nanotechnology to healthcare is referred to as nanomedicine and it requires the intersection of many disciplines including physics, biology, chemistry, chemical and mechanical engineering, material science, and clinical medicine. Nanotechnology is the ability to work at the atomic, molecular, and supramolecular levels (on a scale of 1 - 100 nm) in order to understand, create and use material structures, devices and systems with fundamentally new properties and functions resulting from their small structure (Roco, 2003). Relevant processes of living organisms occur basically at the nanometer scale, elementary biological units like DNA, proteins and cell membranes are of this dimension. Nanostructures and particulates are well known in the biological field with natural entities like viruses and artificially constructed imitations of biomembranes such as liposomes (Duncan, 2003). However, with the recent progress in nanotechnology, we are entering a new era with large scale production and application of nanomaterials with a non-biological origin or history (Dreher, 2004). Nanomaterials, the building blocks for nanotechnology, are engineered materials and they include nanoparticles, nanofibers, nanotubes, composite materials, and nano-structured surfaces. Nanoparticles are currently defined as single particles with a diameter less than 100 nm. Nanofibers and Nanotubes have two dimensions measuring less than 100 nm but the axial dimensions can be much larger (Yang et al., 2008). With the development of engineered nanomaterials, such as nanoparticles, nanotube, and nanoscale devices, the biological units are

going to be better comprehended so that they can be specifically guided or directed (Logothetidis, 2006).

Nanoscale devices smaller than 50nm can easily enter most cells, and those smaller than 20nm can move out of blood vessels as they circulate throughout the body. These nanoscale objects have different physical, chemical, and biological properties from those of the corresponding mass materials (Logothetidis, 2006). These properties, together with their nano-size, promise revolutionary potential applications in clinical practice that could contribute to improved health care in the 21st century at many levels including detection of molecular changes responsible for disease pathogenesis, disease diagnosis, and imaging. These properties can also be applied in drug delivery and therapy, in multi-functional systems for combined therapeutic and diagnostic applications, and vehicles to report the in *vivo* efficacy of a therapeutic agent. Use of nanotechnology in medicine may also lead to more durable and better prosthetics, and nanoscale enabling technologies which will accelerate scientific discovery and basic research (Farokhzad and Langer, 2006). Thus, nanotechnology can be applied in various fields in medicine.

An important field of application for nanotechnology in medicine is biomaterials. It could be used to design for example, a more durable hip implant. If the design of the hip implant was to be carried out at nanolevel, it might become possible to construct it to closely mimic the mechanical properties of human bone, thereby preventing stress-shielding and subsequent loss of surrounding bone tissue (Roszek et al., 2005). Furthermore, nanostructuring of materials provides a powerful mechanism to encourage and direct cell behavior, thus enhancing their biocompatibility, by dictating the desirable interactions between cells and materials. Nanotechnology also has various applications in the field of cardiology research not only for diagnostic but also for therapeutic purposes (Vladimir et al., 2005). With the use of nanotechnology, scientists have been able to deliver genes that stimulate the growth of new

blood vessels in rat heart, create muscle-powered nanoparticles having the ability to transfer information into the cells, giving them potential to replace lost biological function of many tissues such as sinoatrial node (Korpanty, 2005). Hence, advances in nanotechnology can make major contributions in diagnosis and treatment of diseases.

Recent advances in colloidal synthesis have resulted in high-quality nanoparticles of various chemical compositions, ranging from semiconductors, metals, metal oxides, silica, and carbon nanotubes (Cao, 2008). Most nanoparticles currently in use have been made from transition metals, metal oxides, silicon, carbon, and various polymers (Ferrari, 2005). The unique physiochemical properties of engineered nanomaterials are attributed to their small size, chemical composition, surface structure, solubility, shape, and aggregation (Nel et al., 2006). The development of these nanomaterials has created new opportunities in biomedical research and clinical applications (Alivisatos, 2004). Drug delivery is one area that has progressed vastly with the advancements made in nanotechnology.

The most advanced drug delivery systems are liposomes; in fact, many liposomal drug forms have entered clinical trials. But, their suspension instability and dry storage incapability contribute to their poor compatibility with therapeutic applications. Therefore, active research and development on alternative form of drug delivery systems was pursued which led to the development of polymer-based drug delivery system. The polymeric nanoparticles can be designed to break down and release drugs at controlled rates (Na and Bae, 2002). Polymeric nanoparticles can be synthesized directly from monomers or by means of deposition of preformed polymers. These nanoparticles can also be engineered to allow differential release of drugs in certain environments, such as acid milieu, to promote uptake in tumors versus normal tissues (Couvreur et al., 2002). Substantial research is conducted in designing and creating biocompatible novel polymers and dendrimers and their application to many areas in medicine.

Dendrimers, complex almost spherical macromolecules with diameter 1-10 nm, have improved physical, chemical, and biological properties compared to traditional polymers. Some unique properties are related to their globular shape and the presence of internal cavities offering the possibility as medical nanovehicles. Dendrimers have a tree-like structure with many branches where a variety of molecules, including drugs can be attached. Less than 5 nm in diameter, dendrimers are small enough to slip through tiny openings in cell membranes and to pass vascular pores and tissues in a more efficient way than bigger polymer particles (Roszek, 2005). While polymeric nanoparticles are developed for use as drug delivery systems, semiconductor nanoparticles such as quantum dots are researched for use in imaging.

Quantum dots, nanometer sized semiconductor nanocrystals with superior fluorescent properties, possess remarkable optical and electronic properties that can be precisely tuned by changing their size and composition, due to their very small size (2-10 nm). Since they are relatively inexpensive and simple to synthesize, quantum dots have already entered the market for experimental biomedical imaging applications. Quantum dots can be made to emit light at any wavelength in the visible and infrared ranges, and can be inserted almost anywhere, including liquid solution, dyes etc. Quantum dots can be attached to a variety of surface ligands, and inserted into a variety of organisms for in-vivo research (Roszek, 2005). In addition to their use in imaging of cells, semiconductor metal oxides have a broader range of applications.

Metal oxides such as titanium oxide nanoparticles and silicon oxide nanoparticles are increasingly being used in many ways in pharmaceutical and cosmetic products (Gelis et al., 2003). Titanium oxide nanoparticles in medicine are being studied for their photocatalytic properties and for cancer therapy (Blake et al., 1999). In addition to serving as additives to cosmetics, and in pharmaceuticals, silicon oxide nanoparticles are being developed for a host of biomedical applications such as cancer therapy, DNA transfection, drug delivery, and enzyme

immobilization (Chen and Mikezc, 2005). Semiconductor nanoparticles composed of carbon also show a great deal of promise for their use in medicine.

Carbon nanotubes and inorganic nanowires are extensively used in electronic industries due to their exceptional optical, thermal, and mechanical properties. The properties of these nanomaterials are being applied in nanomedicine for development of improved biosensors, drug and vaccine delivery vehicles, eradication of cancer cells, and as novel biomaterials for musculoskeletal and neural tissue engineering (Smart, et al., 2006; Wei et al., 2007).

1.1 Nanoparticles and Nanotubes in nanomedicine

Engineered nanomaterials, particularly nanoparticles and nanotubes, Because of their unique properties are being used widely in industrial, electrical, agricultural, pharmaceutical and medical field. As mentioned earlier, nanoparticles and nanotubes currently synthesized are of various chemical compositions but those composed of metal oxides, silicon and carbonaceous are among the most frequently used in medicine. Here are some examples.

Metal oxides represent the most common, most diverse, and probably the richest class of materials in terms of physical, chemical, and structural properties. They include optical, optoelectronic, magnetic, electrical, thermal, electrochemical, photoelectrochemical, mechanical, and catalytic properties (Vayssieres, 2004). Titanium either pure or in alloys, due to its advantageous combination of physio-chemical and biological properties, are widely used in implanted medical devices (Cunningham et al., 2002). Nanosized titanium oxide particles are being used in cosmetics and pharmaceuticals products, and also in medicine (Kang et al., 2008). The crystalline forms of TiO_2, anatase and rutile, are semiconductor with band gap energies 3.26 and 3.06eV, respectively. TiO_2 absorbs UVA light catalyzing the generation of Reactive Oxygen Species (Gurr et al., 2005). Due to their photocatalytic properties, TiO_2 nanoparticles in medicine are being investigated mainly in the fields of sterilization, cancer treatment, and toxicology (Seo et al., 2007).

The photocatalytic properties of TiO_2 were initially studied for removal of organic and inorganic compounds from contaminated water and air (blake et al., 1999). Researchers have used these properties to sterilize the surfaces which may become exposed to pathogens in hopes of disrupting cellular functions and thus viability. Recently, the photocatalytic properties of TiO_2 mediated toxicity have been applied in eradicating cancer cells (Huang et al., 1997). Specifically, after TiO_2 nanoparticles were taken up by HeLa cells (cervical carcinoma), the cells were exposed to light from an unfiltered 500W mercury arc lamp which resulted in the death of these cells. Recently, human U937 monocytic leukemia cells were killed by first incubating them with 1 mg/ml of colloidal TiO_2 followed by irradiation with UV light (300-400nm). Silicon Oxide nanoparticles (SiO_2, nanosilica) are also used in eradicating cancer cells.

One of the most abundant materials on earth is silicon oxide. In order to produce industrial silica products, naturally occurring silica such as quarts sand, rocks, and clays are chemically treated to produce direct-silica sources. Common method of preparation is done in the solution by the Sto¨ber (sol-gel) process that produces silica by the hydrolysis and polycondensation of silicon alkoxide (Coradin, 2003; Wang, 2000). It has various applications in industry and in the medical field. It is used in pharmacy as a booster agent (Alyushin et al., 1971) and as an enterosorbent (chuiko, 2003). Furthermore, gold-coated silica nanoshells are used in treating benign and malignant tumors (Hirsch et al., 2003b).

The potential applications of Nanosized silica is in diagnostics, bioanalysis and imaging, drug delivery, and gene transfer (Chang et al., 2007). Recently, fluorescent silica nanoparticles have emerged as a fascinating fluorescent probe and attracted worldwide interest in biology and medicine (Tan et al., 2004). Highly luminescent silica nanoparticles have been developed for the selective tagging of cancer cells, bacteria, and individual molecules. These nanoparticles have multiplexing capabilities and ease of functionalization, as well as greater sensitivity and photostability when compared with the organic fluorophores. Overall, the advanced optical

features demonstrated by these nanoparticles are critical for ultrasensitive bio-imaging and detection as well as for real time tracking and monitoring of complex biological events at the cellular level, which cannot be accomplished with the regular fluorescent dyes (Ow et al., 2005). Silica nanoparticles are promising candidates for improved drug delivery systems. Surface modified silica nanoparticles to reach specific sites in the body have been loaded with drug molecules and upon target recognition, these nanoparticles can release drug at a rate that can be precisely controlled by tailoring the internal structure of the particles for a desired diffusion/release profile (barbe et al., 2004). Another promising application of Nanosized silica is in cancer therapy. Gold-coated silica nanoshells are being used to treat benign and malignant tumors (brongersma, 2003). Nanoshells have a core of silica that is 100nm in diameter which is coated with about 20nm of gold. These nanoshells are coated with molecular conjugates to the antigens that are expressed on the cancer cells themselves or in the tumor microenvironment. The nanoshells absorb near infra red light and converts it to heat, causing irreversible thermal destruction of the cells. Carbon nanotubes can also transform electro-magnetic energy into heat, causing a temperature increase lethal to cancer cells by irradiation with an external laser source of near infra red light at the very location where these nanoparticles are bound to or internalized within tumor cells (Roszek, 2005).

Carbon Nanotubes are novel materials which exhibit extraordinary mechanical, electric, electronic, thermal, and optical properties offering the electronic industry properties that few materials platforms could ever hope to match. More recently attention has been turned to the unique biological and medical properties of these materials. Carbon nanotubes were first described by Iijima over ten years ago (Wei et al., 2007). Multi-Walled carbon nanotubes were first discovered, a needle-shaped material containing several layers of coaxial tubes. Iijima later described single-walled carbon nanotubes. Single-Walled carbon nanotube and multi-walled carbon nanotube structures may be prepared by several methods; the electric-arc discharge

and the hydrocarbon pyrolysis methods are most widely used. The electric-arc discharge method is the most expensive and it produces highly crystalline nanotubes, whereas the hydrocarbon pyrolysis method produces large quantities of carbon nanotubes at relatively low cost; however, the nanotubes contain a high concentration of undesirable defects and impurities, which may preclude use in biological applications (Wei et al., 2007).

The interaction between carbon nanotubes and deoxyribonucleic acids, materials made up of nucleotides that contain the genetic information for an organism, has been examined by several investigators. Non-covalent DNA–carbon nanotube interactions were first demonstrated when nanotubes whose surfaces were evenly covered with platinated and iodinated helical double-stranded DNA were employed as probes for DNA visualization (Wei et al., 2007). These early results indicated that strong non-specific interactions may occur between DNA and carbon nanotubes. Carbon nanotubes have also been considered for use as scaffolds for cells in tissue engineering. Considerable research has been performed on using nanotubes in nervous system tissue engineering. Current research is focused on preparation of nanotube-based nerve scaffolds that may guide nerve regeneration activities (e.g., growth cones and neurite branches) across the lesion site. Multi-walled carbon nanotubes noncovalently coated with 4-hydroxynonenal act as substrates for growing embryonic rat brain nerve cells. It was observed that multiple neurites with extensive branches developed on the functionalized multi-walled carbon nanotube substrates. Investigators have also examined the effects of carbon nanotubes and carbon nanofibers on growth of bone and muscle cells used in musculoskeleton tissue engineering (Wei et al., 2007). Since these metals oxides and carbon nanotubes may someday be administered into the human body, information about its toxicity is of vital importance.

