Quantum Dots: from Cytotoxicity to Metalloestrogenicity

by

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I dedicate this thesis to my grandparents, who influence all that I am and all that I do, from near and far.

Especially, to Dr. Magan Lal Pancholy, my Nana, thank you for having been the living embodiment of intellectual curiosity, for instilling in me the same wonder that made your eyes sparkle…

Abstract

The fields of Nanotechnology and Nanomedicine are rapidly expanding. Already numerous nanoparticles have entered clinical trials and are gradually being introduced for patient use. However, certain nanostructures, though, therapeutically and diagnostically promising, have also been shown to induce cytotoxicity both *in vitro* and *in vivo*; cadmium telluride quantum dots fall into this category.

Quantum dots (QDs), are highly fluorescent, semi-conducting nanocrystals, that consist of a metallic core and organic capping layer. As compared to traditional fluorophores, QDs have superior optical qualities, including resistance to photobleaching, broad spectrum excitation and narrow emission. Cadmium telluride (CdTe) QDs were the first QDs to be synthesized without the use of organic solvents, and were, therefore, considered suitable for biological applications. However, early studies demonstrated that QDs induced cytotoxicity, morphological changes in organelles and oxidative stress. Although, this QD-toxicity was ascribed to cadmium liberation from the QD-core, no empirical evidence was shown. Due to the attractiveness of QDs for biological imaging applications and other therapeutic applications, it was necessary that the mechanisms involved in QD-toxicity be better understood so that they may be prevented.

Preliminary studies from our laboratory indicated that pretreatment with the antioxidant, N-acetlycysteine could prevent QD-toxicity. We, therefore, hypothesized that QD-toxicity was not exclusively due to cadmium leaching from the QD. To evaluate this, a fluorescent assay measuring free cadmium was adapted for cellular use, such that the cadmium present in both the cellular media and intracellularly could be measured. The cytotoxicity of various QDs was evaluated and correlated with the free intracellular cadmium after 24 hours of treatment. The results of this study showed that no correlation exists between QD-toxicity and the cadmium release from QDs.

We next questioned whether QD-toxicity could be explained as the sum of parts of the toxicity associated with each core constituting metal, and whether the

complexity of the model system used for evaluation influences the cytotoxicity observed. We, therefore, employed three model systems of the peripheral nervous system (an immortalized cell line, heterogeneous primary cultures and a three dimensional tissue model) and evaluated the toxicity of cadmium, tellurium and QDs in each. Findings from this study showed that QDs are not a sum of parts and QD-toxicity is better ascribed to the induction of cellular oxidative stress which can be prevented by application of the multi-modal antioxidant, lipoic acid. Further, the three model systems did not depict QD-toxicity comparably, thus stressing the need for standardization in nanotoxicological studies.

Finally, it had been shown that cadmium can associate with the estrogen receptor in estrogen receptor expressing cells, and activate estrogenic signalling. As such, cadmium is considered a metalloestrogen. Based on our results that QDs liberate cadmium that can be internalized and retained in the cell, and that QD-induced effects vary based on model system, we investigated whether in estrogen receptor expressing cells, QDs may act as metalloestrogens and induce estrogenic signalling. Results *in vitro* showed that QDs exert potent estrogenic signalling, comparable with 17β-estradiol, including cell proliferation, AKT phosphorylation, ERK phosphorylation and nuclear receptor activation. All of these effects could be attenuated via cellular pretreatment with the specific estrogen receptor inhibitor, ICI 182780, affirming that QD-induced estrogenic activity was mediated via the estrogen receptor. To determine whether the estrogenic activity of QDs could also be demonstrated *in vivo*, ovariectomized mice were treated with QDs chronically, for two weeks, prior to being sacrificed. Subsequently, the wet weights of the mice uteruses were taken and in the mice treated with either QDs or 17β-estradiol a comparable 2.5 fold increase in uterine wet weight was observed.

Taken together, these results indicate that CdTe QDs are both cytotoxic and endocrine disrupting, metalloestrogens. Though these nanocrystals may have valuable applications in biology for imaging and diagnostic applications, the implications of QD-use are dangerous to plants, animals, humans and the environment at large. Therefore, it is imperative that cadmium free QDs be

developed that retain the attractive qualities of QDs while preventing the detrimental side effects. Further, more extensive and standardized testing of nanoparticles is imperative for the safe and efficient use of these novel tools.

Résumé

La nanotechnologie et la nanomédecine sont des domaines en pleine expansion. Certaines nanoparticules sont déjà entrées dans des essais cliniques et sont de plus en plus utilisées par les patients. Par contre, certaines nanostructures, bien qu'elles soient prometteuses à des fins cliniques ou diagnostiques, sont capables d'induire de la cytotoxicité *in vitro* et/ou *in vivo*; les boîtes quantiques de cadmium telluride (CdTe) constituent un exemple.

Les boîtes quantiques (BQ) sont des nanocrystaux semi-conducteurs fluorescents qui contiennent un noyau métallique entouré d'une couche organique. Les BQ ont des propriétés optiques supérieures aux autres fluorophores organiques traditionnelles. Par exemple, ils sont plus résistants au photoblanchiment et sont caractérisés par un spectre d'excitation large et d'émission étroit. Les BQ CdTe, les premiers à être synthétisés sans l'utilisation de solvant organique, ont été prometteurs dans certaines applications biologiques. Cependant, les premières études ont démontré que ces BQ induisent de la cytotoxicité, changent la morphologie des organelles et produisent du stress oxydatif. Le cadmium libéré pourrait être à la base de ces effets toxiques, mais cela n'a pas encore été prouvé. Les études préliminaires au sein de notre laboratoire démontrent que le pré-traitement avec un antioxydant, N-acétyl-cystéine, était capable de diminuer le niveau de toxicité associée au BQ. On a avancé l'hypothèse que cette toxicité n'était pas exclusivement liée au cadmium qui est libéré des BQ. Nous avons effectué des expériences fluorométriques où nous mesurions les niveaux de cadmium libres dans la cellule et dans le milieu extracellulaire. Ces expériences indiquent qu'il n'existe aucune corrélation entre la toxicité associée au BQ et le cadmium libéré. Par après, nous nous sommes demandé si les constituants métalliques du noyau des BQ et les modèles dans lesquels les BQ avaient été évalués, étaient impliqués dans la toxicité associée au BQ. Nous avons évalué la toxicité du cadmium, tellurium et BQ dans trois modèles du système nerveux périphérique (lignée de cellule immortalisée, cultures primaires hétérogènes et modèle de tissue tridimensionnel). Les résultats démontrent que la toxicité des BQ

est principalement attribuée à l'induction du stress oxydatif, qui peut être prévenu en appliquant un antioxydant multimodal, l'acide lipoique.

Dans les cellules, cadmium peut s'associer avec les récepteurs d'œstrogènes et activer les voies de signalisation reliées à ce récepteur. Par conséquent, cadmium est considéré comme étant un métallo-œstrogène. Nous avons montré que les BQ libèrent du cadmium et que celui-ci est internalisé et retenu dans les cellules. Les effets induits par les BQ dépendaient du modèle biologique utilisé. Les études *in vitro* montrent que les BQ exercent une forte signalisation oestrogénique comparable à celle de 17β- estradiol et induisent la prolifération cellulaire, la phosphorylation d'AKT et d'ERK et l'activation du récepteur d'œstrogène nucléaire. Ces effets étaient atténués par un pré-traitement avec un inhibiteur du récepteur d'œstrogène, ICI 182780. Ces résultats affirment, donc, que les BQ exercent leurs activités oestrogéniques via les récepteurs d'oestrogènes. Dans nos études *in vivo* nous avons utilisé des souris ovariectomisées qui avaient été traitées avec des BQ ou du 17β- estradiol pendant deux semaines, et par ensuite sacrifiées. Les traitements ont fait augmenter de 2.5 fois le poids de l'utérus des souris. Dans l'ensemble, ces résultats montrent que les BQ CdTe exercent à la fois des effets cytotoxiques et métallo-eostrogéniques. Malgré leurs potentiels d'application en imagerie ou dans les procédures diagnostiques, il est clair que les BQ peuvent nuire aux plantes, aux animaux, aux humains et à l'ensemble de l'environnement. Il y a un besoin urgent de développer des BQ sans cadmium qui possèdent des qualités attrayantes de BQ mais qui sont dépourvus d'effets secondaires détrimentaux. Cet objectif pourra être atteint à l'aide d'essais plus élaborés et sophistiqués pour déceler les risques des nanoparticules.

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List of abbreviations

AKT	protein kinase B
BrdU	bromodeoxyuridine
Cd^{2+}	cadmium
CdSe	cadmium selenium
CdSe/ZnS	cadmium selenium zinc sulfide
CdTe	cadmium tellurium
CdTe/ZnS	cadmium tellurium zinc sulfide
CFQD	cadmium free quantum dots
CLSM	confocal laser scanning microscopy
CMC	critical micellar concentration
CNT	carbon nanotubes
CO_2	carbon dioxide
CTS	charcoal treated serum
Cys	cysteamine
DHLA	dihydrolipoic acid
DMSO	dimethyl sulfoxide
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
E_2	17β-estradiol
ELISA	enzyme-linked immunosorbant assay
EPR	enhanced permeability and retention
ER	estrogen receptor
ERK	extracellular regulating kinase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FRET	fluorescence resonance energy transfer
GNP	gold nanoparticles
GSH	glutathione
HER2	human epidermal growth factor receptor 2

HSP90	hear shock protein 90
IERG	immediate early response genes
LA	lipoic acid
LBD	ligand binding domain
LED	light emitting diode
LSPR	localized surface plasmon resonance
MPA	mercaptopropionic acid
MT	metallothioneins
NAC	N-acetylcysteine
NGF	nerve growth factor
NIR	near infrared
PDT	photodynamic therapy
PEG	polyethylene glycol
PET	positron emission tomography
PNS	peripheral nervous system
RNS	reactive nitrogen species
PSMA	prostate specific membrane antigen
QD	quantum dot
RES	reticuloendothelial system
RLU	relative luminescence intensity
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
SEM	scanning electron microscopy
siRNA	silencing ribonucleic acid
SNP	silver nanoparticles
SPR	surface plasmon resonance
TEM	transmission electron microscopy
TiO_2	titanium oxide
UFP	ultrafine particles
ZnO	zinc oxide

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leaned on, and my most respected colleague. Your work integrity, your ever willingness to help everyone and your incredible friendship are more appreciated than you know! I can honestly say, I would not be here if it was not for you. Efharisto!

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Last, but most certainly not least, to my sister, **Manika**: you've picked me up every time I've fallen, and you've carried me through the toughest times. You lift me higher during times of celebration and fight my battles when I'm not even looking. Most of all, you walk beside me through everything in between – this degree is as much yours as it is mine! Thank you.

Preface

Thesis format

This thesis is presented in a manuscript-based thesis format, which conforms to the "Thesis Preparations and Submission Guidelines" from the Faculty of Graduate Studies at McGill. The thesis is divided in to four chapters, part one of the first chapter is the introduction. Part two of the first chapter is a manuscript to which I contributed integrally. This manuscript, though not one of my core thesis chapters, presents my preliminary cadmium studies, which are the foundation upon which the rest of my thesis work is based. This chapter has been published in Langmuir: Cho SJ, Maysinger D, Jain MP, Roder B, Hackbarth S, Winnik FM. Long term exposure to CdTe quantum dots causes functional impairment in living cells. 2007; 23(4) :1974-80. Chapters two and three comprise text duplicated from papers that I have already published, and they are bridged by connecting texts printed between chapters. Chapter two was published in Nanomedicine (London): Probing and preventing quantum dot induced cytotoxicity with multi-modal, alpha-lipoic acid in multiple dimensions of the peripheral nervous system. Jain MP, Choi AO, Neibert KD, Maysinger D. 2009; 4(3) : 277-290. Chapter three is also published in Nanomedicine (London): Metalloestrogenic effects of quantum dots. Jain MP, Vaisheva F, Maysinger D. 2012; 7(1) : 23-37. Chapter four presents a general summary and discussion of our results and findings, followed by a list of original contributions. An appendix follows at the end with copies of the published manuscripts, including two that were not included in the body of the thesis. Copyright permission from the journals is also provided in the appendix.

Contribution of authors

Chapter 2: Experimental work described here was done primarily by the candidate. Tissue culturing of the dorsal root ganglia explants, the trypan blue exclusion assays, and images of lipid droplet stained cell samples were captured by Angela Choi (a member of our laboratory). Angela Choi also assisted in manuscript revisions. Cell samples were provided to Kevin Neibert, (a member of

our laboratory) in order for him to stain and measure intracellular glutathione. For this reason he is acknowledged as a co-author of the manuscript.

Chapter 3: Experimental work described here was all done by the candidate, with the exception of subcutaneous animal injections, which were done by Farida Vaisheva. For this work, Farida Vaisheva is acknowledged as a co-author of this manuscript, and at the time of submission she was working as a research assistant in our laboratory.

Chapter 1b, preliminary studies: The synthesis and characterization of the nanoparticles was completely done by Dr. SungJu Cho. The candidate's contributions to this study comprised the conception and execution of all the cellular and cadmium related experimental work, data analysis and interpretation, preparation of the figures, and writing of the corresponding text.

Chapter 1:

Introduction

Introduction Part 1

1. An introduction to the world of Nano

1.1 Nanotechnology and nanomedicine

The term nano originates from the Greek word nanos, meaning dwarf or little old man. However, nano in science and technology refers to 10 to the negative order of 9 ($1x10^{-9}$), or one billionth. The term "nanotechnology" was first introduced by Taniguchi in 1974, and defined as the science of manipulating materials on an atomic or molecular level to build a variety of diverse engineered materials and devices for multiple applications [1]. Nanotechnology as an area of research is highly multidisciplinary requiring the amalgamation of complex physics, chemistry, engineering and biotechnology. The last decade has made nanotechnology a household term, as the products of nanotechnology, known as nanoparticles, have become as diverse in nature and form as their potential applications. Referred to as general purpose technology, the breadth of nanotechnology applications already include industries such as electronics, automotive, clothing, defence, agriculture, medicine and numerous more [2, 3]. Progress achieved in physical and engineering sciences has now started to exert an impact on medicine and bio-medical research, creating the new and evolving field of nanomedicine. Nanomedicine utilizes tools that are nano-sized for diagnostic, prophylactic and therapeutic treatment of disease, as well as to gain better comprehension of the underlying pathophysiology of diseases. The ultimate goal of nanomedicine is to improve quality of life [4]. The products of nanotechnology that serve as broadly used tools in countless industries are referred to as nanoparticles. Nanoparticles must have at least one dimension ranging from 1-100 nanometers in size; however, the synthesis of these particles may vary from modified conventional techniques, to molecular self assembly [5]. Various approaches, including top-down, bottom-up have also been employed in the synthesis of nanoparticles [6, 7]. The key feature of nanoparticles is that their physical-chemical properties are fundamentally different from the bulk materials of which they are composed [8]. Additionally, nanoparticles often possess unique photophysical, optical, mechanical and/or other properties. Some of these unique

properties of nanomaterials, particularly nanocrystals, will be presented in the following sections

1.2 Nanoparticles

A nanoparticle is small particle having at least one dimension measuring less than 100nm with properties that differ from the bulk materials that they are made of. Nanoparticles are similar to ultrafine particles (UFPs), in terms of height, width or length [9]. In contrast to bulk materials, nanoparticles have a high surface to volume ratio [10, 11]. The smaller the nanoparticle the larger the surface to volume ratio, thus altering the physical-chemical properties of the nanoparticle. Nanoparticles constitute a plethora of different structures that are not restricted to any particular shape; even the size of particles may vary greatly, provided one dimension of the particle measures less than 100nm. Spheres, rods, urchins, roses, worms, typical and atypical structures are all common of nanoparticles [12, 13]. Although, nanoparticles are made of many different materials and may serve many different functions, based on the nanoparticle composition they may be divided into two major categories: non-metallic and metallic nanoparticles. In the sections that follow some of the most common metallic and non-metallic nanoparticles will be introduced and discussed.

1.2.1 Non-metallic nanoparticles

Several different kinds of non-metallic nanoparticles are currently being researched and adapted for multiple applications. Introduced below are three examples of such nanoparticles.

Carbon nanotubes (CNTs) are allotropes of carbon, like oxygen (O_2) and ozone (O_3). Cylindrical in shape, the walls of CNTs consist of one atom thick walls of graphene. CNTs have a diameter of 1nm, and a length to diameter ratio of 132 000 000:1; which is greater than any other known material [14]. Resembling a honeycomb crystal lattice made of graphene, there are actually three principal CNTs, which differ based on the alignment of the carbon rings; the three configurations are: arm chair, zigzag, and chiral. The properties of the CNT vary

based on the configuration [15]. Two further variations on the CNTs are single-walled CNTs and multi-walled CNTs. Multi-walled CNTs are several layers of single-walled CNTs placed concentrically one inside the other [16]. CNTs are currently both the stiffest and strongest known material, estimated at being over a hundred times stronger than steel wool and yet capable of repeat bending.

Micelles are self-assembled structures composed of amphiphilic molecules [17]. In an aqueous solvent when the colloidal solution reaches the critical micelle concentration (CMC), due to thermodynamic laws and the balance between entropy and enthalpy, the monomers attract one another and spontaneously form micelles to reduce surface tension. These self-assembled nanoparticles can range between 10 – 100nm in diameter. The lengths of the hydrophobic and hydrophilic portions of the polymer determine the size and shape of the micelle; which is not necessarily spherical. Several other factors influence micelle shape and formation, including temperature, pH, ionic strength or solution, surfactant/polymer geometry and concentration. In general, the lower the CMC of a colloidal solution, the more stable the micelle is likely to be. Conversely, a high CMC is indicative of less stable micelles, which would be susceptible to destabilization likely even from mild dilutions of the colloidal solution [18].

Dendrimers are repeatedly hyperbranched, approximately spherical macromolecules in the nano size range. The term dendrimer originates from the Greek word “Dendron”, which translates to “tree” in English. Dendrimers are highly symmetrical, monodisperse compounds that unlike many polymeric structures can be rendered hydrophilic by the addition of charged species or modification of the surface functional groups [19]. The potential to modify the surface of the dendrimer permits tremendous functionalizaition of the dendrimers; which is of particular interest for drug delivery. Further potential for functionalization exists within the dendrimer, as dendritic encapsulation of functional molecules can also be achieved.