CHAPTER 2

CELL TOXICITY ASSOCIATED WITH NANOPARTICLES AND NANOTUBES

There has been a great deal of interest in the scientific and general community in nanotechnology. Nanotechnology, as mentioned earlier, is the manipulation, precise placement, measurement, modeling, or manufacture of sub-100 nanometer scale matter. This manipulation of matter at the nanoscale will have diverse effects in manufacture, engineering, environmental and information technology, and health and pharmaceuticals. The activity of these materials at nano-scale is enhanced, and the potential risk of these nanomaterials to human health has been of concern. Currently there is production of a wide range of nanoparticles of different types and different properties which will be tested for their utility in various applications, and those that are found useful will be further developed into large scale manufacture. Any technology before it is introduced to the marketplace and into the product chain needs careful evaluation with regard to its sustainability and risk perception (Donaldson et al., 2004).

Materials used in medicine must exhibit several properties, these include: biocompatibility, non-toxicity, non-carcinogenicity, non-mutagenicity, non-antigenicity, and non-tetragenocity. Since most of the engineered nanomaterials currently being used are from transition metals, metal oxides, silicon, and carbon, it is essential that their effects on biological cells and tissues are utterly known. Although human exposure to TiO_2 nanoparticles, SiO_2 nanoparticles, and carbon nanotubes is currently limited to small-scale manufacturing environments, commercial production and wider use of these nanoparticles will likely occur in coming years (Wei et al., 2007). Some investigators have proposed that solid materials that are able to penetrate cells can induce a process that is known as a foreign body transformation. It is

believed that foreign body transformation results from mechanical or oxidoreduction damage to the chromosomes or to the nucleus (Wei et al., 2007).

Some studies suggest that nanoparticles are not inherently benign and that they affect biological behaviors at the cellular, subcellular, and protein levels (Nel et al., 2006). The size of nanoparticles can modify the physiochemical properties of the material as well as create the opportunity for increased uptake and interaction with biological tissues. Moreover, some nanoparticles readily travel throughout the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria, and may trigger injurious responses. This combination of effects can generate adverse effects in living cells that would not otherwise be possible with the same material in larger form (Nel et al., 2006). Therefore, the particle size and surface area are important material characteristics from a toxicological perspective.

The change in the physicochemical and structural properties of engineered nanoparticles with a decrease in size could be responsible for a number of material interactions that could lead to toxicological effects. As the size of the particle decreases, its surface area increases and greater proportion of its atoms or molecules are displayed on the surface rather than the interior of the material (Nel et al., 2006). Shrinkage in size may create discontinuous crystal planes that increase the number of structural defects as well as disrupt the well structured electronic configuration of the material, so as to give rise to altered electronic properties. This could establish specific surface groups that could function as reactive sites. The extent of these changes and their importance strongly depend on the chemical composition of the material. These surface groups can make nanoparticles hydrophilic or hydrophobic, lipophilic or lipophobic, or catalytically active or passive. Studies have shown that exposure to quartz, mineral dust particles (e.g., coal and silicates), asbestos fibers, and experimental instillation of titanium oxide and carbon black nanoparticles in animal lungs, induce oxidative injury, inflammation, fibrosis, Cytotoxicity, and mediator from target lung cells (Nel, et al., 2006). Many

animal studies to date have confirmed an increase in pulmonary inflammation, oxidative stress and distal organ involvement upon respiratory exposure to nanoparticles (Zhou et al., 2003; Lam et al., 2004; Warheit et al., 2004). In vitro studies have not only confirmed the physiological responses found in whole animal models, but also provided data indicating an increased incidence of oxidative stress in cells exposed to various nanoparticles (Cui et al., 2005; Lin et al., 2006; Stone et al., 2007; Wang et al., 2007).

2.1 Role of reactive oxygen species

Recent studies have shown a direct relationship between the surface area, Reactive Oxygen Species-generating capability, and pro-inflammatory effects of nanoparticles in the lung. From a mechanistic perspective, ROS generation and oxidative stress is the best developed paradigm to explain the toxic effects of inhaled nanoparticles. A number of in vitro studies have reported that TiO_2 nanoparticles, SiO_2 nanoparticles, and multi-walled carbon nanotubes cause oxidative stress mediated toxicity. Oxidative stress is a state of redox disequilibrium in which ROS production overwhelms the antioxidant defense capacity of the cell, thereby leading to adverse biological consequences. It is often expressed in terms of the glutathione (GSH) to glutathione disulfide (GSSG) ratio in the cell. This ratio not only serves as a chief homeostatic regulator of cellular redox balance but also functions as a sensor that triggers cellular responses, which depending on the rate and level of decline, could be protective or injurious in nature (Xia et al., 2006). Nanoparticles containing transition metals and organic compounds were found to generate ROS by fenton-type reactions (Kumagai et al., 1997; Shvedova et al., 2003).

Studies in which titanium oxide nanoparticles were exposed to human lung epithelial cell lines and human bronchiolal epithelial cell lines showed an increase in ROS generation (Singh et al., 2007; Park et al., 2008). Furthermore, brain cultures of immortalized mouse microglia exposed to titanium oxide nanoparticles responded with an immediate and prolonged release of

reactive oxygen species (Long et al., 2007). In *vivo* studies have also shown that oxidative stress resulting from experimental instillation of TiO_2 nanoparticles resulted in airway inflammation and interstitial fibrosis (Nel et al., 2006). ROS generation capability of silicon oxide nanoparticles and carbon nanotubes was seen when treatment of these particles to primary mouse embryo fibroblasts at various concentrations induced ROS generation (Yang et al., 2008).

Oxidative stress is known to cause lysosomal membrane permeabilization (LMP) by oxidation of membrane lipids, resulting in the destabilization of lysosomal membrane (Brunk et al., 2001). Lysosomal membrane Permeabilization is the perturbation of lysosomal membrane function that leads to the translocation of catabolic hydrolases from the lysosomal lumen to the rest of the cell, which can mediate caspase-dependent apoptosis, caspase-independent apoptosis-like cell death or even necrosis following high levels of LMP (Jaattela, 2004). To best of our knowledge only one study has reported lysosomal injury which was in response to silicon oxide nanoparticles exposure to mouse alveolar macrophage cell line (MH-S) (Thibodeau et al., 2004). Oxidative stress is also known as a stimulus for intracellular pathways that contribute to upregulation of interleukin-8 (IL-8) synthesis (Lakshminarayanan et al., 1998).

Interleukin-8 is a member of the chemokines family and it is one of the first chemokines discovered. The chemokines are specialized cytokines produced and secreted by a variety of normal and neoplastic human cell types generally secreted in response to inflammatory cytokines, growth factors, and pathophysiological conditions. Interleukin–8 plays an important role in autoimmune, inflammatory, and infectious diseases. Due to its pro-inflammatory properties, it is tightly regulated, and its expression is low or undetectable in normal tissues (Brat et al., 2005). Studies have revealed that exposing cells to TiO2 nanoparticles, SiO2 nanoparticles, and multi-walled carbon nanotubes results in the increased release of interleukin-8, suggesting cell irritation (Singh et al., 2007; Ovrevik et al., 2006; Panessa-Warren et al.,

2006). The effects of titanium oxide on interleukin-8 expression in A549 human lung epithelial cells were investigated because these cells are an important source of interleukin-8 in the lungs, and this chemokine is an important mediator for pulmonary inflammation in humans. It was observed that following 24 hours titanium oxide exposure to A54 cells causes a significant increase in interleukin-8 from these cells along with enhanced mRNA expression of interleukin-8 in the cells (Singh et al.,2007). Interleukin-8 is shown to be induced in A549 cells via a mechanism involving oxidative stress and activation of the redox-sensitive transcription factor nuclear factor kappa-B. In the same study it was observed that interleukin-8 release paralleled the effects on ROS generation (Singh et al., 2007). Similar effects were seen in humans exposed to crystalline silica in the form of silica dust in the course of mining operations, foundry work, mineral processing, and construction sites. Inhaled crystalline silica is shown to induce chronic obstructive pulmonary disease, silicosis, or even lung cancer (Hnizdo, 2003). Epidemiological studies have shown that exposure to crystalline silica leads to airflow obstruction (Humerfelt et al., 1998). Studies have uncovered several potential mechanisms by which silica particles can initiate cell injury leading to chronic obstructive pulmonary disease, these include: cytotoxicity (Castranova, 2000) leading to generation of reactive oxygen/ nitrogen species (Vallyathan et al., 1997), and secretion of proinflammatory factors, cytokines, chemokines, elastase, (Li et al., 1996) and fibrogenic factors (Dai et al., 1998). Silica nanoparticles have caused injury to epithelial cells which facilitated penetration of the particles through the walls of small airways causing localized fibrosis (Dai et al., 1998). They also initiate toxic and inflammatory processes in conducting and peripheral airways, and alveolar tissue characterized by release of mediators leading to increased production of oxidants, cytokines, chemokines, and elastase, inducing airways inflammation (Castranova, 2000) and emphysema (Li et al., 1996). Mechanisms involved in silica-induce release of interleukin-8 from A549 cells were investigated and it was shown that mitogen-activated protein kinases p38 and extracellular

signal-regulated kinases-1 and -2 played important roles in the regulation of silica-induced generation of interleukin-8 (Ovrevik et al., 2006). It was also shown that silica induced activation of Src family kinases appears to be crucial to interleukin-8 release. Nanoparticles composed of metal oxides are not the only class that has shown to cause upregulation of interleukin-8 in cells. Exposure of multi-walled carbon nanotubes at various concentrations to human neonatal epidermal keratinocytes showed a dose dependent cytotoxicity with higher doses resulting in higher toxicity (Wei et al., 2007). These results were similar for when multi-walled carbon nanotubes were exposed to human epidermal keratinocytes (HEK) at various concentrations and for various exposure times, induced the release of interleukin-8 from HEK cells in a time dependent and dose dependent manner (Monteiro-Riviere et al., 2005).

2.2 DNA damage, upregulation of p53, and apoptosis

As mentioned in chapter 2.1, nanoparticle-cell interaction results in the generation of reactive oxygen species. The generation of reactive oxygen species may cause a breakdown of membrane lipids, an imbalance of intracellular calcium homeostasis, and DNA breakage (Clutton, 1997). DNA breakage triggers the DNA damage response which consists of orderly sequence of signal transduction events that can induce the accumulation of p53, which plays a critical role in responding to various stresses that cause DNA damage especially reactive oxygen species (Zhang et al., 2005). Studies have shown that multi-walled carbon nanotubes have induced expression of non-phosphorylated and phosphorylated p53, induced in response to DNA damage (zhu et al., 2007).

Previous studies have shown that titanium oxide nanoparticles exposure induces reactive oxygen species to cause DNA lesions (Kang et al., 2008). Western-blot analysis was performed for proteins involved in p53-mediated response to DNA damage which revealed accumulation of p53 and activation of DNA damage checkpoint kinases in titanium oxide nanoparticles treated lymphocytes. The nanoparticles exposure, however, did not affect downstream targets of p53,

indicating that titanium oxide nanoparticles do not stimulate transactivational acitivity of p53. The generation of reactive oxygen species was also observed and overall results suggested that titanium oxide nanoparticles induces ROS generation in lymphocytes, thereby activating p53-mediated DNA damage checkpoint signals (Kang et al., 2008). Silicon oxide nanoparticles, however, have shown to induce p53 transactivation via induction of p53 protein expression and phosphorylation of p53 and that p53, which plays a crucial role in the signal transduction pathways of silica-induced apoptosis (Wang et al., 2005). Multi-walled carbon nanotubes have shown to elicit similar responses when they interacted with cells. Multi-walled carbon nanotubes incorporated into human embryonic stem cells induced DNA damage and expression of non-phosphorylated and phosphorylated p53 (Hoet and Boczkowski, 2008). Along with DNA damage, the increased generation of reactive oxygen species, if excessive can activate caspase 8 and its downstream effectors, caspase 3/7, inducing apoptosis through extrinsic pathways (Long et al., 2007). A study investigating TiO_2 nanoparticles interaction with brain cultures of immortalized mouse microglia reported significant increases in caspase 3/7 activity, signaling the cell's entry into apoptosis (Long et al., 2007). Short-term nanoparticles exposure to mouse macrophage cell line (MH-S cells) elicited activation of caspase 3 and caspase 9, leading to cell death via apoptosis (Thibodeau et al., 2003). While exposure of TiO_2 and SiO_2 nanoparticles to cells resulted in significant increases in caspase-3/7 activity, exposure of MWCNTs to mouse macrophages (J774.1) did not significantly activate common apoptosis pathways such as caspase 3 and PARP (Hirano et al., 2008). To the best of our knowledge very few studies have attempted to monitor caspase 3/7 activity, but studies have demonstrated that these nanoparticles cause cell death via apoptosis (Panessa-Warren et al., 2006).

Numerous cytotoxic studies have investigated multiple pathways of nanoparticle-induced cell death. Results from most of these studies suggest oxidative stress to be the initiating factor that leads to increase in release of pro-inflammatory cytokines, damage and eventually cell

death via apoptosis. Some studies suggest the destabilization of lysosomal membrane initiates cell death. Researchers investigating the effects of oxidative stress on cells have reported damage to lysosomal membrane. It has been reported that ROS generated by quartz-silica leads to destabilization of lysosomal membrane, but it is still not know whether nanoparticles with different surface composition, such as titania and carbon, elicit same responses. To date, no one has attempted to investigate the role of the size of various diameters of carbon nanotubes in cell toxicity. Therefore, we have chosen to focus this project on titanium oxide (TiO_2) nanoparticles, silicon oxide (SiO_2) nanoparticles, and multi-walled carbon nanotubes (MWCNTs) with three different diameters. The goal of this study is to investigate the underlying mechanism of nanoparticle-induced cytotoxicity using 3T3 fibroblasts as the model cell type.