Dendrimers have been used to deliver drugs and genetic materials [20, 21]. We have used fluorescent dendrimers to investigate their fate in cells [22]. Several

other laboratories have investigated their interactions with cells and their internalization processes [23].

The properties and application of selected non-metallic nanoparticles are summarized in the following table (Table 1)

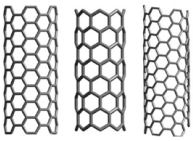
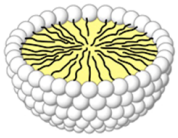
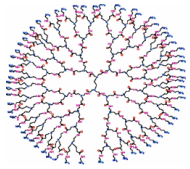
Nanoparticle	Structure	Property	Application	References
Carbon Nanotubes (CNTs)		• Allotropes of carbon • Highest length to diameter ratio • Strongest and stiffest known material	Imaging: • CNTs can be an x-ray source for new CT scans to better visualize small animals Drug Delivery • Targetd delivery of doxorubicin containing, angiopep-2 functionalized MWCNTs across BBB for glioma therapy	Cao *et al.*, 2009 Ren *et al.*, 2012 Liang *et al.*, 2010
Micelles		• Composed of amphiphilic molecules • Commonly spherical in shape • Hydrophobic core & hydrophilic exterior, or hydrophilic core & hydrophobic exterior	Imaging • NIR probes encapsulated in micelles allow for high tumor delineation Drug delivery • Paclitaxel micelles FDA approved for breast cancer therapy	Cho *et al.*, 2012 Oerlemans *et al.*, 2010 Sutton *et al.*, 2007
Dendrimers		• Hyperbranched structures • Highly functionalizable • Polymeric structures • Classification based on generation	Imaging • Dendrimer nanoprobes that are both MR & optical imaging reporters, traverse the BBB, target & effectively image neuroblastomas Drug delivery • Vivagel (dendrimer conjugated antimicrobial agent for preventing STDs) in phase II clinical trials	Yan *et al.*, 2011 Price *et al.*, 2011 Mintzer *et al.*, 2011

Table 1. Non-metallic nanoparticle properties and applications: carbon nanotubes [24-26], micelles [27-29], dendrimers [30-32]

1.2.2. Metallic nanoparticles

A variety of metallic nanoparticles have been prepared, characterized and utilized for a wide variety of medical and non-medical applications. Two examples of these are gold and silver nanoparticles.

Gold nanoparticles: For centuries artisans have exploited the optical properties of colloidal gold for purposes such as staining glass [33]. Gold chloride was mixed into molten glass, a process that allowed tiny gold particles to be suspended in the glass. The concentration of gold introduced determined whether the glass would stain a brilliant shade of red, yellow or mauve. The superior optical properties of

gold nanoparticles can be ascribed to the extraordinarily efficient light absorbing and scattering ability of gold and the surface plasmon resonance (SPR), or localized surface plasmon resonance (LSPR), in the case of metallic nanosized structures [34].

Gold nanoparticles (GNPs) are often prepared as fluid suspensions, ranging in colors depending on their sizes, morphologies and concentrations. GNPs are synthesized in a variety of shapes, including: spheres, rods, urchins, roses, worms, nanocages, and many other structures as determined by the specific fabrication method [35].

GNPs have been used for medical purposes for centuries [36]. Today, they are extensively studied in biology and experimental medicine as diagnostics, therapeutics and even theranostic nanotechnological products (Table 2) [37].

Silver nanoparticles: For centuries silver has been known to have antimicrobial and disinfectant properties. In ancient times silver was used for maintaining hygiene and for medicinal purposes [38]. Even vessels to store and preserve water and wine were made of silver, so as to avail of the antimicrobial effects of silver. In fact, during World War I, prior to the advent of antibiotics, silver compounds were highly utilized to prevent wound infection [39]. Topically, silver sulfadiazine cream has been used as an antibacterial treatment for burn wounds. However, its use had been reduced due to irreversible changes in the skin, eye, as well as other toxicities caused by silver deposition. In contrast, fewer side effects have been noted with the use of silver nanoparticles (SNPs). A new method for SNP production provided “green” SNPs, both biocompatible and stable for up to 90 days [40].

SNPs are clusters of silver atoms or silver oxide. Like GNPs, SNPs can be fabricated in many different shapes and sizes [41], and possess similar photophysical and optical properties [42].

The use of gold and silver compounds for medical purposes dates back to ancient times, as even centuries ago both metals were recognized for having medically beneficial effects, alongside certain limitations and issues of toxicity relating particularly to the ionic and salt forms of the metals. Gold and silver nanoparticles

were therefore conceived to overcome some of the limitations associated with larger gold and silver compounds, while exploiting and optimizing their positive qualities. Investigation of gold and silver nanoparticles led to the study of other metals and the production of other unique metallic nanoparticles, including quantum dots (QDs), which will later be discussed in much greater detail.
The properties and application of the selected metallic nanoparticle are summarized in the following table (Table 2).

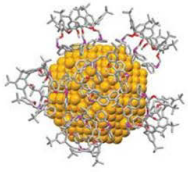
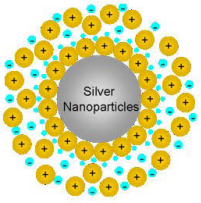
Nanoparticle	Structure	Property	Applications	References
Gold Nanoparticles		• Prepared as a colloidal suspension • Variety of shapes • Efficient light absorption & scattering abilities • Localized surface plasmon resonance & tunability of particles permits functionalization & light scattering in specific wavelengths	Imaging • Anti-epidermal growth factor receptor conjugated to gold nanoparticles used for small tumor diagnosis via CT scanning Drug delivery • Gold nanoparticles linked to tumor necrosis factor-α to halt solid tumor growth completed phase I clinical trails	Reuveni *et al.*, 2011 Libutti *et al.*, 2010 Boisselier *et al.*, 2009
Silver Nanoparticles		• Potent antimicrobial & antibacterial agent • Efficient light absorption & scattering abilities • Localized surface plasmon resonance & tunability of particles permits functionalization & light scattering in specific wavelengths	Imaging • Silver nanoplate contrast agents can be used for *in vivo* photoacoustic imaging Drug delivery: • Silver nanocrystals as topical antibacterial for dermatitis, phase II clinical trials	Homan *et al.*, 2012 Keck *et al.*, 2009 Chaloupka *et al.*, 2010

Table 2. Metallic nanoparticle properties and applications: gold nanoparticles [43-45] , silver nanoparticles [46-48]

2. Quantum dots

Quantum dots (QDs) are semi-conducting nanocrystals comprised of a metal core, capping agent and numerous potential surface modifications [49, 50]. The metal core of the QD ranges between 2-10nm in diameter. In principal, QDs contain excitons that are confined in all three spatial dimensions; and due to this quantum confinement, QDs have properties that resemble both bulk semi-conductors and discrete molecules [51, 52]. QDs also cannot conduct energy due to the quantum

confinement of excitons, however, their unique electrical properties are determined primarily by their size and shape.

QD can both absorb and emit energy. The amount of energy emitted and absorbed is dependent on the distance between energy levels of the QD, better knows as the band gap of the QD. Smaller QDs, have larger band gaps and, therefore, the energy difference is much greater between the lowest conduction bands and highest valence bands; thus greater amounts of energy are required to excite the QD and subsequently greater energy is also emitted as the QD returns to its resting state [53]. With respect to QDs as fluorescent molecules, the energy absorbed by the QDs are frequencies of light energy from the visible spectrum, and the specific wavelengths of light absorbed are determined by the size of the QD (Figure 1). Energy absorption leads to the excitation of the QD followed by an emission of energy in the form of photons of a different wavelength. For example, smaller, green QDs absorb and emit higher frequencies of energy (green QDs: frequency - 526-606, wavelength: 495-570nm) in the visible light spectrum. Conversely, larger red QDs absorb and emit lower frequencies of energy (400-484 THz) and fluoresce in the red (wavelength 620- 750 nm) region of the visible light spectrum. There is an inverse relationship between the size of a QD and the energy of the band gap. Further, given that the size of QDs is tunable, so is the desired color of emitted fluorescence. Consequently QDs have broad spectrum excitation but very specific and narrow emission wavelengths [54].

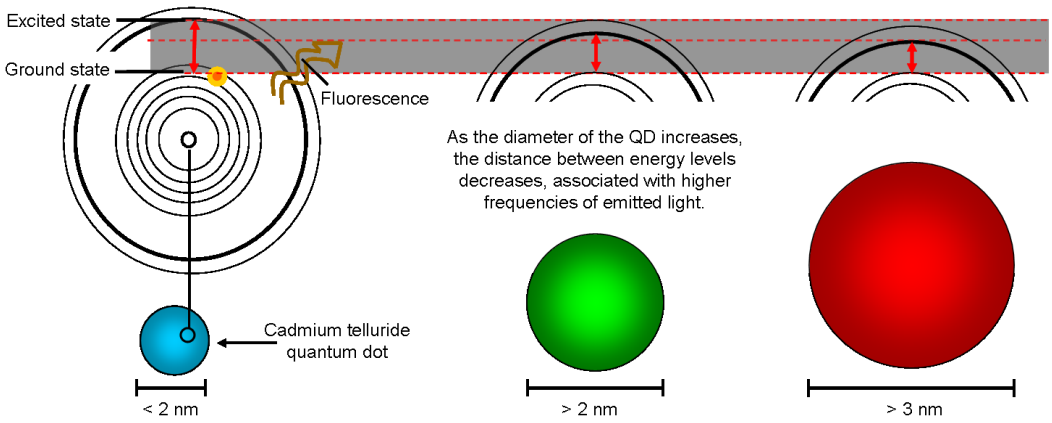


Figure 1. QD-fluorescence is QD-size dependant. Excited excitons move from one energy level to the next. Eventually these excitons return to the ground state and emit fluorescence in the form of different wavelengths (colors). The wavelength, or color of emitted fluorescence depends on the distance between the energy levels, or the band gap (as indicated by the red double headed arrows). The smaller the QD, the larger the band gap and the higher the frequency of light emitted (QDs smaller than 2 nm emit florescence in the blue spectrum of the light). The larger the QD, the smaller the band gap and the lower the frequency of light emitted (QDs larger than 3 nm emit fluorescence in the red spectrum of light).

2.1 Quantum dots as fluorophores

QDs possess numerous superior optical properties as compared to traditional fluorophores; they are resistant to photobleaching and highly luminescent. Additionally, as mentioned above, QDs have a broad spectrum excitation and narrow emission, allowing for the simultaneous imaging of multiple colored QDs [54]. Further, QDs can be tuned and modified to possess either a cationic or anionic surface charge based on the capping agent used to assist in the aqueous dispersion and stabilization of the QD. Additional molecules can also be conjugated to the surface of the QD, including targeting molecules or various drugs, allowing for more specific imaging or therapeutic applications, respectively.

2.2 Applications of quantum dot

Due to their superior electrical and optical properties, QD applications are vast and diverse, ranging from technology to medicine to clothing [55]. QDs are being researched as efficient and economical solutions for the construction of photovoltaic devices to generate solar power [56]. The use of QDs for the development of light emitting devices, as well as photodetector devices for applications in surveillance, spectroscopy and fluorescent biomedical imaging is also the subject of much investigation [57]. QDs are also being applied to develop

better electroluminescent displays [58] and efficient solid state lighting that consumes minimal energy. In the laboratory QDs are already used as a more stable replacement for many organic fluorophores [59]. Conjugation of QDs to antibodies and targeting moieties are gaining popularity for use in many assays and techniques such as ELISAs, Western blots, and immunocytochemistry/immunohistochemistry [60].

Additionally, QD use for *in vivo* imaging of animals is becoming more prevalent as due to the photostability of QDs, repeat imaging is possible. QDs were described in 1998 by Alivisatos' group to have a strong potential to be used as a bioimaging tool in live cells and animals due to their superior properties [61, 62]. Reports investigating the use of QDs for targeted tissue imaging in live animals began to appear as early as 2002 [63]. In this study, QDs coated with a lung-targeting peptide were injected into the animal via the tail vein, and subsequently found to be localized in the lung. In a later study in 2006, the same group showed that these cadmium-based nanocrystals accumulate in the liver, spleen and kidney of mice 90min post intravenous injection [62, 64]. Ballou *et al.* also showed that non-functionalized QDs can be detected in the injected mice for up to four months [65]. In fact, a major concern regarding the use of QDs *in vivo* is their accumulation and the lack of information about the pharmacokinetics of these nanoparticles, post entry into the body. Furthermore, studies from our group have shown that QDs administered intranasaly, not only reach the brain, but can induce inflammatory effects via activation of the glia (astroglia and microglia) in the olfactory bulb and other posterior brain regions [34, 66, 67]. Thus, despite the widespread applications of QDs, there is also much concern regarding QD-toxicity and exposure to heavy metals such as cadmium.

3. Cadmium

Cadmium is one of the integral, core-constituting components of QDs [68]. Its presence in the QD as well as the liberation from the QD-core was considered the cause of QD-induced cytotoxicity. Previous studies have shown that cadmium is also an endocrine disruptor, which is the focus of Chapter 3. The role of cadmium

in mediating QD-induced effects is the central theme of this thesis. In order to better comprehend and compare QDs and cadmium, we present in the following sections various properties of cadmium and its effects on cells *in vitro* and in *in vivo*.

Cadmium is a bivalent metal, with the atomic number of 48. Naturally present in the earth's crust at an approximate concentration of 0.2mg/kg [69], cadmium is considered a relatively rare element. The ubiquitous presence of cadmium in the environment (including our air, water and food) originates from the natural erosion and abrasion of soil and rocks, as well as from events such as volcanic explosions and forest fires [50, 51]. Similar to zinc, cadmium prefers the oxidation state of +2 in most of its compounds, and as such is not found naturally present in its pure state. Cadmium is present in nature as a minor component of zinc, lead and copper sulphide ores and as such is a by-product of zinc production [70].

3.1 Historical use of cadmium

First discovered in 1817 by Friedrich Stromeyer, the name was derived from the Latin, *cadmia* and the Greek, *kadmeia*. Extracted during the mining, refining and smelting of non-ferrous metals, since its discovery, cadmium has been utilized for diverse applications. During the 1930s and 40s cadmium was used for the coating of steal and steal alloys to prevent corrosion. Cadmium is highly resistant to corrosion, has a low coefficient of friction and exhibits excellent plating characteristics on a wide variety or substrates [69]. During the 1940s and late 50s, nearly 60% of all cadmium in the United States was used for this purpose. In the 1950s, cadmium was also utilized for yellow and red pigmentation; and in 1956, approximately 24% of the cadmium in the United States was used for pigmentation purposes. The use of cadmium as a plastic stabilizer gained popularity during the 1970s and 80s, and led to a tremendous increase in the usage of cadmium. However, from the 1980s onwards, a significant decrease in popular cadmium applications began, due to the emergence of health and environmental reports dedicated to cadmium regulation and safety. Though, the distribution of

cadmium use for diverse applications saw a dramatic decline, the overall use of cadmium was maintained; by 2006 while cadmium application as a metal alloy and for pigmentation purposes declined to 7% and 10% , respectively, of the cadmium in the United States. The remaining 81% was utilised to keep up with the growing demand for nickel-cadmium batteries [51]. Today, however, the overall consumption of cadmium has been reduced nearly ten times in comparison to what was used in the 1970s.

3.2 Cadmium toxicity

The reduction in the usage of cadmium has led to an overall decrease in the prevalence of this environmental pollutant. Consequently, the levels of cadmium in air, water and food that people are currently exposed to (10-25 μg/day) is significantly less than the provisional tolerable daily intake set by the WHO (60-70 μg/day) [69]. However, cigarette smoking and tobacco use drastically increase cadmium consumption. As such, cigarette smoking is currently the leading, non-occupational source of cadmium exposure.

Cadmium toxicity has most commonly been ascribed to occupational exposure and is an occupational hazard of working in industrial processes dealing with metal plating and the production of cadmium containing batteries, pigments, plastics or other such synthetic materials. Incineration of waste and burning of fossil fuels also lead to pollution of the environment with the toxic metal [71].

The effects of toxic exposure to cadmium have been grouped into two major categories, acute and chronic. Acute effects tend to be limited to inhalation exposure, and consequently side effects most notable pertain to the lungs; these include metal fume fever, pneumonitis, pulmonary and bronchial irritation, and in extreme cases death. Even short term, acute exposure can cause long term lung function impairment. Further lending to its toxicity, cadmium possesses an extremely long half-life, and is retained in tissue between 15-30 years. Chronic exposure to cadmium has been associated with the accumulation of cadmium in the kidneys, leading to kidney diseases such as proteinuria. In the lungs, long term exposure can lead to bronchiolitis and emphysema. According to the United

States Environmental Protection Agency (EPA), chronic exposure to cadmium via either oral consumption or inhalation can result in deleterious effects to the lungs, liver, kidneys, bones, nervous system, immune system and even blood [71]. Cadmium has been listed in the European Union's Restriction on Hazardous Substances (RoHS) as one of six banned substances, due to it being associated with reproductive and developmental side effects in both humans and animals.

3.3 Cadmium as a carcinogen

The EPA has listed cadmium as a class B1 probable human carcinogen. Many studies, ranging from *in vitro* to *in vivo* and even clinical, have been conducted to better understand the potential carcinogenicity of cadmium. This heavy metal has, thus far been associated with hyperlastic growth, tumors and cancers affecting numerous organs and various systems of the body. Summarized below is the current understanding of cadmium induced carcinogenicity and cytotoxicity, beginning with the *in vitro* findings and mechanistic evidence, and following through to *in vivo* data and clinical studies. Finally, cadmium as an endocrine disruptor or metalloestrogen is discussed.

3.3.1 *In vitro* and mechanistic evidence of cadmium carcinogenicity

Cadmium has long been investigated in cellular model systems as a cancer inducer, promoter and toxic metal. Numerous cellular studies have attempted to ascertain the mechanistic events involved in cadmium-induced carcinogenicity, but thus far have been relatively unsuccessful. Nonetheless, the scientific literature currently available points to certain cellular events that are likely involved in cadmium carcinogenicity [72].

An additional issue surrounding studies of cadmium carcinogenicity is the use of *in vitro* concentrations of cadmium, far surpassing the metal's LD_{50}; only the cells that survive these extreme concentrations of cadmium are then studied, and the resulting cellular transformations are investigated. However, these high concentrations may be masking the initiating events occurring at lower concentrations. Furthermore, concentrations that kill 50% of the population of

cells are rather irrelevant for *in vivo* and human studies, as carcinogenicity studies require the organism to survive post cadmium dosing.

Another key observation that has led to an inconsistent understanding of cadmium induced cancer, is the lack of homogeneity between cell types, tissues and even animal species studied [53-55].

Cadmium, like other carcinogenic metals was assumed to bind to DNA or form crosslinks between DNA strands or proteins surrounding DNA. But it was found that cadmium actually binds DNA with very low affinity, and as such is more likely to bind numerous other biomolecules within the cell, before binding to DNA (as shown in ex vivo studies) [56, 57]. Metallothionien (MT) is the biomolecule cadmium has the greatest affinity for [72]. In fact, the presence of cadmium within the cell signals the gene at the transcriptional level to produce more MT, leading to a rapid marked increase in the protein. Cell expressing higher levels of MT were shown to be less susceptible to cadmium exposure. It was found the mouse lungs expressed significantly more MT than rat lungs, and consequently it was noted that the incidence of lung cancer in mice was much lower than in rats [58, 59]. MT can, however, also be a double edged sword; it was observed in a study by Muller *et al*. that MT proteins that bind both zinc and cadmium are capable of causing DNA strand breaks *in vitro* [73]. The MT gene is under the umbrella of stress genes activated in response to cadmium, along with glutathione (GSH) and heat shock proteins. Heat shock proteins are induced by cadmium exposure as an adaptive cellular response to this metal [74].

Cadmium exposure activates immediate early response genes (IERGs) in a variety of cellular model systems [60]. This category of genes includes *c-jun*, *c-fos* and *c-myc*; the carcinogenicity of cadmium can be ascribed, at least in part, to the over expression of these genes, as they are found to be overexpressed in tumors and cells undergoing either proliferation or differentiation. IERGs undergo early transcriptional activation in response to early mitogenic signalling. IERGs were found to be potently activated at concentrations of cadmium as low as 0.1μM, and their overexpression can be either temporary or sustained [61].

Several transcription factors (TF) are also activated upon cadmium exposure including: AP1, a TF in the promoter region of several genes involved in cell proliferation [60, 62]. Other transcription factors affected include metal regulatory transcription factor 1 (MTF1), nuclear factor κb (NFκB), NF-E2 related factor (NRF2) as well as several others [53].

In general, cadmium is considered a weak genotoxic substance [75]. This effect is largely an indirect one ascribed to oxidative stress induced by the metal. These effects are attenuated by antioxidant treatment [76]. Depletion of GSH by buthionine sulfoximine, increased the genotoxicity of cadmium and led to the formation of 8-OHdg, a marker for DNA damage via oxidative stress.

Cadmium can prevent the repair of DNA damage, leading to the accumulation of DNA mutations, and carcinogenicity. One of the likely mechanisms precluding DNA repair is the substitution of zinc by cadmium in key enzymes required for and involved in repair processes [65].

Oxidative stress is one of the leading explanations and hypotheses for cadmium toxicity and carcinogenicity. Although cadmium is not a Fenton metal, it can generate reactive oxygen species (ROS) [75] [60], leading to lipid peroxidation of plamsma membranes constituents, and of cellular organelles [66] [67, 68]. Iron and copper ions present in bioligands and biomolecules can be displaced by cadmium, resulting in free intracellular copper and iron ions; capable of initiating Fenton reactions and generating ROS contributing to cellular impairments and cell death [69].

In contrast, cadmium can facilitate the proliferation of cancer cells. For example, non-invasive prostate cancer cells became highly invasive post cadmium treatment. Cadmium treatment killed nearly 65% of cells with low MT levels by apoptosis; and led to the transformation of surviving cells with high MTs into genotoxically damaged cells, resistant to apoptosis. These transformed cells had both enhanced proliferative capacity and high expression of anti-apoptotic genes [77].

Taken in concert, these findings show that cadmium can induce cytotoxicity and promote malignancy.

3.3.2 *In vivo* evidence of cadmium carcinogenicity

The earliest evidence suggesting cadmium induces carcinogenicity in rodents was reported by Haddow *et al*. [78]. Cadmium compounds were injected either intramuscularly or subcutaneously into rats and mice. Subsequently, it was observed that sarcomas developed in the rodents at the sites of injection. Other early studies showed cadmium to be a testicular tumorigen; where single high dose injections of cadmium were found to induce a high rate of benign testicular interstitial tumors [79]. Numerous studies since, have also shown inhaled cadmium to be a potent pulmonary lung carcinogen in rats.

Rat testes, are extremely sensitive to cadmium administered either parenterally or orally [79]. High parenteral doses were found to induce severe hemorrhagic necrosis in the testes, chronic degeneration and subsequently high occurrences of interstitial testicular tumors. Cadmium mediated degenerative effects on the testes leads to loss of androgen production, inevitably causing hyperstimulation of the surviving cells by the pituitary [74].

Cadmium has also been associated with prostate cancer. However, it was observed that at high does of cadmium, the tumorigenic and pre-neoplastic effects of cadmium were actually lost; and as such the prostatic tumor correlation to cadmium exposure does not follow a dose dependent pattern [75]. Studies revealed the proliferative effects seen in prostatic cells are only observed at lower concentrations of cadmium than those inducing interstitial testicular cell death. At concentrations below the cytotoxic threshold, there exists a dose dependent relationship between prostatic tumorigenesis and cadmium. Both the prostate and prostatic tumors are highly dependant on testosterone for growth and maintenance [80]. Therefore, high doses of cadmium leading to loss of androgen production, likely cause the prostate to atrophy, thereby rendering it non-tumorigenic.

Two other salient points to note with regards to cadmium exposure and prostatic turmors: studies have found that even oral exposure to cadmium can lead to prostatic tumors, [79] and that in animals exposed to other carcinogenic chemicals, cadmium enhanced the appearance of other chemically-induced tumors [81]. In rats, repeated subcutaneous injections administered at the same site, leads

to a moderate increase in sarcoma incidence, but these sarcomas are found to develop more rapidly, be more aggressive, while being more proximally invasive, and distally metastatic [78]. These effects show cadmium to be a potent promoter of cancer, and thus, cadmium exposure in combination with another carcinogen or cancer initiator could prove highly dangerous.

Other cancers noted *in vivo* in rodents post cadmium treatment include leukemia, [72], and even pancreatic cancer when cadmium is administered with calcium [79]. Other sites of tumorigenesis include the kidneys, the pituitary, and the liver. Thus with respect to *in vivo* studies of cadmium carcinogenicity, Waalkes *et al*, state that "accumulated data indicate cadmium is an effective, multi-route, multi-site, multi-species carcinogen in rodents" [72].

3.3.3 Human studies exploring cadmium carcinogenicity

Various agencies have deemed cadmium likely to be a human carcinogen. This conclusion has resulted from numerous repeat findings, suggesting a link between lung cancer and occupational exposure to cadmium. Further, the epidemiological data was corroborated by *in vivo* studies in rodents. While the data and studies linking cadmium to lung cancer are the most abundant and broadly accepted, an increasing number of studies are also indicating that cadmium may lead to cancers of the prostate and kidney [79]. In fact in recent years, several case control studies have been published reporting renal cell carcinoma development to be associated with occupational cadmium exposure. These studies are in accordance with previously published epidemiological studies. However, though the number of studies linking cadmium to an non-pulmonary cancers (liver, bladder, hematopoietic, stomach and pancreas) is rising, there remains no definitive link. Two studies are accurate depictions of our current state understanding of cadmium carcinogenesis in humans. The first, a case-control study, published by Chen *et al*. in December of 2009 recruited 261 prostate cancer cases and 267 controls with benign disease. Information was obtained from all subjects regarding lifestyle, dietary intake, occupation and disease state [82]. Blood cadmium levels and urinary cadmium levels were measured in all subjects to

assess whether cadmium exposure could be correlated with an increased incidence of prostate cancer. Results found no difference in mean cadmium concentrations of either the blood or urine of case and control groups. In subjects of the case group, with high blood and urine cadmium levels, patients' prostate cancer was found to be at a more advanced stage.

In a second study published by Beveridge *et al*, a two population-based-case control study was conducted evaluating subjects living in Montreal who had been exposed to heavy metals such as cadmium, nickel and chromium. The purpose of the study was to ascertain etiological links between occupational metal exposure (to lower levels of metals than in historical cohorts) and lung cancer. The case group consisted of 1598 subjects and the control group of 1965 subjects [83]. Detailed life history was recorded to evaluate lifetime occupational exposure to the heavy metals of interest. The findings of this study indicate that the carcinogenic effects of metal exposure were only discernible in non-smoking subjects but not did not increase the risk of lung cancer in smokers.

Taken together these studies depict the current complications in deeming cadmium a human carcinogen. There is ample data suggesting a causal relationship, however, concrete evidence is lacking. Further, there are many confounding factors plaguing the published studies. Thus, the current classification of cadmium is that of a probable human carcinogen.

3.4 Cadmium as an endocrine disrupter

Over the past few years, increasing studies have coined cadmium a metallohormone [81], as it is capable of mimicking estrogens and androgens *in vitro* and *in vivo* [84]. The estrogen-mimetic effects of cadmium have been more extensively studied, and cadmium has been found to bind the estrogen receptors, specifically, estrogen receptor α (ER α) and the membrane estrogen receptor, GPR30. Cadmium is currently believed to bind the cysteine residues of the in the ligand binding domain of ER α [83]. Subsequently, cadmium activates genomic and non-genomic estrogenic signalling, including AKT and ERK1/2 pathways and nuclear receptor transcription [84], leading to enhanced cell proliferation *in*

vitro, and hyperplasic growth of the uterus (overectomized female mice) and mammary glands *in vivo* [85]. Numerous studies have discussed the link between cadmium and breast cancer. Concentrations and doses of cadmium eliciting such responses are generally lower than those associated with toxicity. As such cadmium can increase the overall estrogenic burden in cells and tissue, leading to possible carcinogenicity.

Cadmium is therefore, an endocrine disruptor, probable carcinogen, and metal capable of inducing toxicity *in vitro*, *in vivo* and in human populations.

4. Applications of nanoparticles in medicine

Nanoparticles have been used as imaging tools [86, 87], drug delivery [88, 89] and theranostic purposes (i.e. combined imaging and therapeutic application) [90, 91]. These multimodal applications of nanoparticles make them both lucrative and interesting. Some examples illustrating the application of nanoparticles for imaging, drug delivery and therapy in oncology and other medical fields are presented in the subsequent sections.

4.1 Nanoparticles for biomedical imaging

Non-invasive imaging of the whole body to diagnose diseases in early stages is the ultimate goal of biomedical imaging. The advent of highly sensitive and specific imaging techniques is approaching, necessitating more advanced tools and modalities for imaging functional changes at the tissue, cellular and subcellular levels. One promising approach in refining and ameliorating the field of biomedical imaging, is the introduction of nanoparticles as novel contrast agents.

4.1.1 Current imaging approaches and nanoparticles

Currently, most of the imaging tools and agents are contrast agents utilized to enhance the visibility of specific tissues by increasing the signal to noise ratio (SNR) relative to the surrounding tissue [92]. However, numerous of these agents have serious limitations relating to resolution, tissue specificity and the ability to

detect small lesions or disorders. Specific nanoparticles may be able to overcome these limitations, and increase the scope and imaging potential of various approaches.

Several factors must be considered when designing contrast agents, namely the pharmacokinetics and pharmacodynamics [93]. Examples of these include: a reasonable half-life, minimal nonspecific binding, specific binding to desired location, appropriate elimination, and no toxicity. These must all be extensively evaluated and characterized without compromising a high SNR.

The following table (Table 3) summarizes the advantages and disadvantages of various imaging modalities and lists the nanoparticles that can be employed for each modality, along with the advantages associated with nanoparticle application.

Imaging Modality	Advantages	Disadvantages	Nanoparticle	Nanoparticle Advantage	References
Optical imaging	• Broad spectrum to NIR imaging • Inexpensive detection of low energy photons • Good spatial resolution	• Poor tissue penetration • Tissue scattering of photons • High noise & tissue autofluorescence	• Carbon nanotubes • Quantum dots	• Emit in the NIR • better tissue penetration • No autofluorescence • No high background	• Liang *et al.*, 2010 •Kosaka *et al.*, 2010 • Gao *et al.*, 2010
Magnetic resonance imaging	• Unlimited scanning depth • Safe procedure • Good spatial resolution	• High concentrations of contrast agents required leading to issues of elimination & toxicity	• Gadolinium incorporated: Nanodiamonds & carbon nanotubes • Or magnetic iron-oxide	• High magnetic resonance contrast • ten-fold increase in relaxivity • Functionalizable	• Matson *et al.*, 2010 • Xie *et al.*, 2011 • Manus et al., 2010
Positron emission tomography	• Minimal background • No signal amplification needed • Unlimited scanning depth • High sensitivity	• Poor spatial resolution • No timing information • Use of radioactive nuclide tracer	• Isotope incorporated or chelated to: iron-oxide nanoparticles & carbon nanotubes	• Combination probes including multiple contrast agents that can be utilized for multiple scanning modalities to gather more accurate diagnostic information	•Tassa, *et al.*, 2011 •McDevitt, *et al.*, 2007 • Hahn *et al.*, 2011
Computed tomography	• Contrast agents rapidly cleared • Low molecular weight agents • High contrast resolution; better delineation between tissue density • Scan Images can be viewed as axial, coronal or saggital images	• High doses of radiation required for high resolution images, lower doses of radiation results in high noise and blury images. • Potential complications and discomfort due to contrast agent •Risk of certain artefacts on scans	• Dendrimer entrapped gold nanoparticles • Tantalum oxide nanoparticles	• Improve contrast • High-Z nanoparticle contrast agents reduce radiation needs • Surface conjugation of nanoparticle allows active targeting of cells/tissue. • Molecularly targeted contrast agents enable structural & functional imaging	• Wang *et al.*, 2012 • Oh *et al.*, 2011 • Shilo *et al.*, 2012
Ultrasound	• Non-invasive • Easy to use & less expensive • Provides clear images of soft tissues • Real-time imaging	• Waves disrupted by air or gas • Waves cannot penetrate adult bone • Larger patients harder to image; too much tissue attenuates sound waves	• Gold nanorods, nanocages & nanoshells	• Improve contrast • High-Z nanoparticle contrast agents reduce radiation needs • Surface conjugation of nanoparticle allows active targeting of cells/tissue. • Molecularly targeted contrast agents enable structural & functional imaging	• Olafsson *et al.*, 2010 •Yang *et al.*, 2009 •Jokerst *et al.*, 2011

Table 3. The current status of different imaging modalities and the advantages of nanoparticles application. Optical imaging: [26, 94, 95], magnetic resonance imaging: [96-98], positron emission tomography: [92, 99, 100], computed tomography: [101-103], ultrasound: [104-106]

4.1.2 Advantages and disadvantages of nanoparticles in imaging

The application of nanoparticles for biomedical imaging offers numerous advantages over traditional contrast agents, but also poses some disadvantages.

The size of nanoparticles is one of the most advantageous qualities for imaging purposes as they are subject to wider *in vivo* circulation and distribution, than larger molecules of similar composition [87]. Nanoparticle are even able to cross the blood brain barrier, which may be desirable for neuroimaging [107].
The high surface to volume ratio of nanoparticles allow for greater functionalization [87], thereby permitting better tissue specificity which leads to a decrease in the SNR. Such that the images generated could be used for both qualitative and quantitative analysis. The adjunct addition of nanoparticles to form multimodal imaging probes enables the use of multiple imaging modalities to provide both high sensitivity and high resolution images [92]. Such an approach would enable more accurate images and ultimately better diagnoses. Additionally, nanoparticles can undergo a thousand modifications to ensure optimal tuning, without risking the loss of biological function [108]. Conversely, antibodies, for example, lose their antigen recognition after only ten substitutions [109]. Finally, it is predicted that nanoparticles may eventually facilitate the imaging of biological processes [87]. Such an approach would require a nanoparticle to be associated with a highly specific targeting moiety and a reporter system. Biological processes such probes may be able to image include, enzyme activity, protein-protein interactions, gene expression, ion-channel activity and even receptor density.
Unfortunately, thus far there also exist several disadvantages for using nanoparticles as contrast agents. Firstly, the pharmacokinetics and pharmacodynamics of nanoparticles are not entirely understood. Thus assessing factors such as the half-life, elimination, non-specific binding and binding to desired location, all critical for establishing the safety and dosing of nanoparticles is currently challenging [110]. Further, potential opsonization poses a deterrent to using nanoparticles as imaging tools [111]. Opsonization refers to a pathogen-surveillance process by which molecules and/or organisms circulating in the blood are altered by opsonins (molecules that target antigens for immune responses). Opsonization can modify the nanoparticle surface, size, shape, and/or interfere with the nanoparticle targeting and functionalization, thereby altering

nanoparticle fate *in vivo* [87]. Another complication in using nanoparticles is that they have been shown to fall trap to the reticuloendothelial system (RES) The RES sequesters inert particles and colloids in the circulatory system and delivers them to the liver and spleen [112]. Such non-specific phacogytic uptake of nanoparticles by the RES is undesirable as it generally leads to loss of targeting, and potentially hazardous accumulation of the nanoparticles in the liver and spleen. Finally, the greatest current disadvantage of nanoparticle use for *in vivo* imaging is the poorly understood issues of toxicity associated with nanoparticle use [87].

Thus while there are compelling arguments both for and against the use of nanoparticles for biomedical imaging thorough studies and extensive characterization of the nanoparticle are first required.

4.2 Nanoparticles for drug delivery

The field of drug delivery has been moving towards miniaturization for several decades [89]. Thus the superior physical and chemical properties of nanoparticles, and their promising scope as drug carriers has generated much interest in nanoparticle mediated drug delivery.

4.2.1 Current approaches in drug delivery using nanoparticles

The purpose of drug delivery technologies are to alter the drug release profile via the pharmacokinetics and pharmacodynamics of the drug, with the aim to improve drug efficacy, maximize the therapeutic effect and ensure patient safety, convenience and compliance. However, the fundamental paradox plaguing the use of many drugs is the constant compromise between maximizing therapeutic effects and minimizing side effects, is why nanoparticles are attractive tools for drug delivery. In general, nanoparticles are suitable for drug delivery in terms of size and porosity, allowing for the appropriate entrapment and surface association of drug molecules [88]. Further, nanoparticles can be administered via different routes, and may be extensively tuned for optimal drug delivery. Typically drugs

of interest are conjugated, dissolved, entrapped, adsorbed, attached or encapsulated into or onto the nano matrix.