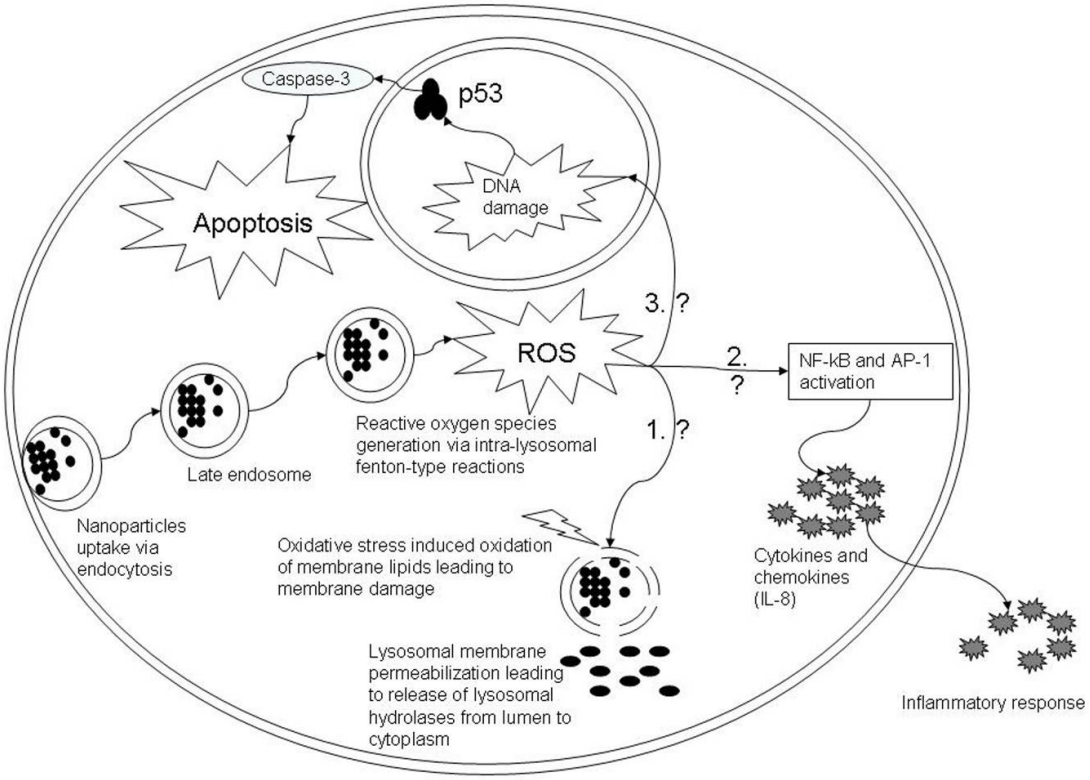

Figure 1. Illustration of intracellular responses to nanoparticles. Three potential mechanisms are demonstrated once the nanoparticles are endocytosed.

Potential Mechanism I: Endocytosed nanoparticles trigger the generation of intracellular reactive oxygen species (ROS) via intra-lysosomal fenton-type reaction. Such reaction causes oxidation of membrane lipids that leads to lysosomal membrane damage. This causes the release of lysosomal hydrolases into the cytoplasm of the cell resulting in caspase-dependent apoptosis.

Potential Mechanism II: Endocytosed nanoparticles trigger the increase generation of intracellular ROS triggering activation of nuclear facto-kappaB and AP-1. This results in the increase of interleukin-8 (IL-8) in the cell and also release of IL-8 from cell triggering an inflammatory response.

Potential Mechanism III: Endocytosed nanoparticles trigger increase in ROS, causing breakage in DNA and initiating DNA damage response. This response consists of orderly sequence of signal transduction events that can induce the accumulation of p53, then activation of caspase 3 that leads to cell death via apoptosis.

CHAPTER 3

HYPOTHESES OF NANOPARTICLE AND NANOTUBE-INDUCED CELL TOXICITY

In order to establish an underlying mechanism of cell death that results due to nanoparticle exposure to the cells, reasonable proposals predicting probable cause of the observed phenomena must be made. These proposals also known as hypotheses must be based on previous observations or on extension of scientific theories. A useful hypothesis might predict the outcome of an experiment either in laboratory settings or phenomena observed in nature. Furthermore, it will enable predictions by reasoning. This chapter describes the hypotheses that are tested in the study. Therefore, this chapter will be divided into four sections with each section dedicated to a single hypothesis.

3.1 Hypothesis 1: Cytotoxicity varies based on nanomaterial composition

Earlier cytotoxic studies have suggested that the surface chemistry of the nanoparticles plays a role in their toxic effects to cells. Results from these studies show that cytotoxicity of the nanoparticles seems to vary with their material composition. For the first study, we have chosen to investigate the Cytotoxicity of TiO_2, SiO_2, and MWCNTs to see whether changing the material composition of the nanoparticles, and nanotubes have any effect on toxicity to the cells. For this study, 3T3 fibroblasts were chosen to investigate the Cytotoxicity of TiO_2, SiO_2, and MWCNT <8nm at concentrations of 1, 10, 100 and 1000 µg/ml.

3.2 Hypothesis 2: Cytotoxicity varies based on nanotube diameter size

Particle size has been shown to play role in the toxic effects of the nanoparticles on cells. Generally as the particle size decreases, it's toxicity to cells increases. To date researchers have compared effects different diameter size nanoparticles of same composition (ex:

carbonaceous or metal oxides) but not the same type of nanomaterial (ex: MWCNT). In one study, the effect of MWCNTs length on activation of human acute monocytic leukemia cell line THP-1 and inflammatory response after subcutaneous implantation was investigated. The change in length of the MWCNTs did not significantly affect the monocyte activity nor was there a difference in inflammatory response between the two. However, the effect of changing the diameter of same nanomaterials of cell toxicity has not been investigated. In this study, we have chosen to investigate the cytotoxicity of MWCNT of fixed length, but different diameters: <8nm, 20-30nm, and >50nm to see whether changing the diameter of MWCNTs has any effect on survival rates of the cells. For this study, 3T3 fibroblasts were chosen and nanoparticles concentrations of 1, 10, 100 and 1000 µg/ml were used.

3.3 Hypothesis 3: Cytotoxicity is dependent on nanoparticle and nanotube exposure time

Results from various cytotoxic studies show that cell viability is dependent on the amount of time cells are exposed to the nanoparticles. Studies investigating cytotoxic effects of nanoparticles after 24, and 48 hours of exposure observed that cell viability decreased with increase in time of exposure. To date no studies have investigated the earliest exposure time which results in a significant reduction in cell viability. Therefore, we have chosen to investigate the influence of exposure time of the nanoparticles on Cytotoxicity to the cells. By doing so, we can determine the Onset Cytotoxicity of TiO_2, SiO_2, and MWCNT <8nm to 3T3 fibroblasts as well as establish a relationship between the change in diameter of Multi-Walled Carbon Nanotubes and the Onset of Cytotoxicity. For this study, 3T3 fibroblasts were chosen to investigate the influence of exposure time of the TiO_2, SiO_2, and MWCNTs at a concentration of 100 µg/ml on cytotoxicity to the cells because all of the nanoparticles exhibited similar cytotoxicity at this concentration. Therefore, this will be the ideal concentration to use to investigate and determine whether different nanoparticles elicit different response when they interact with cells.

3.4 Hypothesis 4: Generation of reactive oxygen species by nanoparticles and nanotubes induces cell death

After conducting a literature review regarding the effects of selected nanoparticles' interaction with the cells, we believe that the nanoparticles exposure may result in increased production of intracellular reactive oxygen species. Studies have shown that reactive oxygen species, an intracellular second messenger, causes lysosomal membrane permeabilization. Lysosomal membrane permeabilization results in the release of catabolic hydrolases that can mediate caspase-dependent apoptosis, caspase-independent apoptosis-like cell death or even necrosis. Therefore, we believe that the increase in intracellular reactive oxygen species will cause damage to lysosome's membrane which results in leakage of lysosomal hydrolases leading to apoptosis. Intracellular ROS production and lysosomal membrane permeabilization may be enhanced in cells that are exposed to carbon nanotubes when compared to titanium oxide nanoparticles and silicon oxide nanoparticles.

Therefore, we aim to detect intracellular ROS production, lysosomal membrane permeabilization, and apoptosis in 3T3 fibroblasts in response to the nanoparticles exposure at a concentration of 100µg/ml.

CHAPTER 4

MATERIALS AND METHODS

4.1 Test Materials

Table 1. List of test subjects and their dimensions

Test Material	**Size**
Titanium Oxide (TiO_2)	5-10 nm in diameter
Silicon Oxide (SiO_2)	30 nm in diameter
Multi-walled carbon nanotube (MWCNT)	<8 nm in diameter; 0.5-2 µm in length
Multi-walled carbon nanotube (MWCNT)	20-30 nm in diameter; 0.5-2 µm in length
Multi-walled carbon nanotube (MWCNT)	>50 nm in diameter; 0.5-2 µm in length

The test subjects used in all the studies listed in Table 1 were acquired from Sun Innovations, Inc. (Fremont, CA). The particle solutions were prepared in Dulbecco's Modified Essential Media (DMEM) supplemented with 10% Calf Serum (CS) and 1% antibiotics at a concentration of 1 mg/ml. Prior to culture, the particle solutions were sonicated and/or vortexed to resuspend the particles, and the solutions were then added to cell seeded well plates at the desired concentrations.

4.2 Reagents

3T3 cells were purchased from ATCC (Manassas, VA). DMEM was purchased from Sigma-Aldrich (St. Louis, MO). Calf Serum and antibiotics were purchased from Atlanta Biologicals (Lawrenceville, GA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt (MTS) was purchased from Promega Corporation

(Madison, WI). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for reactive oxygen species and Live/Dead Viability/Cytotoxicity Kit were purchased from Invitrogen (Carlsbad, CA). Acridine Orange (AO) for lysosomal stability was purchased from Merck (Darmstadt, Germany). Sensolyte Homogeneous AMC Caspase-3/7 Assay kit was purchased from Anaspec (San Jose, CA).

4.3 Cell Culture

3T3 cells are mouse fibroblasts cells used as a model cell in various cell studies. Cells were cultured in Dulbecco's Modified Essential Media (DMEM) supplemented with 10% Calf Serum and 1% antibiotics. Unless stated otherwise, for all the studies 5000 3T3 fibroblasts were seeded per well in a 96-well plate. The experiments were performed on the following day.

4.4 Test Methods

4.4.1 Cell viability study

For all viability studies, 5000 cells per well were seeded in a 96-well plate. On the following day, the cells were incubated with the different nanoparticles at concentrations of 1, 10, 100, and 1000 µg/ml for 24 hours. Four samples were used in each treatment condition. The viability was conducted using The CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay. It is a colorimetric method for determining the number of viable cells in proliferation or Cytotoxicity assays. This solution reagent contains a novel tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.

After 24 hours of incubation with nanoparticles, the culture media containing the nanoparticles was removed. After removal of the culture media, the cells were rinsed three times in PBS followed by the addition of 100 µL of culture media. Assays were performed by adding 20 µL of the CellTiter 96 AQ_{ueous} One Solution Reagent directly to culture wells containing 100 µL of culture media and incubating the 96-well plate for 2 hours and measuring the absorbance. Samples were compared to control, untreated sample optical densities (OD). Sample optical densities were read at 490nm with background at 630nm. Background readings were taken of culture media with 20 µL of the CellTiter 96 AQ_{ueous} One Solution Reagent and subtracted from sample readings. The quantity of formazan product as measured by the absorbance at 490nm directly proportional to the number of living cells in culture. Four samples were used in each case and percentages of live cells were calculated with respect to the controls.

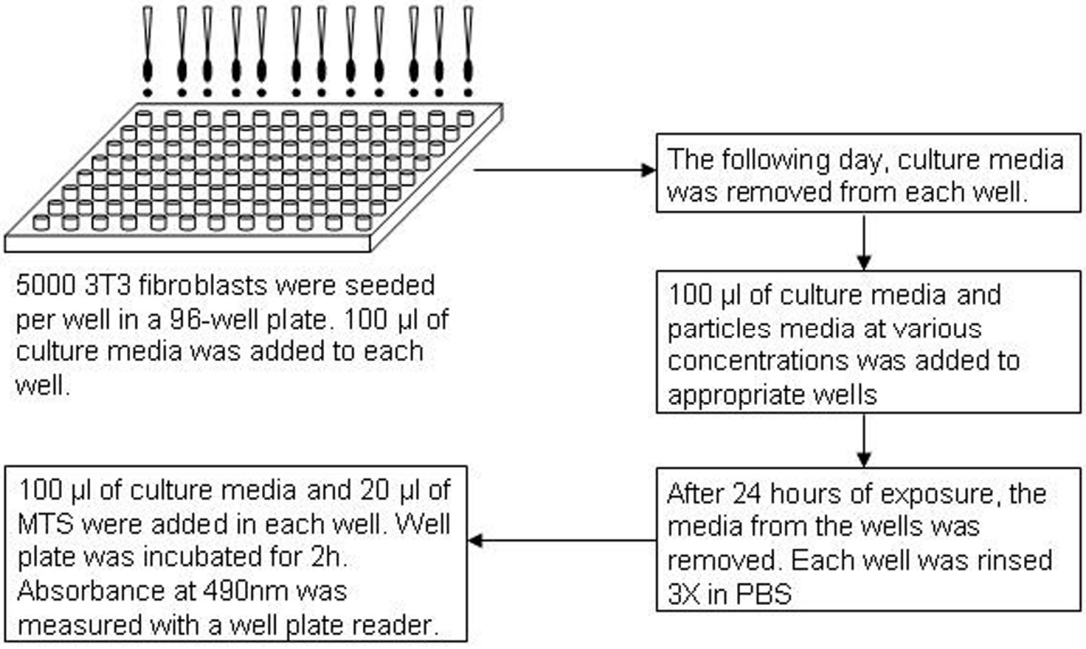

Figure 2. Schematic of the method followed for cell viability study after 24 hours of exposure to nanoparticles and nanotubes.