The particle size of drug delivery vehicles such as nanoparticles is most important to determine their biological fate, and *in vivo* distribution. The smallest capillaries in the body range between 5-6μm in diameter, thus to ensure ubiquitous circulation the size of drug, carriers must be smaller in diameter [113]. Additionally, the smaller the drug carrier, the more efficient the cellular uptake and subsequent drug accumulation at target sites. Certain nanoparticles have even entered the nucleus, [114] thus enabling drug and gene therapy. However, nanoparticle access to the nucleus can also be dangerous as issues of timely clearance, nuclear or DNA-damage and toxicity may arise. Conventional drug delivery tools are both larger in size, and have a smaller surface to volume ratio. These carriers possess large cores in which the drugs are trapped; often leading to slower drug release. Conversely, greater surface to volume ratio of nanoparticles leads to more of the drug being present at the surface of the particle, and consequently associated with more rapid release [115]. For purposes of drug delivery nanoparticles may be either actively or passively targeted to sites of therapy [116]. Passive targeting is quite effective when targeting tumors as the leaky vasculature and enhanced permeability and retention of the tumor facilitates rapid uptake.

Different categories and kinds of nanoparticles are utilized in differing ways for drug delivery. The choice of nanoparticle employed ensures certain uptake characteristics; thus far in terms of cellular uptake it has already been shown that nanoparticles may be internalized via clathrin and/or caveolin-mediated endocytocis, pinocytocis or in certain cases phagocytocis [117-120]. Below two categories of nanoparticle mediated drug delivery are briefly discussed [121].

Polymeric nanomaterials and dendrimers exhibit several attractive qualities for use as drug delivery agents; they are biocompatible, biodegradable and can be easily functionalized. Further, via functionalization and structural manipulation, drugs can be incorporated, encapsulated, conjugated and associated with the nano-

carriers. Dendrimer may even have a degradable link incorporated that could allow further control over drug release [21].

Metal structures composed of gold, silver and platinum and palladium that are hollow, have been investigated for use as drug delivery materials. It was demonstrated that metals were linked to, or embedded in polymeric drug carriers, so that metal nanoparticles may be used as thermal release triggers when irradiated with near infrared light or excited with alternating magnetic fields [122].

4.2.2 Advantages and disadvantages of nanoparticle use for drug delivery

The purpose behind introducing new technologies into any field is to resolve issues and improve upon the previous modalities employed. However, new technologies are not without flaws. Below some of the advantageous and disadvantages of using nanoparticle as drug delivery tools are discussed.

Issues such as poor drug solubility are easily resolved with the introduction of ampiphillic and polymeric nanoparticles [113], potentially decreasing the need for invasive injections. Greater stability and longer shelf-lives are an added advantage of using nanoparticle drug formulations [110]. The ability for nanoparticles to carry numerous drug molecules can be exploited to ensure that the drug to matrix ratio is very high, allowing maximal drug and minimal matrix is administered to the patient [123]. Additionally, the direct coupling of a drug to a targeting ligand can prove inefficient, as it restricts the coupling capacity to a few molecules; however, nanoparticle-drug formulations may be coupled to a targeting ligand, allowing the transport of thousands of drug molecules via each targeting ligand [113]. Due to the enhanced pharmacokinetics and pharmacodynamics, nanoparticle formulations may improve the absorption of insoluble drugs, thereby improving the overall bioavailability and release rates of the therapeutic agent and decreasing off target side effects. Drugs conventionally associated with first-order kinetics, may be altered via nanoparticles drug carriers to exhibit more zero-order-like kinetics [124]. In addition, nano-carriers may act as a protective covering for the therapeutic cargo, preventing rapid *in vivo* degradation. Finally, patient

compliance may improve due to slow, sustained release, as certain nanoparticle-drug formulations can provide therapy for 24hours and more [113].
In terms of the disadvantages associated with nanoparticle use for drug delivery many are similar to those discussed for imaging purposes, particularly the pharmacokinetics and pharmacodynamics being inadequately characterized. For example, controlled release dosage forms present a challenge in terms of predicting the pharmacodynamics of a drug as ascertaining a well defined relationship between the systemic concentration and therapeutic window of a drug [113]. Therefore, estimating the drug input rate for optimal response at the target site can be a rate limiting factor in the use of controlled release and other new drug delivery systems. Also nanoparticle-drug carriers detected by the host immune system and subsequently cleared from circulation, may results in potentially toxic doses of the drug being deposited in the liver or spleen. Finally, nanoparticles often have a tendency to aggregate, thereby altering their size and reducing the efficacy of their functionalization [113].

5. Nanoparticles and the environment

Having discussed the various engineered nanoparticles and their applications in medicine, it is important to note that, numerous nanoparticles exist in our environment; these can be either naturally occurring or the by-product of industrial processes. Over the years these two categories of nanoparticles have been extensively researched and have assisted in the better understanding of each other. Therefore, in this section the environmental burden of both laboratory and environmental nanoparticles are discussed.

5.1 Natural nanostructures and synthetic nanoparticles

Particulate materials are found everywhere in the environment and can be categorized as: naturally occurring nanoparticles, anthropogenic ultrafine particles (UFPs) or synthetic, engineered nanoparticles [125].
Naturally occurring nanoparticles include volcanic ash, ocean spray, viruses, mineral composites, clouds, and forest fire smoke to name a few. UFPs are

unintentional particles, often the by-products of various processes; they include diesel exhaust particles (such as polycyclic aromatic hydrocarbons and quinones), environmental tobacco smoke particles, cooking/frying, biomaterial degradation and sandblasting, to list a few [125-127]. Engineered, synthetic nanoparticles include structures such as quantum dots, micelles, carbon nanotubes, fullerenes and many more. The last two categories include both organic and inorganic particles and are both associated with air pollution and issues of toxicity [9]. There are several differences and similarities between ambient UFPs and engineered nanoparticles. The fundamental difference being that nanoparticles are single unit, functional particles engineered and produced for specific uses [128], whereas UFPs are produced and released via industrial emissions and everyday life processes [125]. Their shapes, sizes and composition are not uniform and, therefore, can cause a variety of toxic effects. UFPs are not intentionally synthesized or intended for any use, rather they are by-products of combustion, cooking and smoking, and thus classified as air pollutants. However, due to the similar size range, other commonalities also exist between nanoparticles and UFPs [126]. Perhaps the most significant similarity is the high surface-area to volume relationship. This surface reactivity becomes both advantageous and concerning with respect to biological matter, as nanoparticles are able to penetrate through various barriers and be internalized [118]. In summary, UFPs can be especially deleterious as these particles can also carry other hazardous materials into the body, such as bacterial toxins [126].

The most common route of exposure to UFPs and nanoparticles are through inhalation, ingestion and dermal [125]. Nano-sized particles in the air are easily inhaled and thus gain entry into the respiratory tract [126]. Larger particles, around 10μm in diameter, do not effectively penetrate and deposit in the lower respiratory tract; they are cleared by the nasal and ciliary mucosa of the airways. However, smaller particles are able to penetrate the lower respiratory tract far more effectively, and are able to diffuse into the tissue and cells more easily. UFPs can undergo endocytosis and be internalized by the cells of the respiratory

tract [129]. Once in the cells, these nano-sized particles can induce ROS and a subsequent inflammatory response.

Nanoparticle and other UFP pollutants that are intentionally or unintentionally combined with either food or water, rapidly gain entry into the gastrointestinal tracts (GI), where these particles are exposed to the highly absorptive, grand surface area of the gut [130, 131]. Once in the GI tract, the nano-sized particles can both damage the tract, or enter the circulatory system, thereby exposing all the organs to potential nanotoxicity [132]. A third route of entry is dermal, from either ambient exposure to UFPs and particulate matter resting on the skin, or in the form or creams, sunscreens and other cosmetic products that contain nanoparticles [133]. Once the particles penetrate the skin, they can be transported to the lymphatic system, eventually reaching systemic circulation and being exposed to multiple organs [125]. Additionally, it is being investigated whether dermal exposure to nanoparticles can lead to anterograde transport via the nerves, such that eventually nanoparticles may gain access to the central and peripheral nervous system [134].

In certain ways UFPs and engineered nanoparticles are diametrically opposite; nanoparticles are synthesized and intended to be highly valuable tools, with widespread applications [135], whereas UFPs are unintentional by-products of combustion and industrial processes, that serve no medical or practical purpose [9]; rather they are classified as air pollutants. Nonetheless, due to similar physical-chemical properties, the two categories of nano-sized particles share numerous commonalities, including toxicity. Understanding and studying this toxicity is imperative if it is ever to be overcome.

5.2 Nanotoxicity and the environment

Ambient UFPs and engineered nanoparticles are present everywhere. The introduction of nanoparticles in electronics, cosmetics and countless other everyday items, translates to widespread exposure and potential ecotoxicological effects [136]. Further, UFPs have been present in the air, water and soil for centuries. However, side effects of these existing particles are now being

extensively investigated, and numerous studies are being conducted to ascertain what the toxic implications of these nanoparticles may be. Below, nanoparticles are discussed with respect to the environment and their subsequent biological and pathological consequences.

5.2.1 Nanoparticles in soil

Nanoparticle production has been increasing rapidly over the last decade and is projected to increase more steeply in the years to come [127]. As such, from an environmental perspective, soil and water are likely to become highly contaminated; translating to the entire ecosystem being exposed to nanoparticles [128]. Of particular concern, with respect to soil, is the threat that the entire food chain will become contaminated [125]. Two recent studies demonstrate the potential deleterious effects of soil being laden with nanoparticles.

In a study conducted by Du *et al.*, soil was mixed thoroughly with either titanium oxide or zinc oxide (TiO_2 and ZnO respectively) nanoparticles to reflect a low level of soil pollution [137]. Wheat sown in this nanoparticle-polluted soil was harvested several months later, prior to analysis of both the wheat and soil. Microscopy of the wheat roots showed that in wheat grown in the presence of TiO_2, dark particles were present. Further, analysis of the dark particles revealed TiO_2 nanoparticles ranging in size from 25nm – 85nm. In addition, analysis of the titanium content showed that only the titanium found in the root was detectable. Conversely, roots of wheat grown in the presence of ZnO displayed no dark spots. With respect to the biomass of the harvested wheat, as compared to control, wheat grown in the presence of nanoparticles was significantly lower in weight; the lowest weight corresponding to TiO_2 laden soil. Interestingly, the titanium content of the TiO_2 exposed wheat plants was not significant; however, the zinc content of wheat grown in ZnO exposed soil was significantly higher than control. Lastly, analysis revealed that in nanoparticle-polluted soil, enzymatic activity (protease, catalase, peroxidase) was much lower as compared to control soil. Taken together, these findings demonstrate that nanoparticles can penetrate the cell wall and be deposited in the plant. Though it seems the mechanism by which each

nanoparticle damaged the wheat plant (represented as a decrease in biomass) may differ, nonetheless, both were harmful. TiO_2 accumulated on the cell walls, and its presence was sufficient to induce changes in the microenvironment, and likely induce reactive oxygen species, thereby precipitating cell membrane damage. Perhaps more than to the plants, the deleterious effects of the nanoparticles were to the soil, as assessed by a decrease in certain enzymes in the soil. It can be hypothesized that nanoparticle metal reactions with sulfhydral groups may lead to metal-sulfides that can inhibit or inactivate enzymes. Perturbations to the soil microenvironment by nanoparticles can be damaging to the soil ecosystem. Up to 40% of QDs applied to soil were retained there and in larvae and moths, such that – fluorescence was detected in their gut and fecal matter, as well as elevated cadmium levels [138]. Thus excessive amounts of toxic-metal-containing nanoparticles in the environment can disrupt our ecosystem.

5.2.2 Nanoparticles in water

The impact and potential threat of nanoparticles in water and on aquatic life are the focus of much research. The increased use and production of nanoparticles, as well as the presence of countless UFPs all eventually lead to the contamination of water. Whether from waste and sewage, soil contamination or air pollution, the ecosystem is intertwined and thus, the ecotoxicity of water and aquatic life warrants careful monitoring.

Countless studies have been reporting the deleterious effects of nanoparticles, endocrine disruptors and other chemicals on aquatic life. In a recent review article published by Shaw *et al.*, the authors tried to ascertain the difference between the danger of metallic nanoparticles (gold nanoparticles, copper nanoparticles, ZnO and TiO_2 nanoparticles) and the better known metal ions to aquatic biota [139]. The authors hypothesize that due to having highly reactive surfaces, nanoparticles pose a greater danger than ionic metals in water, and may be more acutely toxic to fish. In a previous study, it was shown that the 48 hour lethal concentrations (LC_{50}) of copper ions versus copper nanoparticles in adult zebrafish were 250 μg l−1 and 1.56 mg l−1, respectively. Analogous results in juvenile zebrafish

show a 48 hour LC_{50} of about 0.71 and 1.78 mg l−1 with nano-copper and the dissolved form, respectively. This discrepancy between adult and juvenile zebrafish also highlights a greater developmental susceptibility to nanotoxicity. Another study conducted on zebrafish showed a significant disruption in reproduction post chronic exposure to TiO_2. After 13weeks of TiO_2 exposure, nearly a 30% reduction in zebrafish eggs was reported [140]. In the above mentioned study examining the sub-lethal and toxicological difference to rainbow trout post 48 hour exposure to either CdS/CdTe QDs or dissolved cadmium, QDs were found to be far more dangerous [141]. The nanocrystal exposure led to more significant reductions in immunocompetence than dissolved cadmium. Both forms of cadmium, however, were able to induce metallothionein gene expression, but in the microarray examining altered gene expression, QDs and cadmium affected different genes. Taken together, all these results indicate that nanoparticles and UFPs could present a much greater danger to aquatic biota than many traditional pollutants.

5.2.3 Nanoparticles in the air

UFPs, as previously stated, are categorized as air pollutants; additionally, nanoparticles that are being produced on larger spectrums each year, share many size related properties with UFPs; therefore, the release of these nanoparticles into the environment may also deem them air pollutants. The risk associated with UFPs and engineered nanoparticles are primarily due to internalization via inhalation [9, 125, 127]. As discussed above, the respiratory tract can be compromised by inhalation of air pollutants. Inhalation studies in rats, with single-wall CNTs have shown that even short term exposures, can lead to a series of dose-dependent pathological events: early inflammatory response and oxidative stress precipitated the appearance of multifocal granulomatous pneumonia and interstitial fibrosis [125]. From studies done by Poland *et al*, it has been shown that perhaps due to certain similarities in fibrous structures, CNT may induce asbestos like pathologies in the lungs, including lung cancer [142].

The risks induced by inhalation of nanoparticles are unfortunately not exclusive to CNTs; other nanoparticles, such as TiO_2 have been shown to potentiate asthmatic-like effects in young rats, but not in adults. Increased bronchial responsiveness, inflammation and upregulation of chemokines were all noted post inhalation by newborn and two week old rats [143].

Inhalation of UFPs and nano-sized particles is also associated with cardiopulmonary system injury. Apolipoprotein E-deficient mice (genetically susceptible to atherosclerosis), post exposure to concentrated UFPs experienced a larger size and greater number of atherosclerotic plaques, as compared to control apolipoprotein E-deficient mice [144]. The authors speculate that the propensity for UFPs to influence atherosclerotic plaques is due to their stimulation of oxidative stress, and potentially liver lipid peroxidation. The mice in this experiment were exposed to levels of UPFs comparable to 2-6 times higher than those associated with typical highway traffic exposures.

The potential injuries from inhalation of UFPs and nano-sized matter are even speculated to affect the CNS, more specifically the brain. Inflammatory and neurodegenerative effects were noted in the olfactory mucosa, olfactory bulbs and other associated brain structures in dogs from a highly polluted region of Mexico City [125]. Similar neurological changes were not observed in the brains of dogs from other less polluted, and more rural areas near Mexico City.

The injury associated with UFPs and nanoparticles is long reaching. The respiratory tract, cardiovascular system, CNS and circulatory system are all susceptible to air pollution related toxicity. Therefore, understanding and attempting to minimize this risk warrants additional research and attention.

6. Nanotoxicology

Having established the applications and advantages of nanoparticle use, as well as the presence of naturally occurring nanoparticles in the environment, we next examine the undesirable aspects, and major roadblock preventing nanoparticle use: nanotoxicity. Nanotoxicology was described by Oberdorster in 2005 as the study of the effects of engineered nanomaterials on biological systems [125]. As

previously discussed, the unique physical chemical properties of nanoparticles combined with their reactivity under biological conditions, leads to much unpredictability when comparing nanomaterials to their larger counterparts. With the exponential increase of nanoparticle applications in biomedical research, one of the most rapidly emerging fields of study is nanotoxicology.

6.1 Complications with nanotoxicological studies

Although, size is an important factor in determining the potential toxicity of a particle, other properties including chemical composition, shape, surface structure, surface charge, aggregation, solubility, and the absence or presence of functional groups of other chemicals, can also be greatly influential. The large number of variables influencing toxicity renders it difficult to generalize the health risks associated with exposure to nanomaterials. Each new nanomaterial must be assessed individually and all material properties must be taken into account.

A standardization of toxicology tests between laboratories is much needed, but yet to be implemented. Different cell lines have different properties and respond to particles in a different manner. Similarly, nanoparticles in dispersed and aggregated forms are internalized by cells in a different manner and activate different signaling molecules. Manufactured nanostructures are often not well-characterized and should be characterized in individual laboratories before biological experiments are initiated. Many nanoparticles agglomerate or aggregate when exposed to environmental or biological fluids. The terms agglomeration and aggregation have distinct definitions according to the standards organizations, ISO and ASTM, where agglomeration signifies more loosely bound particles, and aggregation signifies very tightly bound or fused particles. Recently, our lab proposed the implementation of the asymmetric flow fractionation method to complement other techniques in assessing and delineating between the aggregation and agglomeration of nanoparticles (unpublished data). Agglomerated nanoparticles are frequently found in biological fluids, although, this observation has been commonly ignored in nanotoxicity studies. Recently,

more studies addressing the question of agglomeration and aggregation have appeared [145, 146]. Although, atomic visualization methods such as scanning- or transmission electron microscopy (SEM and TEM), and atomic force microscopy (AFM) analysis are very useful, information on the precise nanoparticle composition within the biological structures is still lacking. The necessity to perform elemental analysis when investigating intracellular nanoparticle localization using TEM was illustrated in the study by Brandenberger *et al.*, as they demonstrated that not all of the nano-sized structures identified by TEM were indeed nanoparticles.

The study of nanotoxicology is a crucial component in the rapidly aggrandizing fields of nanotechnology and nanomedicine. The current *in vitro* and *in vivo* methods used to assess nanotoxicity are insufficient due to the lack of regulations to unify the evaluation process. There is also an urgent need to identify early markers that are sensitive to toxic nanoparticles, which should preclude irreversible cellular changes (such as morphological changes of the organelles). The following sections highlight some of the molecular mechanisms commonly associated with nanomaterial induced toxicity.

6.2 Systemic and cellular mechanisms involved in nanoparticle toxicity

The toxicity of nano-sized particles has thus far been challenging to evaluate. However, certain biological responses are currently accepted as indicators of potential nanoparticle toxicity. Firstly, an immunological response induced by nanoparticles and UFPs is utilized to assess whether nanoparticles may be toxic [126]. For instance, the architecture and density of polymers coated on the surface of nanoparticles can alter the complement system (a part of the larger immune system) which recognizes these particles. The analysis of structure-complement activation relationship showed that a shift in the configuration of polyethyleneoxide on the surface of nanoparticles (e.g. from mushroom-brush to brush), can switch the complement activation from the classical C1q-dependent to the lectin pathway [147]. Nanoparticle-mediated complement activation is of clinical concern, and currently, nanoparticle surfaces that do not induce

complement system activation are scarce. The generation of proinflammatory compounds, such as cytokines, chemokines and various other biochemical markers is also indicative of nanoparticle toxicity. Nanostructures can activate the immune system inducing inflammation, immune responses, allergies, and even alter the immune cells in a potentially deleterious or beneficial way (immunosuppression in autoimmune diseases, or improving the immune response induced by vaccines). More studies are needed in order to decipher the potential deleterious or beneficial effects of nanostructures in the immune system.
Nanoparticles can also induce the reconstruction of phospholipid membranes at the surface of the cell [148]. Negatively charged nanoparticles were shown to induce localized sites of gelation in the fluid bilayer, whereas positively charged nanoparticles produced local fluidization in gelled membranes.
Finally, metal containing nanoparticles, or transition metals that have attached themselves to the surface of UFPs can also induce Fenton reactions via the creation of oxygen rich intermediates. These oxygen intermediates also contribute to the oxidative threat posed by nano-sized particles on the cell [126].

6.3 Molecular mechanisms of nanoparticle-induced cytotoxicity

The toxicity of nanoparticles has been ascribed to their physicochemical properties, including size, shape, core constituents and surface materials [149, 150]. Numerous studies have been conducted probing the molecular mechanisms involved in nanoparticle-induced cytotoxicity, including intracellular events, organelle responses and functional impairments from nanoparticle exposure. Of the different types of nanoparticles, cadmium telluride quantum dots are now known to be amongst the most cytotoxic. Therefore, described here are some of underlying mechanisms of cytotoxicity induced by nanoparticles, specifically, CdTe QDs.
Smaller nanoparticles (< 5nm in diameter), such as the green quantum dots (QDs), are capable of traversing the plasma membrane and accumulating in various subcellular organelles, including the nucleus and lysosomes [114, 151]. It was further shown that surface charge may assist in determining the extent of

nanoparticle cellular internalization; QDs with a greater cationic charge are taken up by the cells to a greater degree [152, 153]. Cellular internalization of nanoparticles can occur by a number of pathways, however, in recent study by our group, it was shown that QDs are internalized mostly via lipid raft-mediated endocytosis, and via specific transporters if the nanoparticle has the corresponding proteins or ligands conjugated to its surface (e.g. cysteine-QDs are internalized by the X_{AG} cysteine transporter) [117]. Upon entry into the cell, the nanoparticles can localize and interact with the organelles, and consequently, affect their physiological functioning.

QDs have been found to localize in the lysosomes, nucleus and mitochondria [114, 153-156]. Colocalization of nanoparticles in these organelles not only results in morphological changes of the organelle, but may impair organelle functioning. Long term treatment (<12 hours) of cells with QDs induces enlargement of the lysosomes, mitochondrial blebbing, loss in mitochondrial membrane potential, and chromatin reorganization in the nucleus [156]. Due to these small nanocrystals easily traversing the membranes of subcellular organelles, it is conceivable that morphological damage observed may be due to direct interactions of the nanoparticles.

In light of the plethora of deleterious subcellular events initiated by exposure to toxic QDs, we investigated whether the application of antioxidant drugs could protect the cells, and organelles, against nanoparticle-induced oxidative stress [134, 151, 152]. N-acetylcysteine (NAC), a powerful antioxidant, was amongst the first found to directly protect against QD-induced oxidative stress [151]. However, a precursor to glutathione, NAC, may also protect the cells via replenishment of the endogenous antioxidant in the cell [157]. Moreover, upon surface modification of QDs with this antioxidant, QD-toxicity was further reduced [134, 152].

7. Formulation of project

The production and applications of nanoparticles are increasing rapidly. Nanoparticles are being introduced into every aspect of everyday life, including technology, food, clothing, hygiene, makeup, automobiles and transportation, medicine and laboratory research, just to name a few. The promise of nanotechnology is limitless and these tiny particles are gradually revolutionizing our world and becoming more and more prevalent all around us. However, to date, the understanding of potential issues of nanoparticle toxicity is still lacking. Particularly with the applications of nanoparticles for medical purposes – diagnostic, therapeutic and as carriers for drug delivery, the lack of understanding surrounding nanoparticle toxicity is frightening.

CdTe QDs are amongst the nanoparticles most known to be toxic. Countless studies examining the toxicity of the individual metals exist in medical literature; however, the popularity of these QDs, particularly for non-medical use, has been increasing. Nonetheless, the production and use of these nanocrystals in any industry implies environmental pollution, side effects to animal/aquatic life, and implications of toxicity in humans. Additionally, from the increased mining and processing of the metals necessary for QD production, the amounts of these metals present in the environment is likely to increase, and given that these nanoparticles are self-assembled structures, there is no way of ascertaining or preventing spontaneous formation of aggregates which could also be cytotoxic. Prior to the studies presented in this thesis, QDs were synthesized in organic solvents considered harmful to living cells. The first synthesis of these nanocrystals in water was reported for cadmium telluride QDs. We, therefore, started investigating these QDs.

When I commenced my studies with QDs, only the very first reports of QD-toxicity had been reported, and that too by our group. However, the underlying mechanisms of toxicity were completely unknown. In fact, the common assumption was that QD-toxicity could be simply ascribed to the presence and liberation of toxic cadmium ions from the colloidal structure. I was interested in evaluating the role of cadmium in QD-induced cytotoxicity, and whether other

mechanisms or mediators may be involved. By examining *in vitro* cadmium ion release from QDs and examining both the resulting cytotoxicity and metalloestrogeniciy, I was able to classify two dichotomous effects of cadmium-containing QDs, both of which may have tremendous implications for human and animal health.

7.1 Hypotheses

1. Quantum dot toxicity cannot be explained as a sum of parts of the toxicity associated with each QD-constituent.
2. Cadmium containing QDs are metalloestrogens and are thus endocrine disruptors

7.2 Objectives

The objectives of this thesis deal with the experiments designed to address the following questions:

1. Does the amount of cadmium released from QDs correlate with the degree of cytoyoxicity observed *in vitro*?
2. Does CdTe QD-toxicity differ from the combined toxicity of cadmium plus tellurium ions?
3. Can antioxidants prevent QD-induced cytotoxicity, and how?
4. Do QDs exert metalloestrogenic effects, and if so, are these effects mediated via the estrogen receptor-initiated signal transduction pathways, *in vitro* and *in vivo*?

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Connecting Text

Initial studies examining CdTe QD-induced cytotoxicity were quick to presume that the toxicity was caused by the liberation of toxic cadmium ions from the QD-core. However, at the time no studies had been conducted that could empirically attest to this fact. Prior to delving deeper into understanding and preventing QD-induced cytotoxicity, we felt it imperative to determine whether or not cadmium released from the QDs was, in fact, responsible for mediating QD-toxicity. These finding would serve as the foundation for all our future studies. Therefore, in our preliminary study, we attempted to show that QD-induced cytotoxicity cannot be explained only by cadmium released from the QD core. The study compares QDs of different sizes and different surface modifications with the respective degree of cytotoxicity induced by each and the amount of cadmium liberated from each. Intracellular imaging studies are also employed to examine the intracellular location of the QD as well as morphological and biochemical changes indicative of QD-toxicity.

Chapter 1b

Preliminary results

Long-term exposure to CdTe quantum dots causes functional impairments in live cells

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1. Abstract

Several studies suggested that the cytotoxic effects by quantum dots (QDs) may be mediated by cadmium ions (Cd^{2+}) released from the QDs cores. The objective of this work was to assess the intracellular Cd^{2+} concentration in human breast cancer MCF-7 cells treated with cadmium telluride (CdTe) and core/shell cadmium selenide/zinc sulfide (CdSe/ZnS) nanoparticles capped with mercaptopropionic acid (MPA), cysteamine (Cys), or N-acetylcysteine (NAC) conjugated to cysteamine. The Cd^{2+} concentration determined by a Cd^{2+}-specific cellular assay was below the assay detection limit (< 5 nM) in cells treated with CdSe/ZnS QDs, while in cells incubated with CdTe QDs it ranged from ~ 30 to 150 nM, depending on the capping molecule. A cell viability assay revealed that CdSe/ZnS QDs were non-toxic whereas the CdTe QDs were cytotoxic. However, for the various CdTe QD samples there was no dose-dependent correlation between cell viability and intracellular [Cd^{2+}], implying that their cytotoxicity cannot be attributed solely to the toxic effect of free Cd^{2+}. Confocal laser scanning microscopy of CdTe QDs treated cells imaged with organelle-specific dyes revealed significant lysosomal damage attributable to the presence of Cd^{2+} and of reactive oxygen species (ROS) which can be formed via Cd^{2+}-specific cellular pathways and/or via CdTe-triggered photoxidative processes involving singlet oxygen or electron transfer from excited QDs to oxygen. In summary, CdTe QDs induce cell death via mechanisms involving both Cd^{2+} and ROS accompanied by lysosomal enlargement and intracellular redistribution.

2. Introduction

Nanomaterials, such as gold nanoparticles and quantum dots (QDs), are the focus of considerable current attention, especially in view of their applications in biology and medicine [1-4]. Their unique features, such as size, shape, chemical composition, and interactions with light or other external stimuli, are a source of concern, as they may have damaging effects on the environment [5]. Whereas it is generally agreed that gold nanoparticles are essentially inert and non-toxic to cells

[6], there is still no consensus in the case of QDs [7, 8]. The cytotoxicity of QDs may be connected to photochemical processes ensuing irradiation of QDs under the aqueous aerobic conditions of *in-vitro* cell imaging. Thus, photooxidation of cadmium telluride (CdTe) QDs is known to occur in live cells [9]. The process may involve an electron transfer from the excited QDs to O_2 to produce superoxide (O_2^-) and an unpaired hole in the QD [10, 11], which can induce ligand oxidation and cleavage with subsequent oxidation and corrosion of the nanoparticle outer surface [12]. Also, excited QDs may transfer energy to another molecule (sensitizer) capable of generating singlet oxygen [13], leading to the formation of reactive oxygen species (ROS).

Alternatively, or concurrently, in the case of cadmium-based QDs, cytotoxicity can be a consequence of the release of highly toxic free Cd^{2+} ions. Several groups have shown that the amount of free Cd^{2+} in solution of QDs correlates well with their cytotoxicity [14-16]. The free Cd^{2+} concentration in a 0.25 mg/mL solution of QDs was reported to range from 6 ppm (or ~ 50 μM) for non-toxic CdSe QDs to as much as 126 ppm (or ~ 1.05 x 10^3 μM) for toxic CdSe QDs [15]. The latter value is within the range of Cd^{2+} levels known to lead to significant cell death (100 to 400 μM) [17]. Free Cd^{2+} has been detected also in fluids in contact with QD-based materials, such as CdTe-doped polyelectrolyte multilayers [18]. However there is no experimental data that correlate QD cytotoxicity to the concentration of Cd^{2+} in the intracellular compartment of cells incubated with QDs.

The chemical composition of the nanoparticle core and the composition of the outer capping layer are important factors affecting QD toxicity. As a rule, CdTe QDs are significantly more toxic than core/shell (CdSe/ZnS) nanoparticles [7]. Thus, hydrophilic CdTe nanoparticles are not widely employed for imaging of live cells, but they are still applied in various luminescence detection systems and devices [19, 20]. All water-soluble QDs are capped either with amphiphilic compounds or with hydrophilic thiols or dithiols that function as passivating agents designed to insulate the semiconductor core from its surroundings. Prolonged contact of hydrophilic QDs with biological fluids can cause partial

desorption of the capping layer, exposing the bare semiconductor surface to the biological milieu, with possible detrimental consequences on the QD environment. In a recent confocal laser scanning microscopy (CLSM) study of live cells treated with CdTe QDs capped with the labile mercaptopropionic acid (MPA), we demonstrated that one of the cytotoxicity mechanism is related to the ability of CdTe QDs to generate reactive oxygen species (ROS), such as hydrogen peroxide and various hydroperoxide radicals [21]. These, in turn, inflict damages to proteins, DNA and lipids, thereby leading to severe cell functional impairments and, eventually, to cell death. While our study was limited to CdTe QDs, other groups have shown that CdSe QDs also generate free radicals in solution [22] as well as *in-vitro* [23]. In contrast, CdSe/ZnS core/shell QDs seem not to produce free radicals in solution [22], although free radical generation has been implicated in the mechanism of double stranded DNA nicking in the presence of CdSe/ZnS nanoparticles [24]. It is evident from the data reported so far that the cytotoxicity of cadmium-containing QDs cannot be ascribed to a single universal mechanism, but that it is a consequence of several phenomena triggered by exposure of QDs to light and the biological milieu. How a given QD sample will respond to these stimuli reflects its composition, surface chemistry, and history. The damage inflicted by a given QD sample depends also on cell type [25].

The study reported here was devised to assess the involvement of free cadmium in QD-triggered cell death and to gauge its importance with respect to other cytotoxicity mechanistic pathways. We carried out a quantitative analysis of free Cd^{2+} in the intracellular compartment and extracellular milieu of cells incubated with QDs. We used CLSM to image live cells treated with the same QD samples in order to detect QD-induced changes in cellular morphology which yield information on the possible involvement of individual organelles in the cellular mechanisms responsible for cell death. We used the human breast cancer MCF-7 cell line and four types of cadmium-based QDs: three samples of CdTe nanoparticles capped with (a) mercaptopropionic acid (MPA), (b) cysteamine (Cys), or (c) N-acetylcysteine (NAC) conjugated to cysteamine, and one sample of CdSe/ZnS core/shell QDs capped with cysteamine (Table 1). These *in-vitro*

studies were complemented by control experiments carried out with QD solutions: (1) a quantitative analysis of the free Cd^{2+} concentration in QD solutions and (2) photophysical measurements designed to detect the generation of singlet oxygen upon QD photoirradiation and to assess the occurrence of fluorescence resonance energy transfer (FRET) between QDs and the organelle-specific organic dyes employed in the imaging experiments. Important conclusions of our study are (i) that free Cd^{2+} is present *in the intracellular compartment* of cells treated with CdTe QDs, but not of those incubated with CdSe/ZnS QDs and (ii) that uptake of CdTe QDs triggers specific changes in cellular morphology, in particular significant lysosome enlargement, a cellular damage known to occur in cells contaminated with toxic doses of cadmium, but also attributable to the presence ROS from other sources.

Table 1: Characteristics of the nanoparticles used in this study.

Sample	Capping agent	λ_{max} (emission)[a]	$[Cd^{2+}]$[b] (μM)
MPA-CdTe	$HS\text{-}(CH_2)_2\text{-}CO_2H$	535 nm	5.62 ± 0.03
Cys-CdTe	$HS\text{-}CH_2CH_2\text{-}NH_2$	554 nm	4.85 ± 0.06
NAC-CdTe[c]	$HS\text{-}CH_2CH_2NHCO(NHCOCH_3)CH_2CH_2SH$	528 nm	3.77 ± 0.03
Cys-CdSe/ZnS	$HS\text{-}CH_2CH_2\text{-}NH_2$	517 nm	0.07 ± 0.02

a in PBS buffer (pH 7.4), QD concentration 0.5 mg/mL, λ_{exc} = 400 nm
b for solutions of QDs (10 μg/ml) in water
c NAC conjugated on Cys-CdTe

3. Materials and methods

3.1 Materials

Tellurium powder, sodium borohydride, cadmium perchlorate hydrate, 3-mercaptopropionic acid (MPA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), cysteamine hydrochloride (Cys), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), trioctylphosphine oxide (TOPO), and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich Chemicals (Saint Louis, MO, USA). LysoTracker Red DND

99, MitoTracker Deep Red 633, Hoechst 33342, and the Measure-iT™ Lead and Cadmium assay Kit were obtained from Molecular Probes (Eugene, OR, USA). Water was deionized with a Millipore Milli-Q water purification system. Centrifugation was performed with Eppendorf centrifuges 5403 (10,000 rpm) or 5415C (14,000 rpm). Dialysis was performed using spectra/por membrane tubing (Spectrum Laboratories, Inc.) with a 6,000-8,000 Da molecular weight cutoff.

3.2 Instrumentation

UV-visible absorbance measurements (MTT assay) were performed using a Benchmark microplate reader (Bio-Rad, Mississauga, ON, Canada). Fluorescence measurements (Cd assay) were performed with a FLUO-Star Optima plate reader (BMG Labtech, Durham, NC, USA). For solution studies a Cary Eclipse Fluorescence spectrometer was used for recording steady state emission spectra. Time-resolved singlet oxygen detection was performed with a system consisting of a Nd-YAG laser pumped OPO (BMI) (excitation) and a 5 x 5 mm² Ge-Pin diode with cooled preamplifier (Northcoast) (detector) described elsewhere in detail [26].

3.3 Preparation of QDs

CdSe/ZnS core/shell nanoparticles were prepared by the established method using high-temperature pyrolysis of organometallic precursors in trioctylphosphine oxide (TOPO) [27]. Cysteamine coated CdSe/ZnS QDs (+) were obtained by ligand exchange following the procedure reported by Hoshino et al [28]. MPA-coated CdTe QDs were prepared as described previously [29]. NAC-conjugated CdTe QDs were obtained by treatment of a solution of CdTe (+) QDs (2 mg) in water (2 mL, pH 5.5) first with NAC (4 mg, 24.5 μmol) and, second, with EDC (12 mg, 77.3 μmol). The mixture was stirred at room temperature for 3 hr under N_2 and dialysed against water for 4 hr. The dialysate was lyophilized and the solid was dispersed in deionized water (2 mg/mL). In all cases the QDs were green-emitting nanoparticles ($\lambda_{em} \sim 530$ nm).