4.4.2 Onset cytotoxicity study

For onset cytotoxicity study, 5000 cells per well were seeded in a 96-well plate. On the following day, cells were incubated with the different nanoparticles at a concentration of 100 µg/ml for 6, 12, 18, and 24 hours. Four samples were used in each treatment condition. After the appropriate incubation period with the nanoparticles, the viability was conducted using The CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay as described in chapter 4.4.1. Briefly, 20 µL of the CellTiter 96 AQ_{ueous} One Solution Reagent was added directly to culture wells containing 100 µL of culture media. After two hours of incubation the absorbance was recorded at 490nm with background at 630nm with a 96-well plate reader. The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture. Four samples were used in each case and percentages of live cells were calculated with respect to the controls. Onset cytotoxicity is the minimum amount of exposure time required for the nanoparticles and nanotubes to cause significant reduction in live cells. It is determined by monitoring the first significant reduction in toxicity from the zero hour time point and previous time point toxicity level.

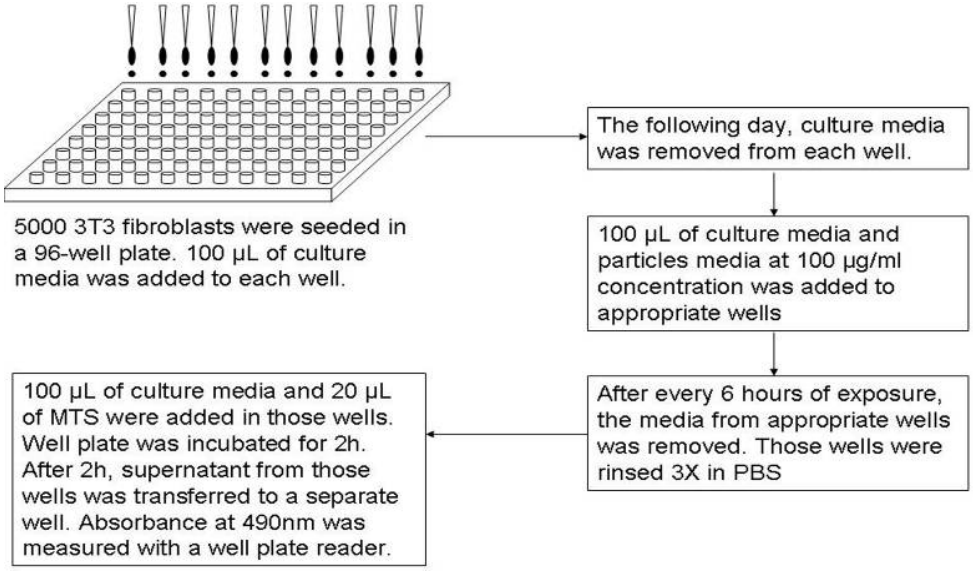

Figure 3. A schematic of the method followed for onset cytotoxicity study

4.4.3 Cell viability imaging

For all viability imaging, 3T3 fibroblasts were grown to sub-confluence on coverslips. The cells were then incubated with different nanoparticles at a concentration of 100 µg/ml for 18 hours. The cells were then stained with Live/Dead Viability/Cytotoxicity Kit according to the manufacture's protocol. It is a quick and easy two color assay to determine the viability of cells in a population. The kit identifies live versus dead cells on the basis of membrane integrity and esterase activity. This kit comprises two probes: calcein AM, a fluorogenic esterase substrate that is hydrolyzed to the green-fluorescent calcein, and ethidium homodimer-1, a high-affinity, red-fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. Thus, green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products, and red fluorescence is an indicator of cells with compromised membranes.

Briefly, 1µM calcein AM and 2µM EthD-1 solutions were prepared in PBS. After 18 hours of incubation with the particles, the cells were rinsed with PBS and the staining solution was added to cover the coverslips followed by 30 minutes of incubation. The cells were then photographed using a Leica fluorescence microscope coupled with a digital camera.

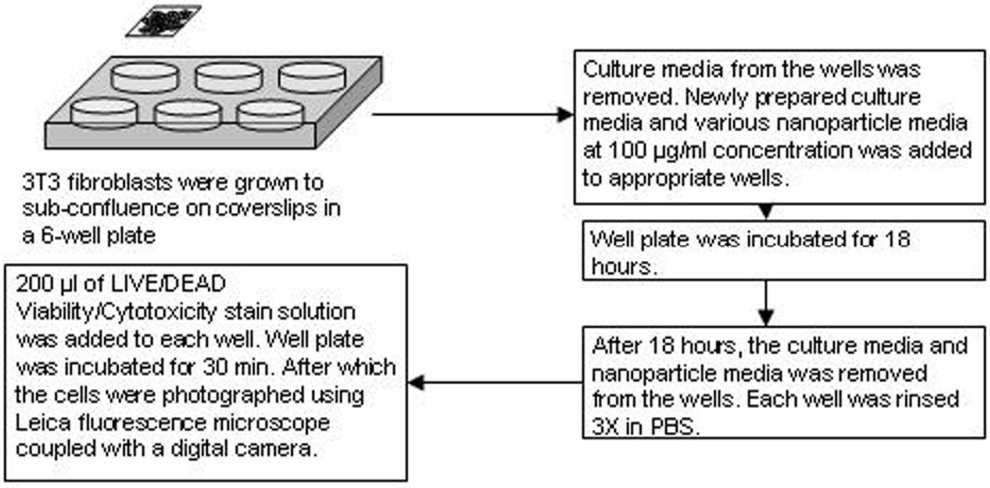

Figure 4. A schematic of the method followed for imaging Live and Dead cells following 18 hours of exposure to the nanoparticles and nanotubes

4.4.4 Assessment of ROS generation

After removing the culture media from the cell-seeded wells of the 96-well plates, 100µl solutions of TiO_2, SiO_2, and MWCNTs at a concentration of 100 µg/ml were added to the appropriate wells and the cells were incubated with the nanoparticles for 4 hours. After exposure, Intracellular Reactive Oxygen Species production was detected using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA is a cell-permeant indicator for reactive oxygen species that is nonfluorescent until the acetate groups are removed by the intracellular esterases and oxidation occurs within the cells, thereby indicating the level of ROS. Esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than is the parent compound. Oxidation of these probes can be detected by monitoring the increase in fluorescence with a flow cytometer, fluorometer, microplate reader, or fluorescence microscope, using excitation sources and filters appropriate for fluorescein (FITC).

After cells were exposed to nanoparticles, the culture media containing the nanoparticles was removed and the cells were washed three times with PBS. Thereafter, 100µl of 5-µM H_2DCFDA diluted in PBS was added to each well, and the cells were incubated for 30 min. The H_2DCFDA was the removed, and each well was washed with PBS. 200µl of PBS was added to each well and fluorescence was measured with excitation set at 485nm and emission at 530nm. Four samples were used in each treatment condition.

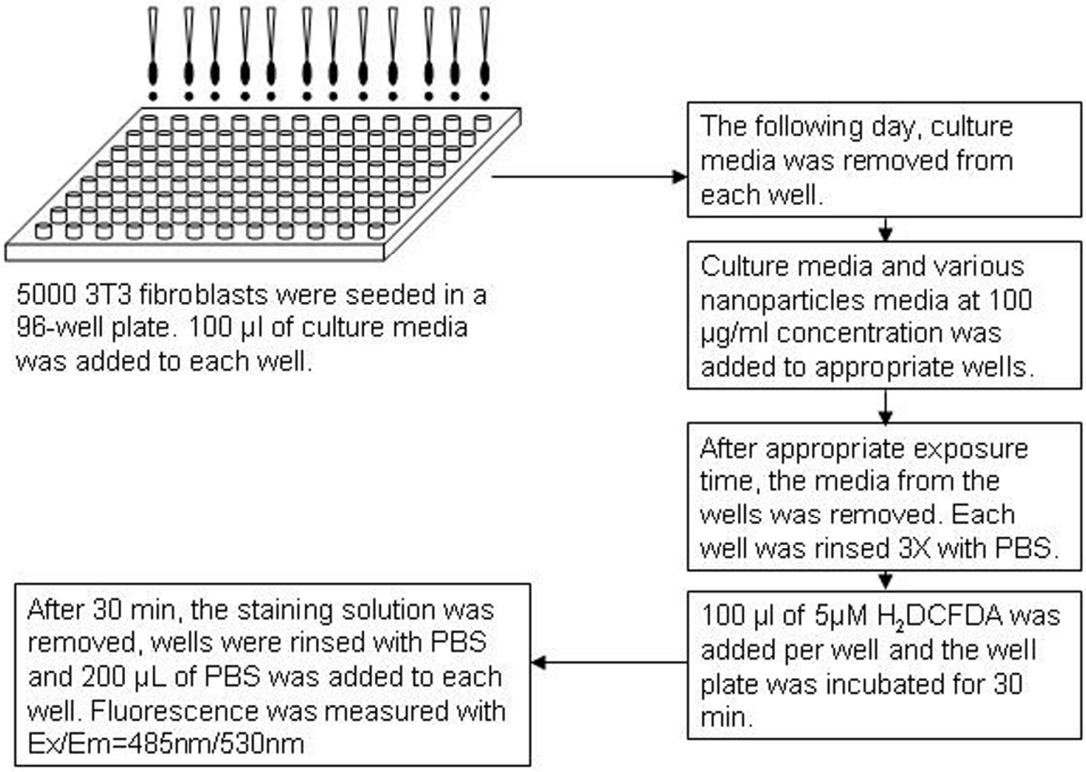

Figure 5. A schematic of the method followed for detecting generation of ROS following four hours of nanoparticles and nanotubes exposure to 3T3 fibroblasts.

4.4.5 Morphological assessment of lysosomes

To visualize the lysosomes, 3T3 fibroblasts were grown to sub-confluence on coverslips. The cells were then loaded with AO (5 µg/ml) in culture media for 15 min at 37^{o}C, rinsed with DMEM and incubated with the nanoparticles at a concentration of 100 µg/ml for 4 hours. Acridine Orange (AO) is a weak base that is able to cross the plasma membrane and due to proton trapping, preferentially distributes within the acidic vacuolar (lysosomal) cellular compartment. AO molecules then become protonated, and trapped by base stacking within the granular membrane. Continuing influx of AO molecules results in a change in the fluorescence emission of the probe from green to red. This results in red fluorescence inside the lysosomes,

where it is highly concentrated, and weekly green in the cytosol and nucleus, where it is much less concentrated. Disruption of the lysosomal membrane and/or marked change in lysosome pH can, therefore, be assessed by measuring the change in emission ratio in comparison to controls and by visual inspection of the cells by confocal microscopy. After 4 hours, the culture media containing nanoparticles was removed and cells were rinsed with PBS and photographed using a Leica fluorescence microscope coupled with a digital camera.

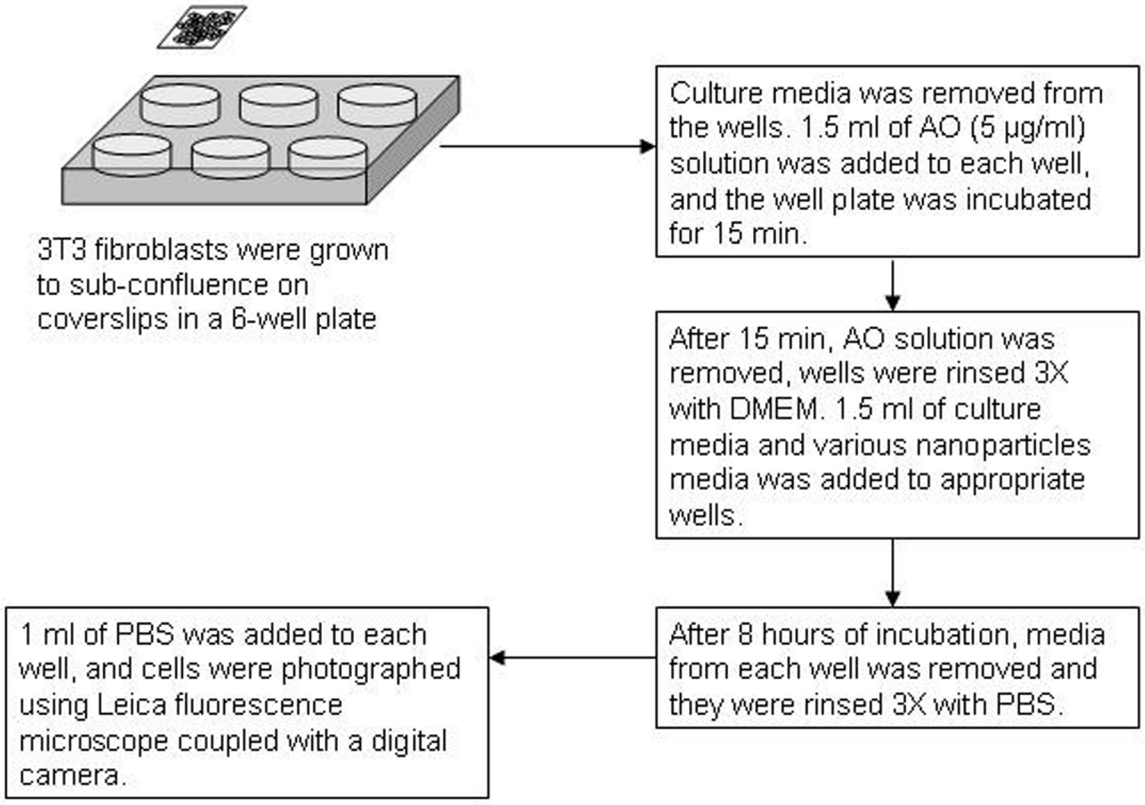

Figure 6. Schematic of the method followed for visualizing lysosomes following four hours of exposure to nanoparticles and nanotubes.

4.4.6 Lysosomal stability assessment

The lysosomal membrane integrity following incubation of nanoparticles with 3T3 fibroblasts was assessed using the acridine orange relocation technique.

For our experiment, cells were loaded with Acridine Orange (5 µg/ml) in culture media for 15min at 37^{o}C, rinsed with DMEM and then incubated with 100 µl of 100 µg/ml concentrated TiO_2, SiO_2, and MWCNTs for 4 hours. Four samples were used in each particle treatment. After exposure, the culture media containing the nanoparticles was removed and the cells were rinsed three times in PBS. 100 µl of PBS was added to each well and the fluorescence was measured with the excitation wavelength set of 485nm and readings made at 530nm (Cytoplasm) and 620nm (Lysosomes). Analysis was done by taking the 530nm/620nm intensity ratio and calculating the percent increase or decrease of this ratio with respect to the controls.

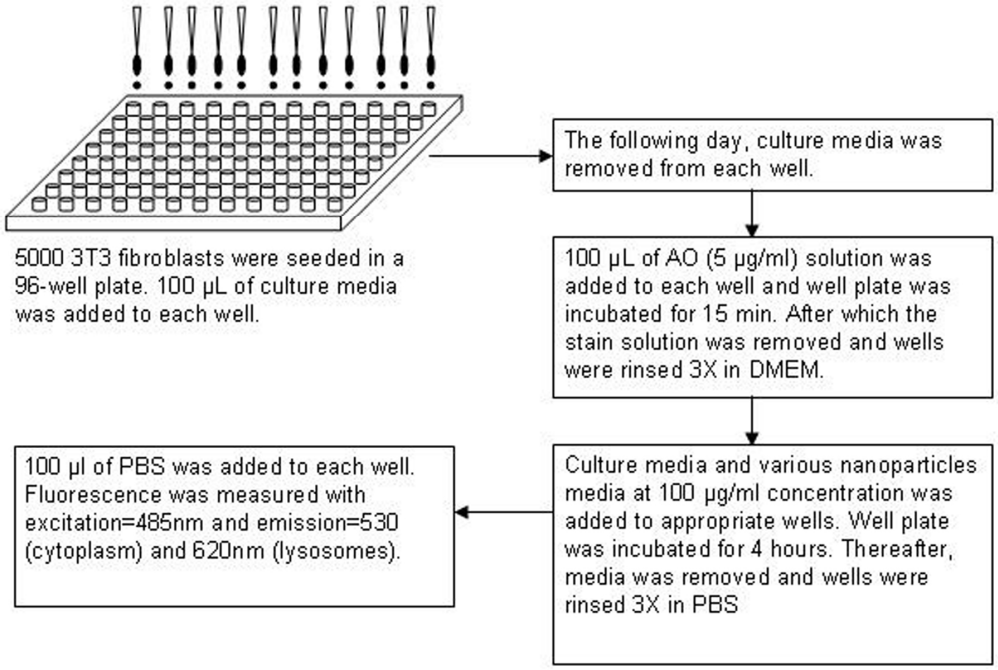

Figure 7. Schematic of the method followed for monitoring lysosomal membrane permeabilization following four hours of exposure to nanoparticles and nanotubes.

4.4.7 Caspase activity assay

The Caspase-3/7 assay was performed with Sensolyte Homogeneous AMC Caspase-3/7 Assay Kit according to manufacturer's protocol. This kit is optimized to detect caspase-3/7 activity in cell culture directly without time consuming cell extraction step. The two closely related caspase-3 and caspase-7 are central to the execution phase of apoptosis. The

Sensolyte Homogeneous AMC Caspase-3/7 Assay Kit uses Ac-DEVD-AMC as the fluorogenic indicator for assaying caspase-3/7 activities. Upon caspase-3/7 cleavage, Ac-DEVD_AMC generates the AMC fluorophore that has bright blue fluorescence and can be detected at excitation/emission=354nm/442nm. It is a bi-functional assay that lyses the cells and provides optimal conditions for measuring enzymatic activity.

In our study, 5000 3T3 fibroblasts were seeded per well in a 96-well plate. On the following day, 150 µl of different nanoparticles' media at a concentration of 100 µg/ml was added to the appropriate wells and the well plate was incubated for 10 hours. After 10 hours, 50 µl of the working solution of the caspase-3/7 that was prepared following manufacturer's protocol was added to each well and the well plate was incubated at room temperature for 6 hours. Thereafter the fluorescence in each well was measure as described above.

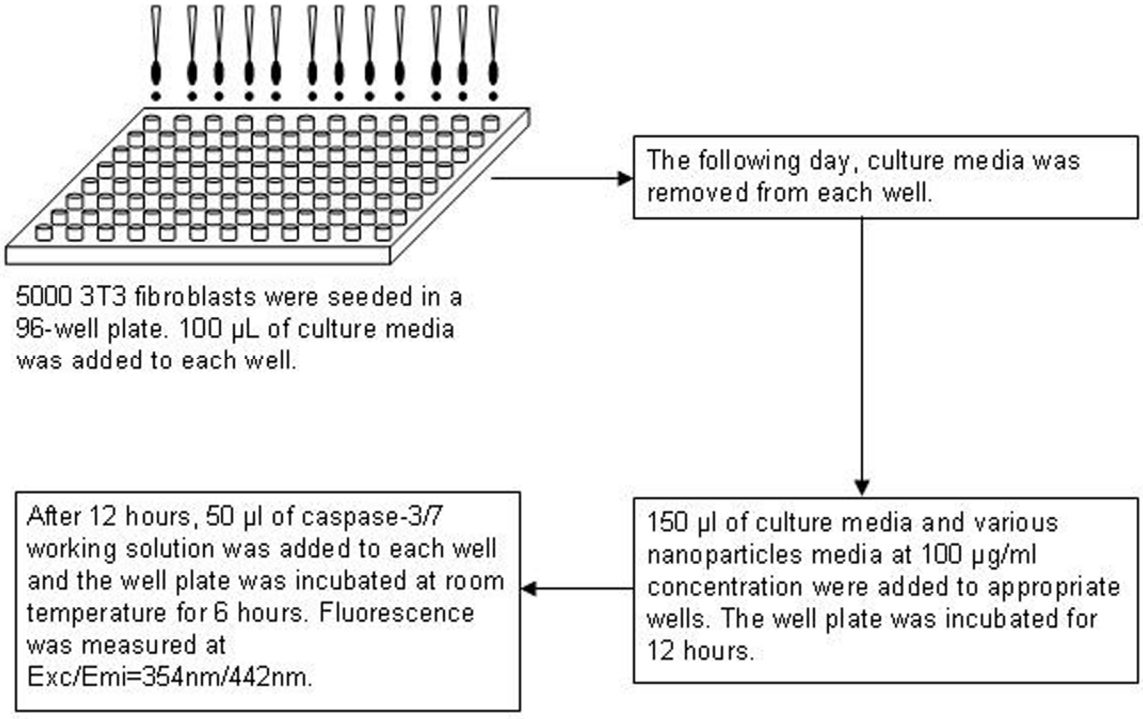

Figure 8. Schematic of the method followed for detecting caspase-3/7 activity due to exposure of nanoparticles and nanotubes.

4.5 Statistical Analyses

Statistical comparisons were conducted using EXCEL equipped with data analysis plugin provided within the software. ANOVA was performed to compare the data of changing material composition, concentration and diameter, and student t-tests were performed to compare means of various exposure times for statistical significance. Differences were considered statistically significant when $p<0.05$.

CHAPTER 5

EVALUATION OF CELL TOXICITY OF TiO_2 NANOPARTICLES, SiO_2 NANOPARTICLES, AND MULTI-WALLED CARBON NANOTUBES

The goal of this study is to establish the toxicity of the selected nanomaterials to the 3T3 fibroblasts, as well as investigate whether changing the diameter of the multi-walled carbon nanotubes has any effect on the cytotoxicity of these carbon nanotubes to the 3T3 fibroblasts.

5.1 Cytotoxicity varies based on nanomaterial composition

5.1.1 Rationale

After conducting a literature review regarding the selected nanomaterials' interactions with the cells, we believe that the toxicity of different nanomaterials at different concentrations differs when they are exposed to the same type of cell.

Therefore, we aim to establish the cytotoxicity of TiO_2, SiO_2, and MWCNT <8nm to 3T3 fibroblasts at concentrations of 1, 10, 100, and 1000 µg/ml as described in chapter 4.4.1.

Hypothesis 1: Cytotoxicity varies based on nanomaterial composition.

5.1.2 Results

Influence of nanomaterials' composition and their concentrations on cell survival: The effect of changing the nanomaterials' composition along with their concentration on cell survival was examined. TiO_2 nanoparticles, SiO_2 nanoparticles and MWCNT <8nm were exposed to 3T3 fibroblasts at concentrations of 1, 10, 100, and 1000 µg/ml for 24 hours. Changing the nanomaterials' composition did affect the cell viability significantly at all

concentrations that were tested. Cell viability was also significantly affected by increasing the concentration of the nanoparticles and nanotubes. Of the three, TiO_2 nanoparticles were the least toxic to 3T3 fibroblasts while MWCNT <8nm were the most toxic. With TiO_2 nanoparticles little to no effect was observed on 3T3 fibroblasts survival up to concentration of 10 µg/ml, but a significant decrease in cell viability was seen at a concentration of 100 µg/ml. In contrast, SiO_2 and MWCNT <8nm affected the cell survival even at the low concentration of 1 µg/ml and exhibited a decrease in cell viability as their concentration was increased.

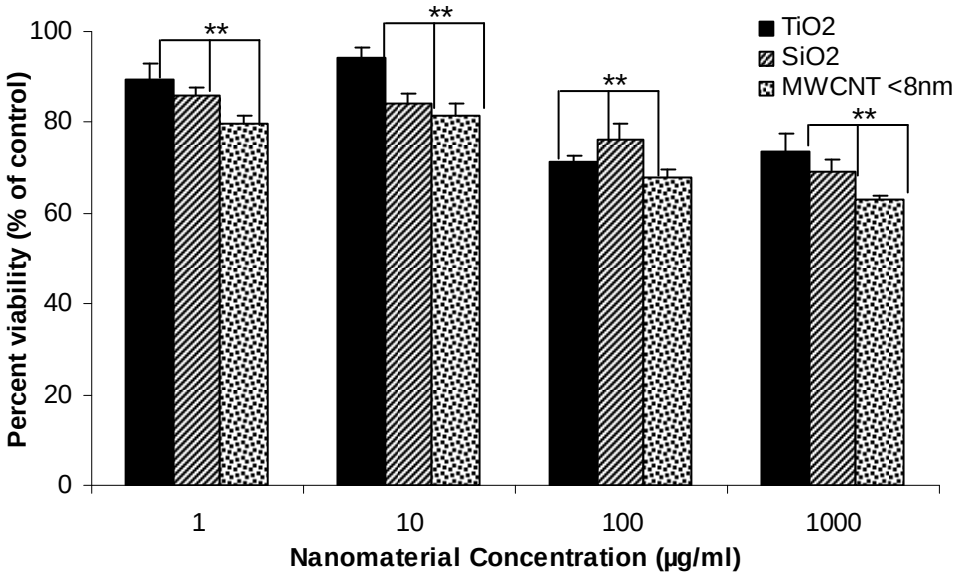

Figure 9. The influence of nanomaterial composition on survival rates of the cells. Vertical lines denotes ± 1SD (n=4 for all test samples and cells). (**) indicates statistically significant difference between TiO_2 nanoparticles, SiO_2 nanoparticles and MWCNT <8nm ($p<0.05$).

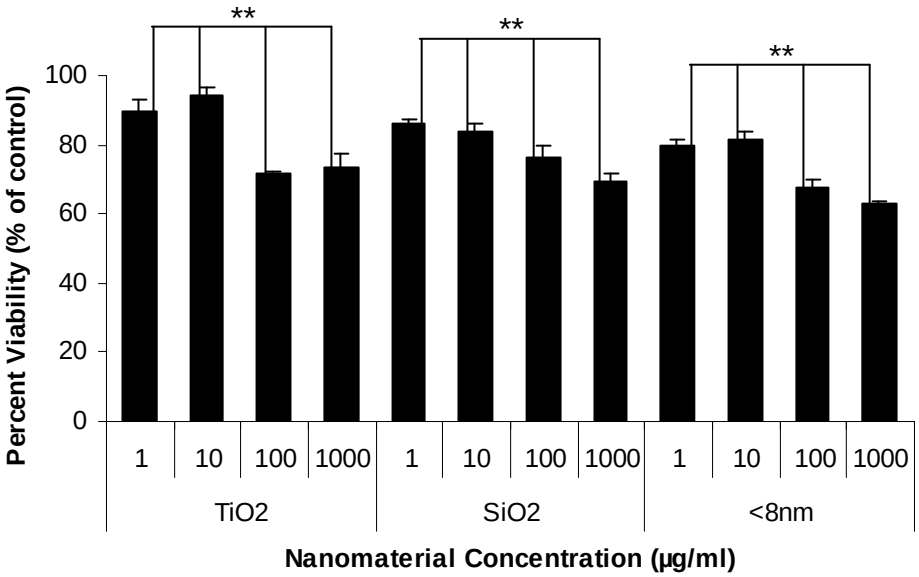

Figure 10. The influence of nanomaterial concentration on survival rates of the cells. Vertical line denotes ±1 SD (n=4 for all test samples and cells). (**) indicates statistically significant difference between different concentrations of each nanomaterial ($p<0.05$).