3.4 Cell culture conditions and treatments

Human breast cancer cells (MCF-7, American Type Culture Collection (ATCC), Rockville, MD, USA) were maintained as per ATCC recommendations. Cells were cultured (37°C, 5% CO_2) in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Burlington, ON, Canada). RPMI 1640 medium was phenol-red free and contained 1% penicillin-streptomycin. For MTT assays, cells were cultured in 24-well plates (Sarstedt, Montreal, QC, Canada) at a density of 10^5cells/cm^2. For Cd^{2+} quantitation, cells were seeded at a density of 2 x 10^5 cells/well. One hour prior to cell treatments, medium containing serum was aspirated, and cells were washed with serum free medium. Fresh serum free medium was added to all wells except to control cells grown in 10% FBS to account for changes in cell morphology and metabolic activity due to the serum withdrawal. All treatments were done in triplicates in three independent experiments. Cells were incubated with QDs up to 24 hr before biochemical analysis or real-time live cell imaging.

MTT assay. MCF-7 cells cultured as described above were treated with either a solution of $CdCl_2$ (final concentrations ranging from 10 nM to 2 μM) or a solution of QDs such that the final QD concentration in each well was 10 μg/mL. The treated cells were incubated for 24 hr. After this time, the medium was aspirated. The cells were rinsed with serum-free medium. Fresh serum-free medium was added to each well as well as a MTT stock solution (50 μL, 5 mg/mL). Cells were incubated for one hour at 37°C. The medium was removed and cells were lysed with DMSO. The absorbance at 595 nm was measured. All measurements were done in triplicates and at least two or three independent experiments were carried out

Cellular cadmium assay. MCF-7 cells cultured as described above were treated in the exact same manner as for cytotoxicity assay with QDs or a $CdCl_2$ solution. After the 24 hr incubation with $CdCl_2$ or QDs, the cell supernatant was collected in 1.5 mL Eppendorf tubes and centrifuged for 5 min (5,000 rpm). To determine the extracellular Cd^{2+} concentration, an aliquot of the supernatant (10 μL) was added to a well of a 96-well plate, followed by an aliquot (200 μL) of the

Measure-iT kit was added to each well. Each treatment was carried out in triplicates and three readings were done for each sample. The fluorescence intensity at 520 nm (λ_{ex}: 490 nm) was recorded for each well. A [Cd^{2+}] calibration curve, drawn following the assay instructions, was used to determine the Cd^{2+} concentration of the extracellular fluid. To determine the intracellular Cd^{2+} concentration, the residual supernatant was aspirated from each well. DMSO (500 μL) was added to each well and then transferred to the respective Eppendorf tubes in order to lyse both the cells in the wells and those pelleted upon centrifugation of the supernatant. Each sample was vortexed. An aliquot (10 μL) of each solution was transferred in a well of a 96-well plate, followed by an aliquot (200 μL) of the Measure-iT kit solution. The fluorescence intensity at 520 nm (λ_{ex}: 490 nm) was recorded for each well. The intracellular Cd^{2+} concentration was obtained using the Cd^{2+} calibration curve. All measurements were done in triplicates and at least two to three independent experiments were carried out.

For measurements of free Cd^{2+} concentration in solutions of QDs, the samples were diluted to a concentration of 10 μg/mL. Aliquots (5 or 10 μL) of the QD solutions were transferred in a well of a 96-well plate, followed by an aliquot (200 μL) of the Measure-iT kit solution and assayed as described above.

3.5 Confocal laser scanning microscopy

Confocal laser scanning microscopy was carried out using a Zeiss LSM 510 microscope equipped with the following lasers: (i) HeNe LASOS LGK 7786 P/Power supply 7460 A: 543 nm, 1 mW, (ii) Argon LASOS LGK 7812 ML-1/LGN: 458, 488, 514 nm, 25 mW, Laser class 3D and (iii) Titanium:Sapphire The Coherent Mira Model 900-F Laser tunable from 710 to 1000 nm for two photon Microscopy set to pulse at 800 nm. Cells for imaging were grown on 8-well chambers (Lab-Tek, Nalge Nunc International, Rochester, NY, USA). QDs were added to designated wells and the cells were incubated for 24 h. Lysosomes were stained with LysoTracker Red DND 99 (0.5 μM, 1 min, λ_{ex} 577 nm, λ_{em} 590 nm). Mitochondria were stained with MitoTracker Deep Red 633 (1 μM, 1 min; λ_{ex} 644 nm, λ_{em} 665 nm) and nuclei with Hoechst 33342 (10 μM, 1h; λ_{ex} 350 nm,

λ_{em} 461 nm). Before imaging cells were washed with PBS or with serum-free medium. No background fluorescence of cells was detected under the settings used. Figures were created using Adobe Photoshop.

4. Results and Discussion.

4.1 In-vitro cytotoxicity of QDs

Based on our previous experience with QDs and on reports from other groups [7], we selected a set of QDs expected to be extremely different in terms of their cytotoxicity and assessed the effect of these QD samples on cellular metabolic activity using the MTT assay. The decrease in metabolic activity of human breast cancer MCF-7 cells treated with QD solutions (10 μg/mL) determined after a 1 hr exposure is presented in Figure 1, together with data recorded with untreated cells used as control and cells subjected to known amounts of Cd^{2+}. Exposure of the cells to Cys-capped CdSe/ZnS QDs led to a very small reduction in metabolic activity. In contrast, MPA- and Cys-capped CdTe QDs caused a substantial drop in metabolic activity. The cytotoxic effect of CdTe nanoparticles protected by a NAC layer was slightly less pronounced. A drastic reduction in metabolic activity occurred also when cells were exposed to free Cd^{2+}. This first set of measurements demonstrates unambiguously, and in accordance to reports from other groups, that CdTe QDs capped with small ligands are toxic to cells, whereas the core/shell CdSe/ZnS QDs are rather inert in the cellular milieu [30-32], causing only marginal reduction in mitochondrial metabolic activity, even when coated only with a short hydrophilic ligand. Cell counting after a 24 hr QD treatment, using the tripan blue exclusion assay, confirmed significant cell death with CdTe QDs, but not with CdSe/ZnS QDs.

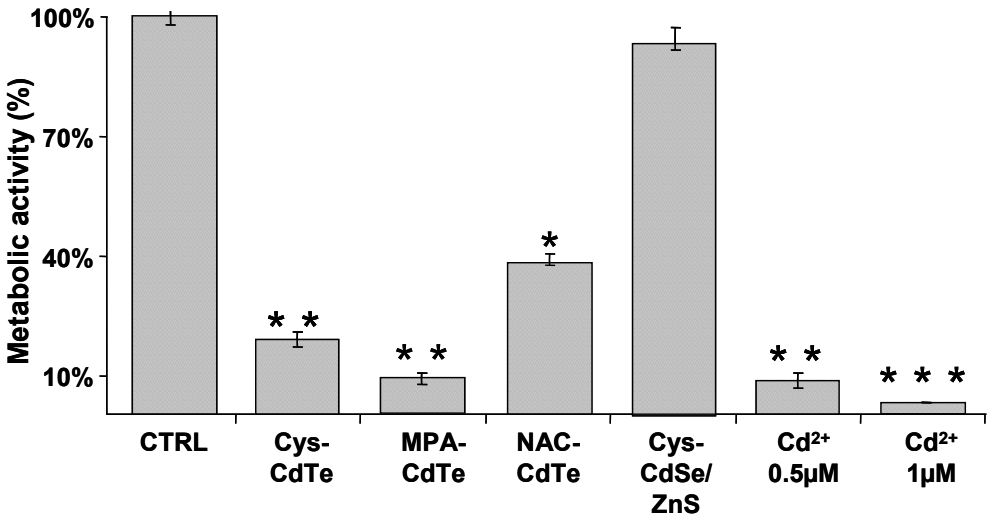

Figure 1. Decrease in metabolic activity of MCF-7cells treated with various QD samples (10 μg/mL), with $CdCl_2$ aqueous solutions and control as measured by the MTT assay.

4.2 Uptake of QDs by MCF-7 cells

We carried out a fluorescence imaging study of live MCF-7 cells incubated for 24 hr with the highly toxic Cys- and MPA-capped CdTe nanoparticles of concentrations similar to those used in the cytotoxicity assay, in order to determine their localization in the cells and to assess the morphological changes of the cells as a result of the treatment. Figure 2 presents fluorescence images of cells incubated with green Cys-capped CdTe nanoparticles and stained with Hoechst 33342 for visualization of the nuclei. Staining the nuclei with Hoechst 33342 reveals shrinkage and deformation of the nuclei with chromatin aggregation (Figure 2A, 2C), indicating significant nuclear damage induced by QDs. The QDs are located predominantly in the vicinity of the nucleus, as illustrated by the overlay (Figure 2C) of the image with nuclear staining (Figure 2A) and the image with QD luminescence (Figure 2B) in the same field of cells. We noted that in the harsh lysosome environment, in particular the acidic pH and the presence of lysosomal enzymes, the green emission emanating from CdTe QDs in the cells, although detectable, was weak. In contrast, the CdSe/ZnS QDs were readily detected in cells Control experiments confirmed that the emission

intensity of CdTe QDs in solution also decreased, by nearly 30 %, upon lowering the solution pH from neutral to ~ 4.5 as observed by Kirchner et al [14].

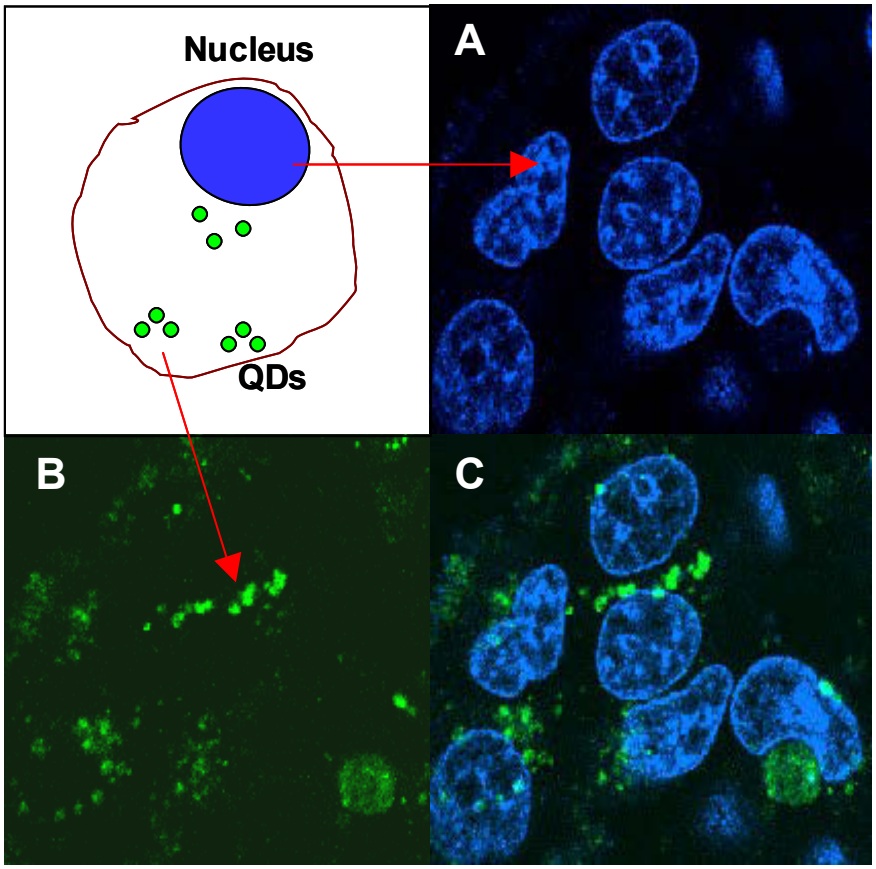

Figure 2. Confocal micrographs of MCF-7 cells treated with green Cys-CdTe QDs (10 μg/mL): (A) schematic representation of the cellular compartments observed; (B) visualization of nuclei stained with Hoechst 33342; (C) visualization of green QDs in the intracellular compartment with highest concentration in the lysosomes; (D) overlay of the two micrographs; a minimum of 50 cells per condition were analyzed from three independent experiments

To gain insight in QD-inflicted cellular morphological changes, we used two organelle-specific dyes, LysoTracker Red DND 99 and MitoTracker Deep Red 633, to visualize the lysosomes and mitochondria, respectively. Striking morphological cellular changes took place within a 24-hr incubation of MCF-7 cells with Cys-CdTe QDs, as seen in Figure 3 where we present fluorescence

images of QD-treated cells (panels C and F) and of control cells stained under the same conditions, but in the absence of QDs (panels B and E). Cells treated with QDs exhibit significant mitochondrial swelling and rounding up, as revealed by MitoTracker Deep Red staining (Figure 3C). In the QD-treated cells (Figure 3C), the mitochondria are seen mostly in the perinuclear area, whereas the mitochondria of cells in the control experiment have a filamentous structure and are more uniformly distributed throughout the cytoplasm (Figure 3B). Lysosomes of QD-treated cells became enlarged or fused and adopted a more perinuclear localization, a response seen in different stressed cells (Figure 3F) [33]. Normal cells grown in the absence of QD show punctuate, uniformly distributed lysosomes (Figure 3E).

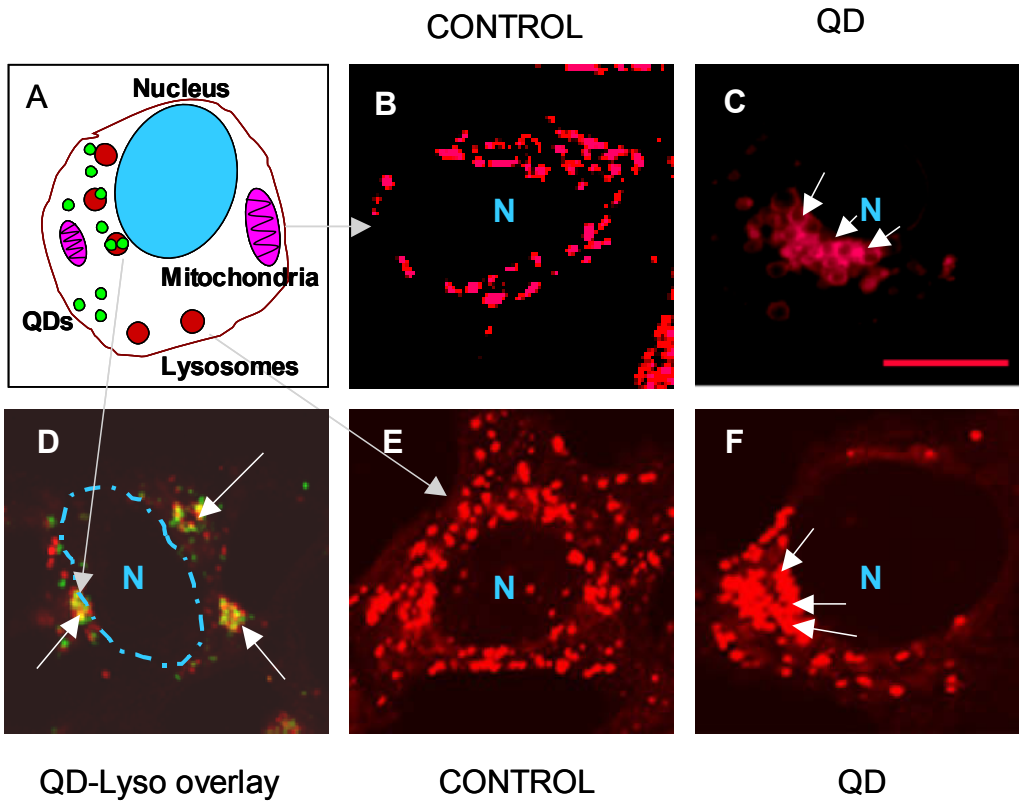

Figure 3. Confocal micrographs of MCF-7 cells: (A) schematic representation of the cellular compartments observed. Cells in B and C were stained with MitoTracker Deep (1 μM) : (B) control (untreated) cells and (C) cells treated with MPA-CdTe QDs (10 μg/mL). Cells in E and D were stained with

LysoTracker DND 99 (0.5 μM) : (E) control cells and (F) cells treated with MPA-CdTe QDs (10 μg/mL) ; (D) Overlay of E and F : commonly observed agglomerates in cells treated with MPA-CdTe QDs (10 mg/mL) show colocalization (yellow) of QDs (green) and LysoTracker Red DND 99. The arrows in (C) and (F) point towards perinuclear regions. Note the rounded and damaged mitochondria in (C) and the enlarged (sometimes fused) lysosomes in (F) as a consequence of intracellular damage inflicted by the CdTe QDs ; N : nucleus.

Panel D in Figure 3 is an overlay of panels E and F. It shows co-localization (yellow) of green CdTe QDs with red LysoTracker Red DND 99 inside the lysosomes, demonstrating that, indeed, QDs reside preferentially in the lysosomes, as seen in Figure 2B. However, it was difficult to demonstrate localization of green QDs in LysoTracker-stained lysosomes in a statistically significant sampling of imaged cells. In fact, in most cases it seemed that the red LysoTracker Red DND emission was stronger in the case of QD-treated cells, compared to normal cells. The absence, or weakness, of QD emission may indicate a gradual degradation of the QDs capping layer as a result of ligand desorption upon prolonged exposure to the cell environment. It could also be due to the occurrence of fluorescence radiative energy transfer (FRET) between excited QDs and LysoTracker Red DND 99 co-localized in high concentration and close proximity within lysosomes. LysoTracker Red DND 99 can act as energy acceptor via FRET from excited green QDs, although the efficiency of FRET is weak for the unconjugated dye/QD pair.

Several studies provide evidence for the susceptibility of lysosomes to photooxidative stress and to insults by oxidative agents [32]. Metallic pollutants, such as cadmium ions, also induce lysosomal enlargement as well as rupture of the lysosomal membrane in mammalian cells [33]. The cytotoxic effects of lysosomal membrane permeability often relay on the activation of the mitochondrial pathway, as it appears to be the case of QD-exposed cells which exhibit significant mitochondria morphological changes. Release of lysosomal

hydrolases, formation of ROS in lysosomes and cytosolic acidification can ultimately lead to classical apoptosis, caspase-independent apoptosis or necrosis-like programmed cell death, depending on the cell type, as a consequence of lysosomal membrane permeabilization [34, 35]. Lysosomes as dynamic organelles participate in cell death, but can also contribute to membrane restoration in response to membrane damage by exocytosing their contents into the extracellular space [34].