5.1.3 Discussion

This study was designed to analyze the cytotoxicity of the nanoparticles and nanotubes to 3T3 fibroblasts and determine the effects of changing the nanomaterials composition and their concentration on survival rates of the cells. Among the three different nanomaterials used in this investigation, we find that TiO_2 nanoparticles have relatively low cytotoxicity to 3T3 fibroblasts at lower concentrations up until 10 µg/ml, whereas the multi-walled carbon nanotubes showed significant decrease in cell survival by up to 20% at 10 µg/ml. In TiO_2 nanoparticles and MWCNT <8nm we see an increase in cell viability from concentration of 1µg/ml to 10 µg/ml and then a decrease from 10 µg/ml onwards to 100 µg/ml. In TiO_2 nanoparticles we also see an increase in viability even though concentration was increased from 100 µg/ml to 1000 µg/ml. This increase in viability even though nanomaterial concentration was increased could be due to errors in the experimental procedure. These samples could have had higher population of cells. The level of toxic effects of SiO_2 nanoparticles fell between that of TiO_2 nanoparticles and

MWCNT <8nm. These results are in agreement with findings that the biological impacts of nanomaterials are dependent on chemical composition and shape along with size, surface structure, solubility, and aggregation. These parameters can modify nanomaterial-cell interaction including its uptake, protein binding, and possibility of causing injury to tissues (Nel et al., 2006). The reactivity of the substances used during nanomaterial synthesis that are contained within the nanomaterials has been shown to affect the toxicity of the nanomaterials. Pulmonary toxicity in mice via intratracheal instillation was investigated of unrefined SWCNT, SWCNT containing nickel, and SWCNT synthesize via elelctric arc discharge. It was observed that SWCNT containing nickel produced the highest mortality rate in mice compared to the other two (Smart et al., 2006).

5.2 Cytotoxicity varies based on nanotube diameter

Based on the cytotoxicity results of TiO_2 nanoparticles, SiO_2 nanoparticles, and MWNCT <8nm (Figure 1&2), we see that indeed different nanomaterials exhibit different levels of toxicity to 3T3 fibroblasts. Since these effects are likely due to the material of the nanomaterial and/or the size, changing its size may lead to changes in the toxicity of the nanoparticles to 3T3 fibroblasts. In section 5.1, we have investigated the cytotoxicity of different nanomaterials at different concentrations. In this section we will investigate whether changing the diameter and concentration of multi-walled carbon nanotubes leads to changes in their toxicity to 3T3 fibroblasts.

Hypothesis 2: Cytotoxicity varies based on nanotube diameter.

5.2.1 Rationale

In the first part of the study we were able to determine that TiO_2 nanoparticles, SiO_2 nanoparticles, and MWCNT <8nm each exhibit different levels of toxicity to 3T3 fibroblasts, especially with SiO_2 nanoparticles and MWCNT <8nm which exhibited decreased viability at relatively low concentrations. In this part of the study, we sought to determine whether changing

the diameter of the carbon nanotubes has any effect on the survival of 3T3 fibroblasts as described in chapter 4.4.2.

5.2.2 Results

Influence of nanotube diameter on cell survival: The effect of three different diameters of carbon nanotubes (<8nm, 20-30nm, >50nm) of fixed length at various concentrations on 3T3 fibroblasts' cell survival was examined. With MWCNT >50nm we see little to no effect at low concentration of 1 µg/ml, but the cell viability drops significantly as the concentration of the nanoparticle is increased. In contrast, multi-walled carbon nanotubes of diameter <8nm and 20-30nm affected cell survival even at very low concentration of 1 µg/ml but the drop in cell survival was not as significant as the concentration increased when compared with MWCNT >50nm. MWCNT 20-30nm affected the cell survival the most. Specifically, more than 30% of 3T3 fibroblasts were killed at a concentration of 1 µg/ml and 40% at a concentration of 1000 µg/ml.

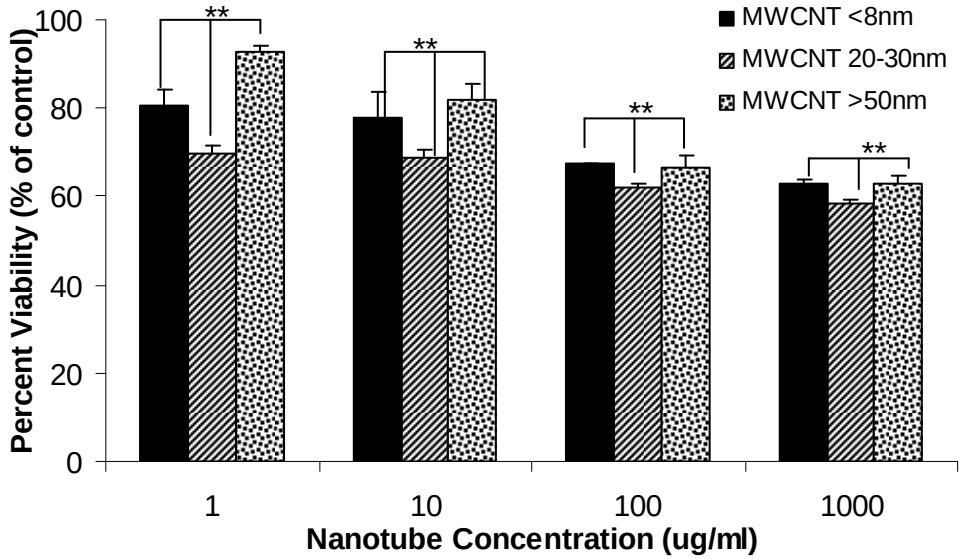

Figure 11. The effect of nanotube diameter on survival rates of the cells. Vertical line denotes ±1 SD (n=4 for all test samples and cells). (**) indicates statistically significant difference between MWCNT <8nm, MWCNT 20-30nm, and MWCNT >50nm ($p<0.05$).

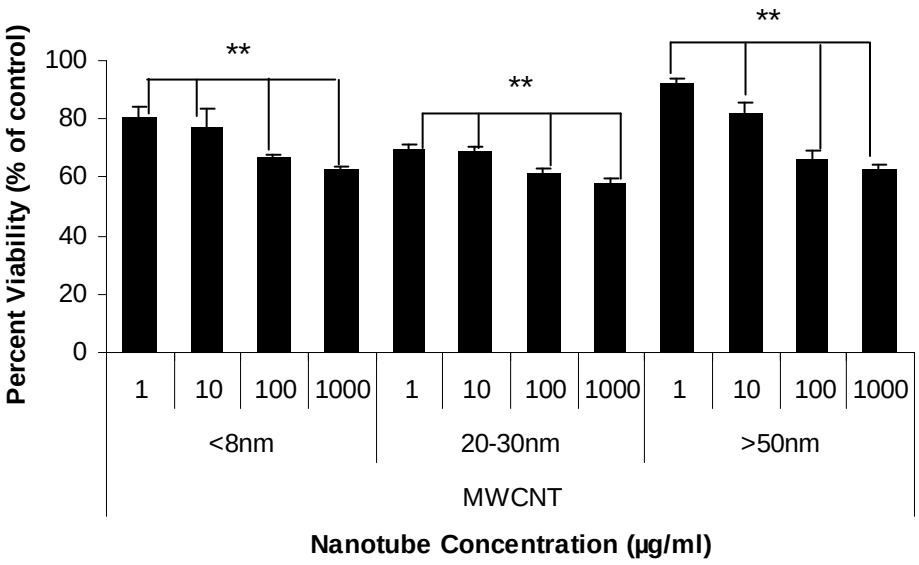

Figure 12. The influence of nanotube concentration on survival rates of the cells. Vertical line denotes ± 1SD (n=4 for all test samples and cells). (**) indicates statistically significant difference between different concentrations of each nanotube ($p<0.05$).

5.2.3 Discussion

The nanoparticle's dimension and composition has been shown to affect the cell toxicity (Grabinski et al., 2007). Studies that have varied the diameter of a single nanoparticle to investigate its effects on cell toxicity are limited. We thus hypothesize that, by varying the diameter of multi-walled carbon nanotubes, cytotoxicity of these particles to 3T3 fibroblasts is altered. Multi-Walled carbon nanotubes with three different diameters (<8nm, 20-30nm, and >50nm) were included in this investigation. Our results have shown that the toxicity of multi-walled carbon nanotubes changes as the diameter of these nanotubes changes. The results support the theory that size is one of factors influencing nanomaterials' toxicity to cells and tissues (Donaldson et al., 2004). Results from studies have suggested that the smaller the nanomaterials are, the greater their level of toxicity to cells (Grabinski et al., 2007). One possible explanation for this phenomenon is that the smaller the nanomaterial, the more surface area it has per unit mass. A larger surface area will provide the nanomaterials with a greater

area of contact with the cellular membrane, increasing the chances of absorption and transport of toxic substances. It has also been suggested that very small particles may not be detected by phagocytic defenses and could enter the blood or the nervous system causing damage, or they could act like haptens, either modifying protein structures, altering their structure or rendering them antigenic, raising the potential of autoimmune effects (Donaldson et al., 2004). We found that multi-walled carbon nanotubes with diameter of 20-30nm were the most toxic to 3T3 fibroblasts when compared with the other two. This is contrary to what is generally expected, the smaller the nanomaterials dimension the greater the toxicity. This could be due to combined effects of low 20nm diameter sized nanotubes which provided more surface interaction with the cells and also due to the high 20nm diameter sized nanotubes which most probably caused membrane damage to intracellular components after their uptake. The cumulative effects of these interactions are most like contributing to the high toxicity of these nanotubes to 3T3 fibroblasts.

CHAPTER 6

CYTOTOXICITY IS DEPENDENT ON NANOPARTICLE AND NANOTUBE EXPOSURE TIME

6.1 Rationale

Three factors are generally accepted which influence the potential of a material to cause harm, they include: the chemical composition of the material, the size of the material, and the retention time of the material. In chapters 5.1 and 5.2 we investigated the effects of varying chemical composition and size of the nanomaterial. Cytotoxicity studies investigating toxicity of nanomaterials after 24 and 48 hours of exposure have reported an increase in cell toxicity with an increase in time. To date no one has attempted to study the minimum exposure time required for the nanomaterials to cause harm to the cells. Therefore, we have chosen to investigate the influence of exposure time of TiO_2, SiO_2, and MWCNT <8 nm to 3T3 fibroblasts so as to see whether nanoparticle toxicity to 3T3 fibroblasts is dependent on their exposure time and also to determine the onset cytotoxicity of these particles with the 3T3 fibroblasts. Onset cytotoxicity, as stated in chapter 4.4.3, is the first significant reductions in toxicity form the zero hour time point and previous time point toxicity level. For this study, 3T3 fibroblasts were used to investigate the onset cytotoxicity of TiO_2, SiO_2, and MWCNT <8 nm at concentration of 100 µg/ml as described in chapter 4.4.3.

Hypothesis 3: Cytotoxicity is dependent on nanoparticle and nanotube exposure time.

6.2 Results

Influence of nanoparticle and nanotube exposure time on cell survival: The effect of nanoparticle and nanotube exposure time on survival rates of 3T3 fibroblasts was

investigated. Among the two different nanoparticles and three different nanotubes, only MWCNT <8nm and MWCNT 20-30nm showed a significant decrease in cell viability by 6 hours of exposure. After 12 hours of exposure, cell viability was significantly reduced in all cases. With TiO_2 nanoparticles the rate of decrease in cell viability seems to level off after 12 hours of exposure. With TiO_2 nanoparticles and MWCNT >50nm, there was a little but non-significant increase in cell viability from 18 hour time point to 24 hour time point. In all the cases, we observed a significant reduction in cell viability from 6 hour time point to 12 hour time point (Figure 15).