4.3 Determination of the concentration of Cd^{2+} in MCF-7 cells treated with various QD samples.

The evidence gathered so far points to the involvement of ROS in the mechanism of QD-induced cell death, but such species could originate from photooxidative pathways ensuing excitation of the QDs as well as from the oxidative stress caused by Cd^{2+} ions. To inflict this damage to cells, free cadmium ions need to be present in sufficient concentration within the intracellular compartment. The Cd^{2+} concentrations in the extracellular fluid and in the intracellular compartment of MCF-7 cells incubated with each of the four QD samples described above were determined by a quantitative fluorometric cellular assay for Cd^{2+}. First, we used the assay to measure the concentration of free Cd^{2+} in QD solutions (10 μg/mL) prior to cell incubation. The three CdTe QD solutions proved to contain free Cd^{2+} (Table 1). In contrast, the $[Cd^{2+}]$ value recorded for solutions of CdSe/ZnS core/shell QDs was at or below the assay detection limit (< 5 nM).

Next, MCF-7 cells were incubated for 24 hr with aliquots of each QD sample under serum-free conditions. Following this treatment, the medium was collected from the cells and used to assess the Cd^{2+} concentration in the extracellular milieu. The cells were then collected, lysed and assayed for Cd^{2+}, yielding the corresponding intracellular Cd^{2+} concentrations. These concentrations are presented in Figure 4, together with values for intra- and extracellular $[Cd^{2+}]$ values recorded in MCF-7 cells incubated with different concentrations of $CdCl_2$ solutions. Note that Cd^{2+} ions contaminate the intracellular compartment of MCF-7 cells treated with the three CdTe QD samples and that the $[Cd^{2+}]_{intracellular}$

values lie within the range of concentrations known to inflict substantial cell death. The $[Cd^{2+}]_{extracellular}$ values exceed the intracellular $[Cd^{2+}]$ by a factor of ~ 5 - 6 in each case. Both intra- and extracellular Cd^{2+} concentrations are very low for MCF-7 cells treated with CdSe/ZnS nanoparticles. It seems that the ZnS shell is highly effective in preventing surface corrosion and release of free Cd^{2+} by CdSe/ZnS nanoparticles ingested by cells.

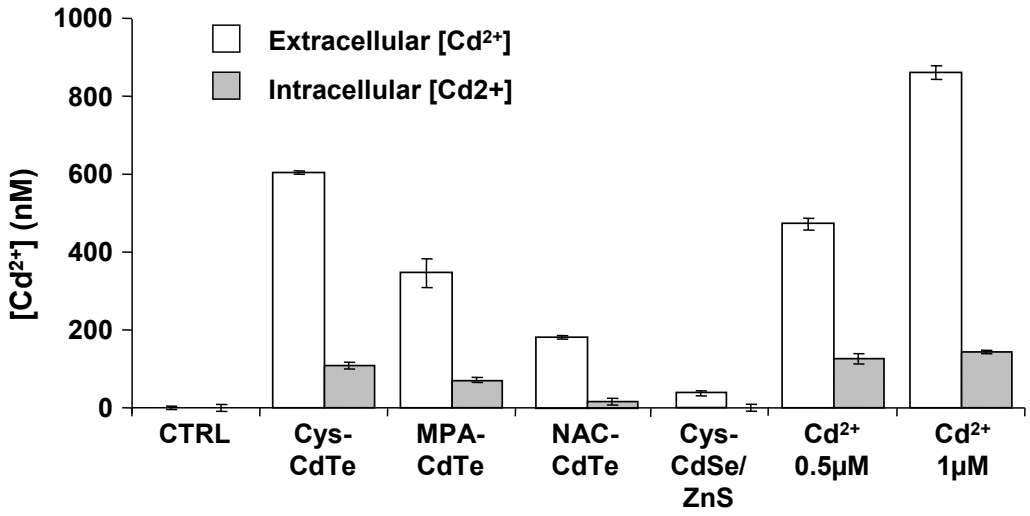

Figure 4. Intra- and extracellular Cd^{2+} concentrations in MCF-7 cells incubated for 24-hr with various QD samples and with $CdCl_2$ solutions.

At this point, it is informative to relate the results of the MTT assay for cell viability and mitochondrial activity (Figure 1) to the corresponding intracellular Cd^{2+} concentrations. For cells incubated with soluble Cd^{2+}, there is a good correlation ($r = 0.868$) between cell viability and $[Cd^{2+}]_{intracellular}$: cell viability decreases linearly with increasing $[Cd^{2+}]_{intracellular}$ (Figure 5, diamonds), in accordance with studies on the toxic effect of Cd^{2+}. Turning now to data recorded for cells treated with QDs, we note that the $[Cd^{2+}]_{intracellular}$ values recorded in cells treated with CdTe QDs are significantly lower than the concentration of soluble Cd^{2+} of identical cytotoxic effect (Figure 6, circles). Also, MPA-capped CdTe QDs are significantly more toxic than Cys-capped CdTe QDs, yet the

$[Cd^{2+}]_{intracellular}$ is significantly lower in the case of Cys-capped CdTe QDs, compared to their MPA-capped counterparts.

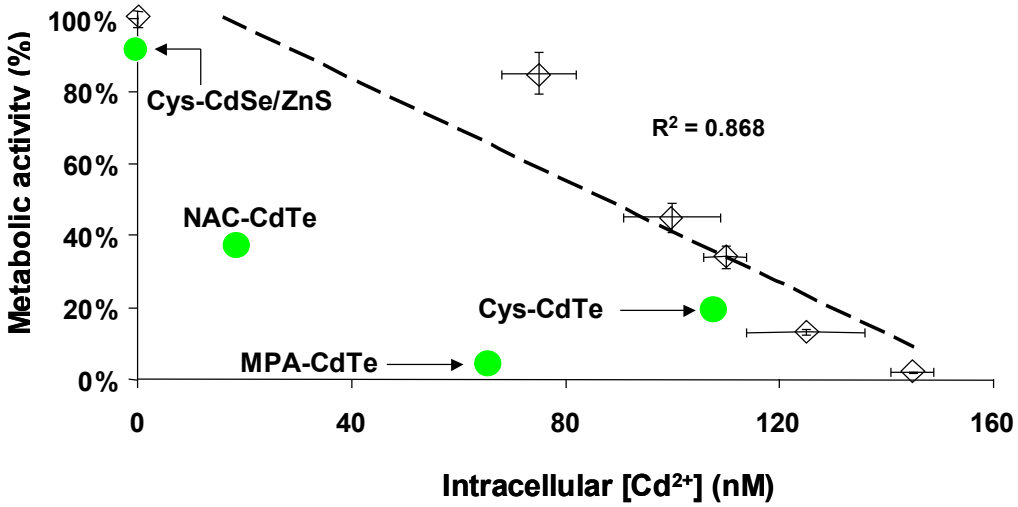

Figure 5. Plot of the changes in MCF7 cell viability as a function of intracellular Cd^{2+} concentration for cells subjected cells with three QD samples (open circles) to Cd^{2+} Also shown are the cell viability/intracellular Cd^{2+} data recorded upon treatment of MCF7 with aqueous $CdCl_2$ solutions (full diamond).

4.4 Photooxidative pathways leading to ROS generation from CdTe QDS.

To reconcile cytotoxicity and $[Cd^{2+}]$ data, one needs to envisage that CdTe QDs are intrinsically cytotoxic via pathways that are not linked to free cadmium ions in a dose-dependent fashion, as is the case for cell death via exposure to Cd^{2+} salt solutions. Recalling that the most prominent morphological changes induced in cells by CdTe QDs, namely lysosomal enlargement and mitochondria rounding, (Figures 2 and 3) found in cells under oxidative stress, we suggest that CdTe QDs contribute to the generation of ROS via mechanisms that are inherent to their size and composition and outlined in Figure 6. Previous photophysical studies have shown that QDs can generate $^1O_2$ in solution, and we were able to detect $^1O_2$ upon irradiation of solutions of MPA-capped CdTe QDs (Figure 7), although the quantum yield was low (< 5%). Whether this photooxidation pathway can occur

also *in-vitro* remains at this time a speculation. We were not able to detect the formation of $^1O_2$ in photoirradiated solutions of CdSe/ZnS core/shell nanoparticles nor of NAC-conjugated CdTe QDs. Also, the electron-hole pairs formed upon excitation of QDs could undergo electron transfer to oxygen, leading to a cationic QD and O_2^-. Strongly polar media, such a water, cause a broadening of the O_2 LUMOs [36], thereby facilitating electron transfer. The resulting highly reactive O_2^- can trigger the cascade of intracellular events in response to oxidative stress, while the QD cation may induce ligand oxidation and cleavage [12], exposing bare QD surface to the environment. The occurrence of electron transfer from QDs conjugated to known electron acceptors has been detected *in-vitro*[37], a similar process may occur between QDs and oxygen in the aerobic cell environment.

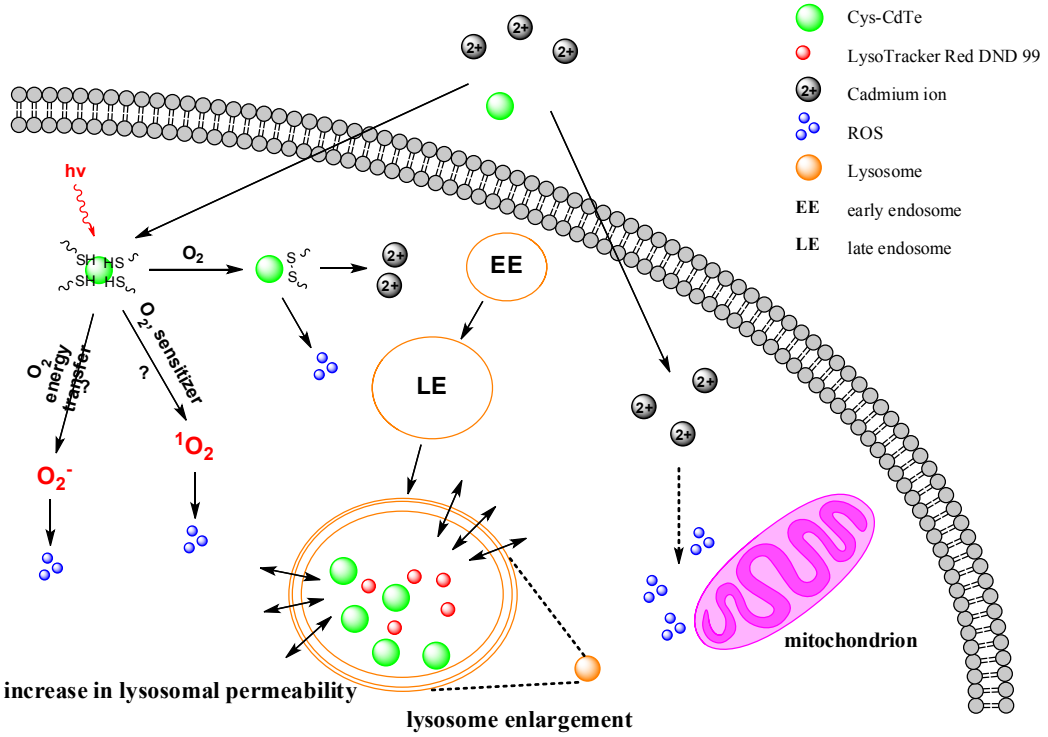

Figure 6. Schematic representation of the mechanistic pathways implicated in the cytotoxicity of CdTe QDs in live cells, highlighting the salient changes in cellular morphology, the chemical species involved, and the chemical reactions that can lead to ROS and free Cd^{2+} ion release.

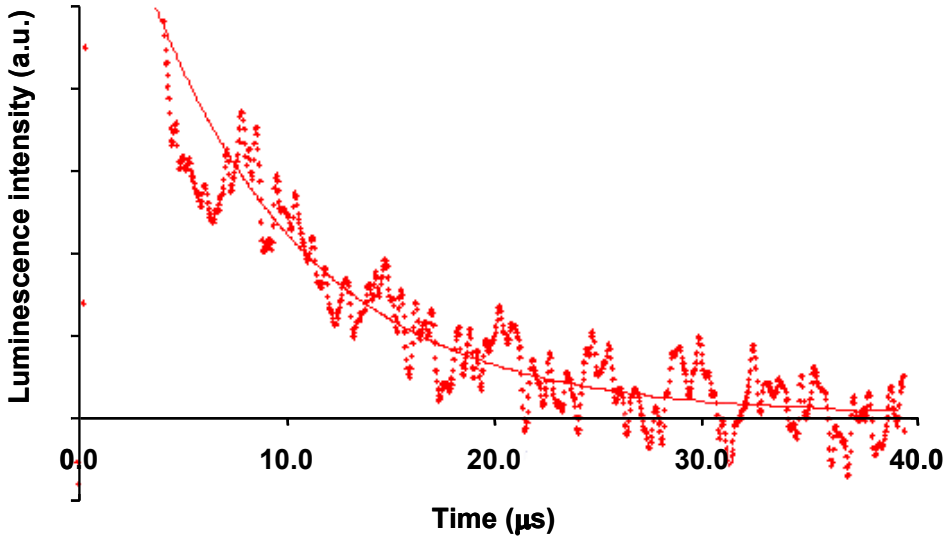

Figure 7. Time-resolved singlet oxygen emission of MPA-CdTe QDs in water

5. Conclusion and Outlook

We have compared the long-term cytotoxicity towards the MCF-7 cell line of four QD samples differing in terms of chemical composition, charge, and surface modification and confirmed that while CdTe QDs capped with small organic ligands are cytotoxic, core shell CdSe/ZnS QDs present little damaging effects to cells, at least under the conditions of our studies. Our results on the low chronic cytotoxicity of core/shell CdSe/ZnS QDs are consistent with conclusions from other studies [38]. We established that the toxicity of CdTe QDs was, in part, a consequence of the contamination of the intracellular compartment of cells by free Cd^{2+} ions, either present in the QD solution prior to incubation with cells or released *in-vitro* by the nanoparticles. We observed that while the percent decrease in cellular metabolic activity correlates linearly with intracellular Cd^{2+} concentration for cells treated with solutions of $CdCl_2$, this correlation does not hold in the case of cells treated with QDs. This observation, together with an inspection of CdTe QD-inflicted cell damages by CSLM in the presence of organelle-specific dyes, suggests that the mechanism of QD toxicity triggers the typical response of cells subjected to oxidative stress, generated both by free Cd^{2+} and by photooxydation processes specific to semiconductor nanoparticles in a polar aerobic environment.

6. Acknowledgments

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Connecting text

In our preliminary studies we show that there is no correlation between the cadmium released from CdTe QDs and the extent of corresponding QD-induced cytotoxicity. As such, QD-toxicity cannot be ascribed solely to cadmium liberation from the QD core. Imaging studies examining lysosomal morphology post QD-treatment, both show co-localization of QDs and the lysosome and show morphological impairments indicative of oxidative stress. This study, however, raises several questions, as to whether QD-toxicity is the result of QD-induced oxidative stress or whether QD-toxicity results from the combination of cadmium plus tellurium ions. We also wanted to investigate the role of different model systems (from simple cell lines, to heterogeneous primary cultures and three dimensional tissue from the peripheral nervous system). The objective was to determine whether QD-induced cytotoxic effects observed in a cell line can be translated into similar changes in a more complex system. The studies clearly show that this is not the case. Thus, cytotoxicity of nanomaterials must be examined in different biological systems in order to set the limits for concentrations and durations of exposure to nanomaterials. Data from studies employing increasingly complex biological systems are required to compare the findings from different laboratories and to standardize the conditions under which nanotoxicological investigations should be conducted.

Chapter 2

Probing and preventing QD-induced cytotoxicity with multi-modal lipoic acid in multiple dimensions of the peripheral nervous system

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1. Abstract

Aim: Toxicity of nanoparticles developed for biomedical applications is extensively debated as no uniform guidelines are available for studying nanomaterial safety; resulting in conflicting data obtained from different cell types. This study demonstrates the varied toxicity of a selected type of nanoparticle, cadmium telluride quantum dots (QDs), in three increasingly complex cell models of the peripheral nervous system (PNS). **Materials & methods:** QD-induced cytotoxicity was assessed via cell viability assays and biomarkers of subcellular damage in PC12 cells and mixed primary dispersed dorsal root ganglia (DRG) cultures. Morphological analysis of neurite outgrowth was used to determine the viability of axotomized DRG explant cultures. **Results & discussion:** CdTe QDs and their core metals exert different degrees of toxicity in the three cell models, the primary dispersed DRGs being the most susceptible. Lipoic acid is an effective, multi-modal, cytoprotective agent that can act as an antioxidant, metal chelator, and QD-surface modifier in these cell systems. **Conclusion:** Complex multi-cellular model systems, along with homogenous cell models, should be utilized in standard screening and monitoring procedures for evaluating nanomaterial safety.

2. Introduction

The toxicity of nanoparticles, including carbon nanotubes, fullerenes, micelles and quantum dots has been tested in a wide variety of stable cell lines [1, 2]. The concern of toxicity is mounting as increasing numbers of nanomaterials are being used in consumer products including sunscreens and cosmetics [3]. However, discrepancies in nanoparticle-induced cytotoxicity have been noted not only due to the nanoparticle composition and concentration, but due to the wide variety of cell lines and cell culturing conditions (including cell density, media composition) utilized, as well as the duration of nanoparticle exposure and differences in nanoparticle concentrations. There is currently no consensus on the necessary criteria for the standardization of nanoparticle toxicity. This deficit of appropriate

nanoparticle toxicity screening processes has limited the development of efficient measures to prevent nanoparticle- induced toxicity.

The majority of nanoparticle toxicity screening has been conducted in stable cell lines, and thus far only a few studies have examined nanoparticle insult in primary cultures and *in vivo* [4-6]. In this study we investigated the cytotoxicity of a specific type of nanoparticle in three cell models, all representative of the peripheral nervous system: a neuronal-like stable cell line derived from rat cells of the adrenal gland (PC12 cells); dispersed dorsal root ganglia (DRG) primary cultures, comprised of both neurons and Schwann cells; and lastly, three dimensional, multilayer and multi-cell, DRG explant cultures.