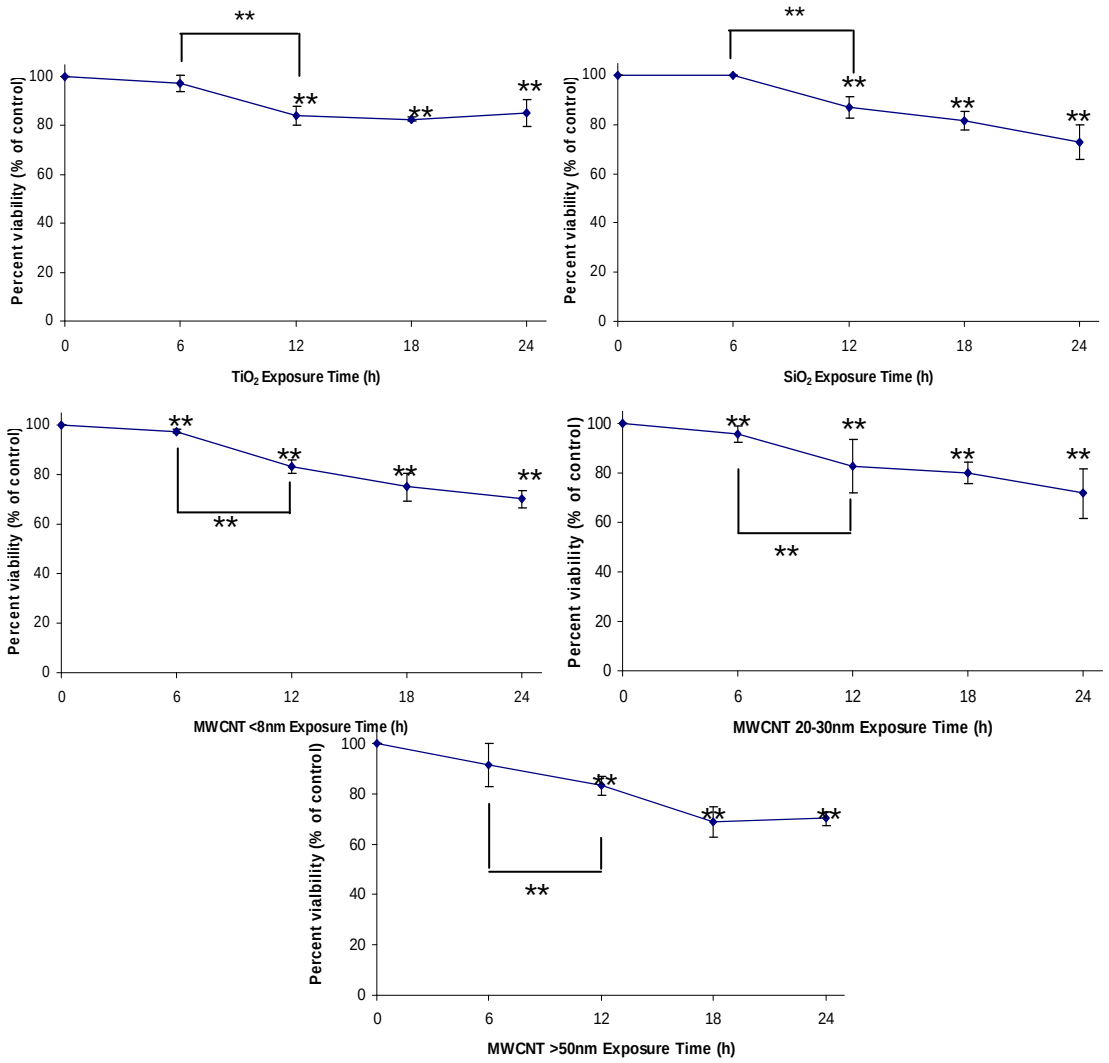

Figure 13. The influence of nanoparticles and nanotubes exposure time on survival rate of the cells. Vertical line denotes ±1 SD (n=4 for all test samples and cells). (**) denotes statistically significant difference between the present and zero hour toxicity time point ($p<0.05$).

6.3 Discussion

Studies have shown that the longer the nanomaterial stays in contact with the cellular membrane the greater the chance for damage (Donaldson and Tran, 2002). Our results are in agreement with this phenomenon. The nanoparticles and nanotubes used in this study caused a significant reduction in cell viability as their exposure time to the cells increased (Figure 15). In the case of TiO_2 nanoparticles, SiO_2 nanoparticles, and MWCNT >50 nm, 6 hours of exposure to 3T3 fibroblasts did not cause a significant reduction in cell viability. However, a significant reduction in the percentage of live cells was observed between 6 hours and 12 hours of exposure. With TiO_2 nanoparticles, the decrease in percentage of live cells after 18 hours of exposure was relatively similar to the percent viability after 12 hours of exposure. Furthermore, we observed a slight increase in cell viability after 24 hours of exposure compared to 18 hours of exposure. This increase could be due to the high variance in the results. This phenomenon was also observed with MWCNT >50 nm. These results indicate that TiO_2 nanoparticles after 12 hours of exposure do not cause any further significant damage to 3T3 fibroblasts, and MWCNT >50 nm after 18 hours of exposure do not cause any further significant damage to 3T3 fibroblasts. SiO_2 nanoparticles on the other hand, caused a significant reduction in cell viability as the exposure time was increased from 6 hours to 12 hours, 12 hours to 18 hours, and even when exposure time was increased from 18 hours to 24 hours. MWCNT <8 nm also caused significant reduction in cell viability as its exposure time to 3T3 fibroblasts was increased, but the MWCNT <8 nm did significantly affect cell viability with as little as 6 hours of exposure. MWCNT 20-30 nm also caused a significant reduction in cell viability in as little as 6 hours of their exposure to 3T3 fibroblasts. These results suggest some intracellular event occurring before 6 hours is causing the significant drop in cell survival that was observed in all cases. The cells were visualized via LIVE/DEAD viability/cytotoxicity stain as described in chapter 4.4.3 to further confirm that they are dying and to visualize the difference seen at 18 h time point in cell viability between the cells exposed to nanoparticles and nanotubes. After performing the stain,

we observed increased number of dead cells in wells that were exposed various nanomaterials (Figure 14). We also see an increase in number of dead cells in cell population that was exposed to MWCNTs than those that were exposed to TiO_2 and SiO_2 nanoparticles, both of which had similar number of dead cells (slightly more dead cells with SiO_2 exposure) at 18 h time point.

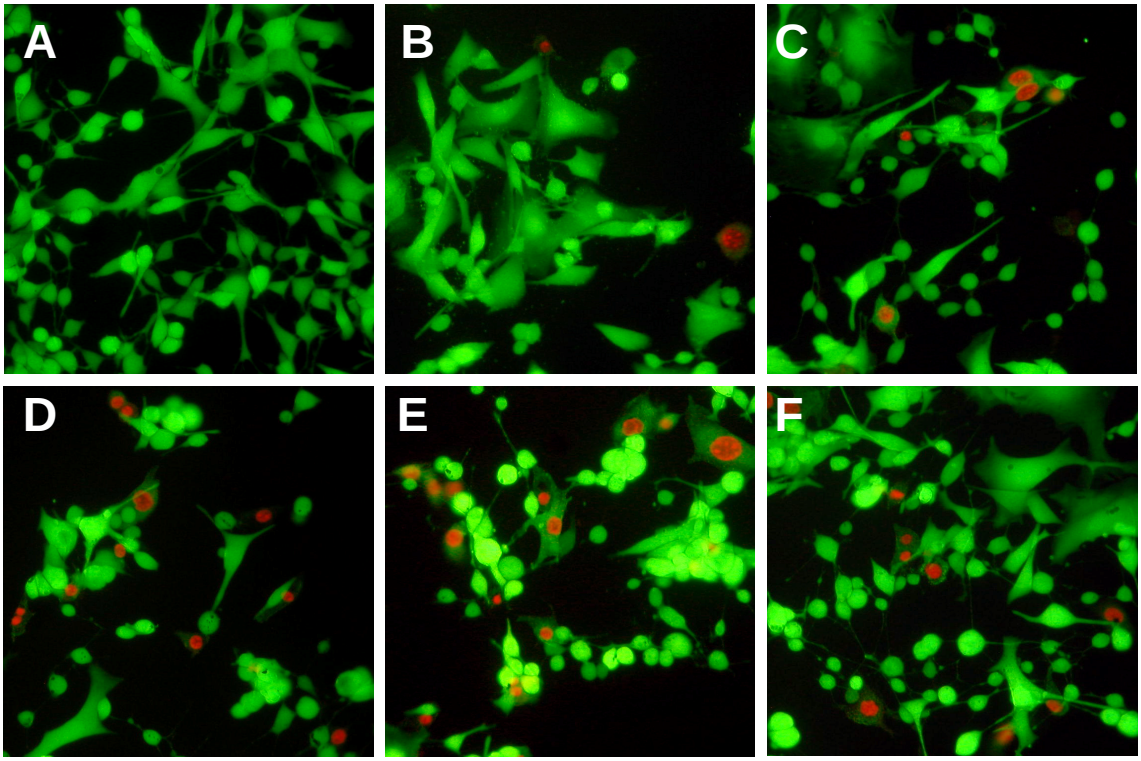

Figure 14. Pictures of viability stains taken at 20X of 3T3 fibroblasts exposed to (A) Control, (B) TiO_2 nanoparticles, (C) SiO_2 nanoparticles, (D) MWCNT <8nm, (E) MWCNT 20-30nm, (F) MWCNT >50nm. Cultures were incubated overnight. Positive live cells appear green, and positive dead cells appear as red stained nucleus.

CHAPTER 7

GENERATION OF REACTIVE OXYGEN SPECIES BY NANOPARTICLES AND NANOTUBES INDUCES CELL DEATH

7.1 Rationale

As mentioned earlier, TiO_2 nanoparticles, SiO_2 nanoparticles, and multi-walled carbon nanotubes exhibit the potential for spontaneous ROS generation based on material composition and surface characteristics. Numerous studies have found that oxidative stress may play a key role in inducing the cytotoxicity of nanoparticles. Intracellular second messengers such as reactive oxygen species are one of the inducers of lysosomal membrane permeabilization which results in the release of lysosomal hydrolases from the lumen into the cytoplasm leading to apoptosis. We hypothesize that exposure of the selected nanoparticles to 3T3 fibroblasts leads to an increase in the generation of intracellular reactive oxygen species and lysosomal membrane permeabilization leading to apoptosis.

We sought to test this hypothesis by measuring the generation of intracellular reactive oxygen species and lysosomal membrane permeabilization using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), Acridine Orange, and Sensolyte Homogeneous AMC Caspase-3/7 Assay Kit, respectively as described in chapters 4.4.4, 4.4.5, 4.4.6 and 4.4.7.

Hypothesis 3: Generation of reactive oxygen species by nanoparticles and nanotubes induces cell death.

7.2 Results

Cytotoxic studies have indicated that nanoparticle and nanotube interaction with the cells results in spontaneous generation of reactive oxygen species. Reactive oxygen species has been shown to cause damage to lysosomal membrane leading to leakage of intra-lysosomal hydrolases leading to apoptosis. To test this hypothesis, we measured the increase in intra-cellular ROS with and without nanoparticle and nanotube exposure.

Increase in intra-cellular ROS was observed in all test subjects after 3T3 fibroblasts were exposed to them for 2 hours confirming the relationship between nanomaterial-cell interaction and ROS generation (Figure 7). To test whether this increase in intra-cellular ROS generation causes damage to lysosome's membrane, morphology of the lysosomes was visualized as described in chapter 4.4.5.

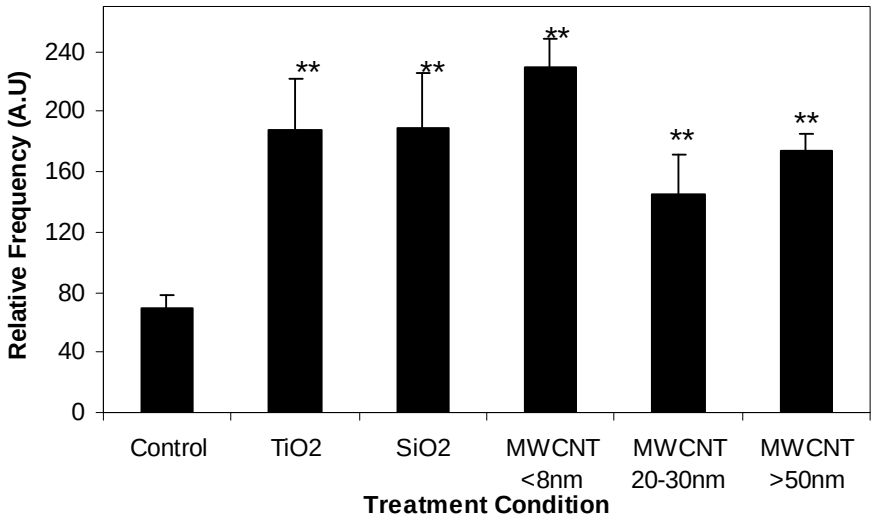

Figure 15. The influence of nanoparticles and nanotubes exposure for 2 hours on generation of intracellular reactive oxygen species in 3T3 fibroblasts. Vertical lines denote ±1 SD (n=4 for all samples and cells). (**) represents statistically significant differences with respect to control ($p<0.05$).

By assessing the morphology of lysosomes we see severe damage to the lysosomes membrane in cells that were exposed to MWCNT (Figure 16). These cells had more enhanced

green fluorescence and very little to no presence of orange granules which represent the lysosomes. This is especially the case with cells exposed to MWCNT <8 nm. In cells that were exposed to TiO_2 and SiO_2 nanoparticles, the lysosomes are still visible although their structure is not as clear as in cells that were not exposed to any nanoparticles or nanotubes. The amount of lysosomal damage observed in each case was then quantified as described in chapter 4.4.6.

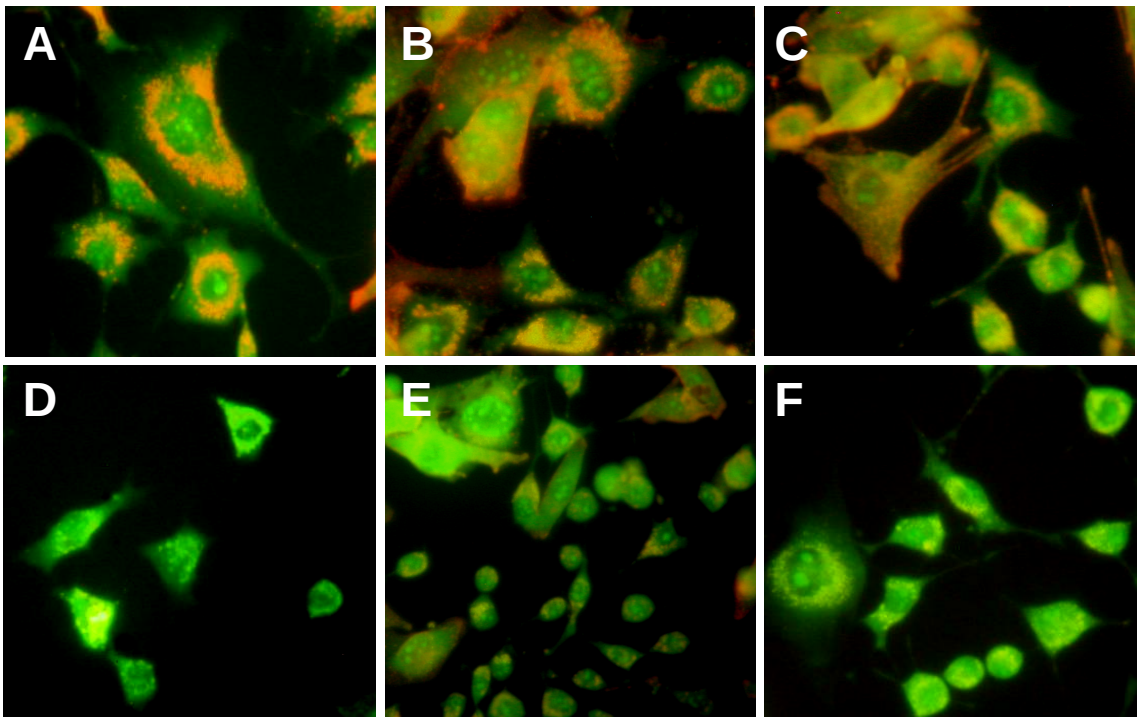

Figure 16. Visualization of the lysosomes of 3T3 fibroblasts A) without exposure to any nanoparticles or nanotubes, and 3T3 fibroblasts exposed to 100 µg/ml of B) TiO_2 nanoparticles, C) SiO_2 nanoparticles, D) MWCNT <8 nm, E) MWCNT 20-30 nm, and F) MWCNT >50 nm. The lysosomes (orange) and cytoplasm (green) can be clearly seen in control cells. Cells exposed to the nanoparticles exhibit slightly damaged lysosomes and those exposed to nanotubes exhibit severe damage to lysosomes shown by enhanced green (cytoplasm) intensity and very low to none orange (lysosome) intensity.

Lysosomal stability assays performed after 4 hours of exposure to the test subjects resulted in the release of lysosomal contents into the cytoplasm indicating damage to the lysosome' membrane (Figure 17). Figure 17 displays the percent increase or decrease in the

ratio of 530nm (cytoplasm) to 620nm (lysosomes) for all the nanoparticles and nanotubes with respect to control. Value higher than 100% represents lysosomal contents leaking out of the lumen of lysosome and into the cytoplasm of the cell, which is evident in all cases. Among the five test subjects, only TiO_2 nanoparticles caused a non-significant increase in the ratio while exposure to the other four resulted in a significant increase. The damage to lysosomal membrane and the resulting release of hydrolases into the cytoplasm has shown to cause caspase dependent apoptosis and even necrosis following high levels of lysosomal membrane permeabilization. Caspase-3/7 activity was tested in 3T3 fibroblasts after 10 hours of exposure to the nanoparticles and nanotubes.

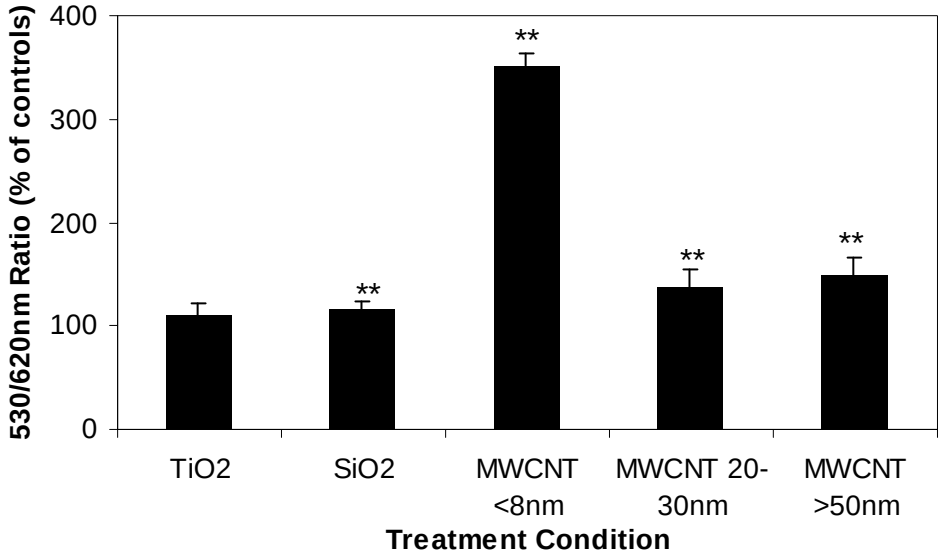

Figure 17. The influence of nanoparticles and nanotubes exposure for 4 hours on lysosomal membrane permeabilization. Vertical lines denote ±1 SD (n=4 for all test samples and cells). (**) represents statistically significant differences with respect to control ($p<0.05$).

Caspase-3/7 assay performed after 10 hours of exposure to the test subjects caused significant increase in caspase-3/7 activity with TiO_2 nanoparticles and SiO_2 nanoparticles only (Figure 17). Exposure to all three different MWCNTs did not cause an increase in caspase-3/7 activity, with MWCNT <8 nm and MWCNT >50 nm showing the least amount of caspase-3/7 activity.

Exposure to MWCNT 20-30 nm caused neither an increase nor decrease in caspase-3/7 activity with respect to control.

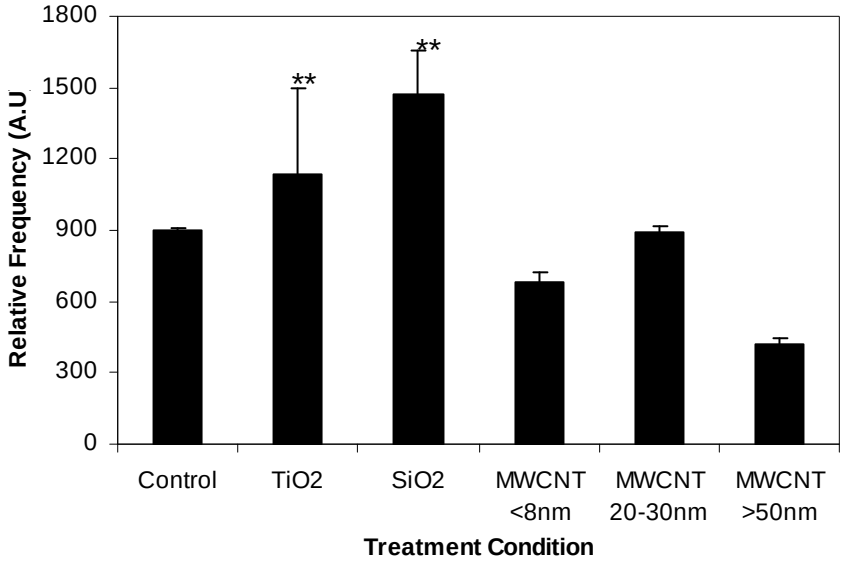

Figure 18. The influence of nanoparticles and nanotubes exposure for 10 hours on caspase-3/7 activity in 3T3 fibroblasts. Vertical lines denote ±1 SD (n=3 for all test samples and cells). (**) represents statistically significant differences with respect to control ($p<0.05$).

7.3 Discussion

It is believed that nanoparticle and nanotube exposure to cells results in the spontaneous generation of reactive oxygen species which then causes damage to other organelles and activates pathways that leads to cell death via apoptosis or necrosis. Our results support this hypothesis. Significant increase in intracellular reactive oxygen species was detected with H_2DCFDA in particle-incubated cells as early as 2 hours (Figure 15). Exposure of MWCNT <8 nm to 3T3 fibroblasts resulted in the largest increase in generation of ROS, while MWCNT 20-30 nm and MWCNT >50 nm caused the least but still significant increase in ROS. TiO_2 and SiO_2 caused similar amount of ROS generation in 3T3 fibroblasts. Even though exposure of MWCNT 20-30 nm caused the greatest amount of cell, it did not cause the greatest generation

of ROS. This could be due to difference in the shape of the nanomaterials. While the TiO_2 and SiO_2 are nanospheres, MWCNTs are tubular. This indicates that exposure to MWCNT 20-30 nm is most likely causing some form of damage to the cells due to its structure in addition to ROS generation. The increase in the generation of ROS has shown to cause lysosomal membrane permeabilization resulting in the release of hydrolases from the lumen of the lysosome and into the cytoplasm that can mediate caspase-dependent apoptosis or even necrosis if large amounts of hydrolases are released (Jaattela, 2004).

Detection of lysosomal membrane permeabilization was seen after 4 hours of nanotubes exposure to 3T3 fibroblasts as shown in figure 16 and figure 17. Comparing the morphology of lysosomes in figure 16 with 530/620 nm intensity ratio in figure 17 we see that exposure of TiO_2 and SiO_2 caused almost no damage to lysosomes membrane, while exposure to MWCNTs especially <8 nm caused damage to lysosomes membrane. Likewise we see similar increases in 530/620 nm ratio with respect to control (100%), indicating lysosomal membrane permeabilization. Exposure of MWCNT <8 nm caused a much higher level of lysosomal membrane permeabilization when compared with TiO_2 and SiO_2 whereas the ROS generation was only about 20% more in MWCNT <8nm when compared with that of TiO_2 and SiO_2. This could be due to the combination of intracellular ROS generation and the shape of the carbon nanotubes. In contrast to TiO_2 and SiO_2 nanoparticles, the carbon nanotubes have two dimensions, diameter and length. While the uptake of the carbon nanotubes caused an increase in intracellular ROS generation leading to lysosomal membrane damage, the accumulation of these nanotubes in the lysosomes might have also caused further damage or even rupture in the lysosomal membrane due to its tubular shape. Thus, combinations of the two events are most likely the cause for the high level of lysosomal membrane permeabilization observed with MWCNT <8 nm. This effect of carbon nanotubes shape is also evident with MWCNT 20-30 nm and MWCNT >50 nm both of which caused lower generation of ROS when compared with TiO_2

and SiO_2, but their exposure led to a greater degree of lysosomal membrane permeabilization when compared with TiO_2 and SiO_2 nanoparticles. This further indicates that the MWCNTs' structure might also be causing damage to the lysosomal membrane. Studies have suggested that the release of lysosomal hydrolases can mediate caspase dependent apoptosis or even necrosis following high levels of lysosomal membrane permeabilization.

In our study, a significant increase in caspase-3/7 activity was observed when 3T3 fibroblasts were exposed to TiO_2 and SiO_2 nanoparticles whereas exposure to the different MWCNTs did not cause an increase in caspase-3/7 activity (Figure 17). These results are in agreement with recent findings. Specifically, exposure of TiO_2 nanoparticles to brain cultures of immortalized mouse microglia, and exposure of SiO_2 nanoparticles to mouse alveolar macrophage cell line stimulated increase in caspase-3/7, and caspase 3 and 9 activity, respectively (Long et al., 2007; Thibodeau et al., 2004). However, exposure of MWCNTs to mouse macrophages did not significantly activate caspase 3 (Hirano et al., 2008). Our results indicate that TiO_2 nanoparticles and SiO_2 nanoparticles exposure to 3T3 fibroblasts triggers an increase in intracellular ROS generation. This most likely causes DNA damage which then upregulates p53. This leads to increased activation of caspase-3 and caspase-7 resulting in cell death via apoptosis. Our results also indicate that exposure of 3T3 fibroblasts to MWCNTs also caused ROS generation which then caused damage to the lysosomal membrane. This damage led to the release of lysosomal hydrolases into the cytoplasm. In contrast to TiO_2 and SiO_2 nanoparticles, MWCNTs did not increase the caspase-3 and caspase-7 activity. Therefore, MWCNTs most likely triggered cell death via necrosis. These findings are similar to the ones reported in studies investigating the effects of reactive oxygen species. These studies suggest that a limited release of lysosomal contents into the cytoplasm due to low levels of lysosomal membrane permeabilization, triggers apoptosis or apoptosis-like cell death. However,

generalized lysosomal rupture or high levels of lysosomal membrane permeabilization, as observed with MWCNTs, results in cellular necrosis (Kagedal et al., 2001).

CHAPTER 8

OVERALL CONCLUSION

- The cytotoxicity of nanomaterials is dependent on their composition to 3T3 fibroblasts.
- Cytotoxicity of nanotubes is dependent on their diameter size to 3T3 fibroblasts.
- Nanoparticle and nanotube-induced cell toxicity is concentration dependent.
- Cytotoxicity is also dependent on nanoparticle and nanotube exposure time.
- A significant reduction in the percentage of viable cells was seen between 6 to 12 hours of nanomaterials exposure to the cells.
- Exposure of nanoparticles and nanotubes to 3T3 fibroblasts caused different levels of increase in the generation of intracellular ROS.
- Along with increase in ROS, exposure to MWCNTs also caused lysosomal membrane permeabilization, whereas TiO_2 and SiO_2 nanoparticles exposure did not cause lysosomal membrane permeabilization.
- Among the five nanomaterials, only exposure to TiO_2 and SiO_2 nanoparticles caused an increase in the caspase-3/7 activity.
- These findings suggest that exposure of TiO_2 and SiO_2 nanoparticles to 3T3 fibroblasts caused oxidative stress leading to DNA damage. This leads to upregulation of p53, increase in caspase-3 and caspase-7 activity and cell death via apoptosis.
- As seen with TiO_2 and SiO_2 nanoparticles, MWCNTs exposure also caused oxidative stress, but it did not cause increase in caspase-3 and caspase-7 activity. However, MWCNTs exposure did cause higher levels of lysosomal membrane permeabilization which caused cell death most likely via necrosis.

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BIOGRAPHICAL INFORMATION

Syed Khaja Sohaebuddin was born in Hyderabad, India on April 11, 1985. He moved to Dallas, Texas in 2000. Syed was accepted in to the University of Texas at Arlington in the Fall of 2005, where he pursued a Bachelor of Science Degree in Biology and a Master of Science in Biomedical Engineering. His research interests are in Tissue Engineering and Drug Delivery.