Cell lines, such as PC12 cells are suitable for initial toxicological screening purposes because they are well characterized and the signal transduction pathways for neuronal differentiation in response to different toxic insults and nerve growth factors have been previously described. On the other hand, although tumor-derived and immortalized cell lines are frequently used in biological studies, they often do not properly represent the biological or physiological systems as they have likely adapted (i.e. via mutations) to their artificial culture environment. Hence, experimental data obtained from such cell systems should be confirmed or studies should be extended to primary cultures, and ultimately, to whole animals. Primary neural cultures represent suitable model systems to explore nanoparticle effects on more than one cell type. Dorsal root ganglia (DRG) dispersed cultures consist of both neurons and Schwann cells, in ratios that are physiologically representative [7]. Sensory DRG neurons connect to both skin and muscles, consequently both are potential target tissues for nanoparticles either from the contaminated environment or deliberate nanoparticle administration. 3D cell systems, such as DRG explants, present a unique tool to study both toxicity as well as functional impairments that may be induced by nanoparticles, prior to *in vivo* studies, where detecting minor functional changes can be extremely difficult. In DRG explants, not only can the toxicological potential of nanoparticles be evaluated by biochemical assays, but functional characteristics such as nanoparticle uptake, axonal transport and neurite outgrowth can also be examined.

While primary neural cultures are a versatile model for screening nanoparticle toxicity, they have not been extensively used in nanomedicine, mainly because they are more complex to work with than cell lines and they still lack the connectivity and actual physical structure of tissue and multicellular systems. DRG explants represent three-dimensional (3D), multilayered cell systems [8, 9]. To date there have been no studies examining nanoparticle toxicity in such systems.

Multiple screening systems with increasing complexity could be particularly useful for resolving or avoiding controversy regarding potential toxicity of quantum dots (QDs) in the PNS. QDs are novel semi-conducting fluorophores with diverse surface chemistry that renders them highly lucrative as both a tool in basic scientific research as well as a potential diagnostic and therapeutic agent [10, 11]. The QD-core, as we have investigated in this study, consists of heavy metals such as cadmium, tellurium and selenium, which are considered responsible for the toxic potential of QDs. Although the mechanisms of QD-toxicity remain to be elucidated, in the past few years, studies have described the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), upregulation of Fas death receptor, induction of epigenetic changes, and activation of p53-depedent mechanisms to underlie QD-toxicity [12-18]. Antioxidant agent, N-acetylcysteine (NAC) was effective in preventing QD-induced cytotoxicity [14, 16] corroborating the findings that ROS are involved in QD-induced toxicity. QD-surface modifications were previously done with dihydrolipoic acid (DHLA), the reduced form of the antioxidant, lipoic acid (LA) [19]. DHLA-QDs have improved solubility in aqueous environments; however, their toxicity was not investigated in primary or other cultures.

α-Lipoic acid (LA) is a naturally occurring, amphipathic compound present in many prokaryotic and eukaryotic cells. LA can readily cross the plasma membrane and blood-brain barrier, and is a metabolic antioxidant, likely due to its sulfhydryl group. LA has a low redox potential of –0.29 V [20, 21], suggesting weak function as an antioxidant in this state. However, intracellular LA is rapidly reduced to dihydrolipoic acid (DHLA), and this reduced form is a strong

antioxidant, and acts to induce and regenerate endogenous antioxidants such as vitamin C, vitamin E, and glutathione (GSH) [20, 21].

Due to the antioxidant potential of LA and the oxidative stress induced by "naked" CdTe QDs, as well as the QD-core components, we investigated the ability of LA to prevent QD, cadmium and tellurium-induced cytotoxicity, in three increasingly complex cell systems representative of the PNS. Examining the cytotoxicity of nanoparticles, in monolayer cell cultures, three dimensional cell systems, and *in vivo*, together will provide complementary information needed for establishing the criteria upon which standardized screening and selection of safe nanomaterials can be based.

3. Material and Methods

3.1 Preparation of CdTe quantum dots

Cysteamine-capped CdTe QDs were synthesized as per the method described by Gaponik [22], and modifications to the synthesis procedure and measurement of QD photoluminescence were as described in our previous studies [14-16]. These green cationic CdTe QDs ranged between 2-3nm in diameter, with an emission maxima of 525-530 nm. Further the zeta potential of these QDs was between +14.2 and +15 mV, and their quantum yield was 0.01. CdSe/ZnS QDs capped with DHLA were also prepared according to the method described by Gaponik's group.

3.2 Cell cultures and treatments

Rat pheochromocytoma cells (PC12), (ATCC, USA) were cultured in RPMI 1640 media containing 5% FBS. Cells were maintained at 37˚C (5% CO_2) in a humidified atmosphere, and all media contained 1% penicillin-streptomycin and were free of phenol-red. For spectrofluorometric and colorimetric assays, cells between passages 20-30, were seeded in 24-well plates (Sarstedt, Montreal, QC, Canada) at a density of 10^5 cells/cm^2.

Dispersed primary neural cultures were established from dorsal root ganglia (DRG) isolated from postnatal day 5 mice and placed in Hank's buffered saline solution Gibco) (HBSS supplemented with Hepes buffer and antibiotics) and kept on ice. Upon centrifugation, the pellet of DRGs was rinsed with HBSS followed by Ham's F-12 media. DRGs were then incubated at room temperature for one hour with 1% collagenase in Ham's F-12 media. After completion of collagenase incubation, the DRGs were first rinsed with Ham's F-12 followed by HBSS and then incubated with 3ml of 0.25% Trypsin/EDTA for 15 minutes at 37˚C. 1ml of high glucose DMEM supplemented with 10% FBS, antibiotics and Hepes buffer was then added to inhibit the trypsin. DRGs were again centrifuged and rinsed with HBSS, followed by DMEM. The cells were then pipetted up and down in DMEM to completely disperse the DRGs. 50µL of this cell suspension was then added to circular coverslips previously coated with poly-L-ornithine and laminin. Cells and coverslips were incubated for 1 hour at 37˚C, after which the coverslips were submerged in 450µL of DMEM in a 24-well plate. After 72 hours, the cells were rinsed with neurobasal A and then placed in fresh neurobasal A media, supplemented with glutamine, antibiotics and B27.

Explant cultures were established by isolating DRGs and placing them in pre-chilled phenol-free RPMI media containing 1% penicillin-streptomycin. A droplet of Matrigel (Becton Dickinson #40234) was placed on a sterilized glass coverslip. Individual DRG explants were embedded into polymerized Matrigel. An additional drop of Matrigel was added to the tissue and the coverslips were placed individually into wells of a 12-well plate (Corning) and incubated for 10 min at 37˚C to allow complete polymerization. Pre-warmed RPMI media was then added to each well to cover the embedded DRG tissues. Explant cultures were maintained at 37˚C, with 5% CO_2 until treatment.

3.3 Cell Treatment

PC12 cells were washed and switched to serum-free media (Ctrl) for 1 hr prior to QD, Cd^{2+}, or Te^{4+} treatment. For the duration of the treatment, PC12 cells were maintained as described for cell culturing (37˚C, 5% CO_2, humidified atmosphere,

1% penicillin-streptomycin). Treatments were done 24 hours after cells were seeded into 24-well plates. QDs were added to wells at concentrations of 5-10 μg/ml and incubated for the times indicated. In experiments involving lipoic acid (LA; Sigma), LA (200 μM) and QDs were added to wells and incubated at 37˚C for 24 hours. In DRG cultures, LA was added at a concentration of 750 μM, followed by treatments with QDs, Cd^{2+}, or Te^{4+} (metal chloride concentrations ranging from 0.05–50 μM as indicated). LA was also combined with QDs in a cell-free environment for 24 hours at 4˚C prior to cell treatment (LA-QD), so that LA may chelate the free Cd^{2+} ions in the QD solution. Treatments were allowed to incubate with the cultures for 72 hrs.

3.4 MTT assay and trypan blue exclusion assay

Colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) assays were performed to assess cell viability. After 24 h treatment (see treatment details above), media was removed and replaced with drug-free, serum-free media (500 μL/well). 50 μL of an MTT stock solution (12 μM) was added to each well and cells were then incubated for one hour at 37°C. Following the incubation, media were removed, cells were lysed and formazan was dissolved with dimethyl-sulfoxide (DMSO, Sigma). Absorbance was measured at 595 nm using a Benchmark microplate reader (Bio-Rad, Mississauga, ON, Canada). All measurements were done in triplicates in three or more independent experiments. Additional cell viability assays, including cell counting using the trypan blue exclusion assay as described previously [17], confirmed the results obtained from the MTT assays.

For imaging experiments of the explant cultures, DRGs from postnatal day 5 animals were pretreated with 750 μM of LA (750 μM), QDs (330 μM), cadmium chloride (2 μM) and tellurium chloride (5 μM) for 4 days. MTT solution (500 μg/ml) was added to cultures and incubated for 3 hours at 37˚C as described previously [23]. Phase contrast pictures of the DRGs were taken with a light microscope at 20X (Olympus BX51) and analyzed with the MCID Elit7 and ImageJ programs.

3.5 Intracellular glutathione determination assay

Following treatment, PC12 cells and DRG cultures were incubated with 50 μM monochlorobimane (mCBI, Calbiochem 475906) for 1 hour at 37˚C. Dye-containing media was then aspirated, cells were washed twice with PBS, and fresh media was given. Fluorescent images were acquired with a Leica DFC350FX monochrome digital camera connected to a Leica DMI4000B inverted fluorescence microscope using a DAPI-1160A filter at 40X. Images were acquired and pseudo-colored using the Leica Application Suite (LAS) software. Fluorescent intensity was measured with the FLUOROstar Optima spectrofluorometer (BGM, Labtech) with the filter set to 380/460 nm and employed 3x3 matrix well scanning.

3.6 Intracellular cadmium determination assay

PC12 cells and DRG dispersed cultures, as described above were treated in the exact same manner as for cytotoxicity assays with QDs or a $CdCl_2$ solution, either with or without LA. Post treatment, the cell supernatant was collected in 1.5 mL Eppendorf tubes and centrifuged for 5 minutes (5,000 rpm) after which the resulting supernatant was aspirated. To the original 24-well plate DMSO (500 μL) was added to each well and then transferred to the respective Eppendorf tubes in order to lyse both the cells in the wells and those pelleted upon centrifugation of the supernatant. Each sample was vortexed. An aliquot (10 μL) of each solution was transferred to a well of a black, 96-well plate, followed by 200 μL of the Measure-iT kit solution (Invitrogen). Each treatment was carried out in triplicates and three readings were done for each sample. The fluorescence intensity at 520 nm (λ_{ex}: 490 nm) was recorded for each well. A [Cd^{2+}] calibration curve, drawn following the assay instructions, was used to determine the Cd^{2+} concentration of our samples. Data was obtained from at least two to three independent experiments. Fluorescence obtained in control, untreated cells was subtracted from all other conditions, as it represented non-specific binding of other charged ions to the fluorogenic component of the kit.

3.6 Lipid Droplet Staining

For the imaging of lipid droplets in PC12 cells and dispersed DRG cultures, BODIPY® 493/503 (Invitrogen D3922; 38 μM for 10 min) and the Nile Red (Invitrogen N1142; 1.57 μM for 15 min) fluorescent dyes were used. Briefly, after treatment, cells were washed once with PBS, and then fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized using 0.1% Triton X-100 for 5 min at room temperature. Anti-β-tubulin (Chemicon, MAB1637; 1:100, overnight) antibody was used to distinguish neuronal cells from Schwann cells in the DRG cultures. The PKH26 dye (Molecular Probes, K1739; 2 μM for 10 min) was used to stain the cell membrane of PC12 cells. Cell nuclei were stained with fluorescent dye, Hoechst 33342 (Molecular Probes, H1399; 10 μM for 10 min). After staining, glass coverslips containing the cells were mounted onto microscope slides (Fisher Scientific) with glycerol-free aqueous mounting media (Vector, Vectashield H-1000). Images were viewed with the fluorescent microscope (Leica DMI4000B) at 40X and 63X. Pictures from at least six random fields were captured from each coverslip with the Leica DFC 350 FX camera, and a representative field was selected and presented in the final figure. Pictures were processed using the Leica Application Suite program.

3.7 Statistical analysis

Data were analyzed using SYSTAT 10 (SPSS, Chicago, IL, USA). Statistical significance was determined by Student's *t*-tests with Bonferroni correction. Differences were considered significant where $*p<0.05$, $**p<0.01$, $***p<0.001$.

4. Results

4.1 CdTe QDs and core metal ions, Cd^{2+} and Te^{4+} induce cytotoxicity in PC12 cells and dispersed DRG cultures at low micromolar concentrations.

Although cadmium telluride quantum dot-induced cytotoxicity has been extensively studied by our group, this is the first demonstration of significant

differences in cytotoxicity by the same QDs (equivalent concentrations and exposure times) in differently complex biological systems. Several QDs of either different core compositions and/or different surfaces were assayed in parallel in PC12 cells (Figure 1A). Only CdTe QDs were found to exert substantial toxicity (~62% cell death as compared to untreated control cells). We therefore, selected these QDs for further investigation to assess whether individual core constituting metals or their combination may induce cytotoxicity or functional impairments similar to the QDs alone. (We found that CdTe QDs can exert different extents of toxicity in monolayers versus 3D cultures Figures 1-3). Earlier studies also demonstrated that the release of cadmium ions is linked, but not proportional to QD-induced toxicity in human breast cancer cells [15]. We here tested QD-core metal ions, namely Cd^{2+} and Te^{4+}, in different concentrations to determine their contribution to the toxicity of QDs. Our findings show that high nanomolar concentrations of cadmium chloride (about 0.4 μM and greater, Figure 1B) and high micromolar concentrations of tellurium tetrachloride (about 20 μM and greater, Figure 1C) induce more than 50% cytotoxicity in PC12 cells. The LD_{50} of $CdCl_2$ in PC12 cells is approximately 750 nM, which is a higher concentration of Cd^{2+} ions than the amount found in toxic doses (≥ 10 μg/ml) of CdTe QDs (~300 nM Cd^{2+} ions). The LD_{50} of tellurium ions in PC12 cells is ambiguous as the toxicity induced by tellurium ions did not increase proportionally beyond 0.3 μM and a "plateau" was reached, in which cell viability did not decrease below 60% until treatment concentrations were 20 μM or higher (Figure 1C).

Dispersed DRG cultures are less susceptible to core metal ion-induced toxicity, as cells treated with $CdCl_2$ and $TeCl_4$ did not show significant cell death at high nanomolar concentrations, but did at low micromolar concentrations (Figures 1D, 1E). The LD_{50} of Cd^{2+} in DRG cultures was approximately 2 μM and Te^{4+} was approximately 25 μM. A similar trend in the Te^{4+}-induced toxicity was observed in DRG cultures, as a plateau was reached at 2 μM (Figure 1E). However, at high micromolar concentrations of $TeCl_4$, cell viability decreased below the plateau in dispersed primary cultures.

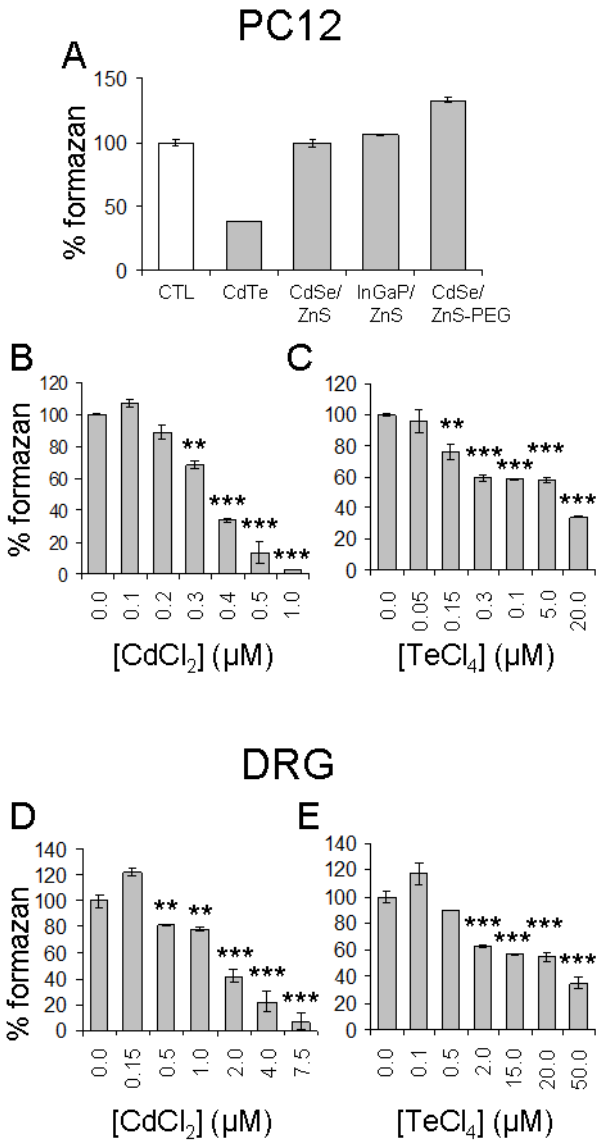

Figure 1. QD core metal ions, Cd^{2+} and Te^{4+} induce cytotoxicity in PC12 cells and dispersed DRG cultures at low micromolar concentrations. PC12 cells (A) were treated with 10 μg/ml of various QDs, with different core metal components and/or different surface modifications. After 24 hours of treatment, the MTT assay was performed and data expressed as a percent of formazan produced by cells relative to untreated cells (Ctl). Only CdTe QDs demonstrated significant cytotoxicity as compared to control. PC12 (B, C) and DRG cultures (D, E) were treated with increasing concentrations of cadmium chloride ($CdCl_2$) and tellurium chloride ($TeCl_4$) for 24 and 72 hours respectively. Again the MTT assay was utilized. Data points represent the means ± SEM from three independent

experiments. Statistically significant differences compared to untreated cells (Ctl) are indicated by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

QD core constituents, Cd^{2+} and Te^{4+}, induced toxicity at relatively high concentrations and our interest was to examine whether lipoic acid (LA) could act as a cytoprotective agent when cells were exposed to QDs or metal ions. The fact that antioxidants can reverse the damage induced by QDs affirms the role of reactive oxygen species in QD mediated toxicity [14] (supplementary material). If the cytotoxicity induced by CdTe QDs could exclusively be ascribed to core metal ion release, then like the combination of LA + QDs, the combination of Cd^{2+} and Te^{4+} ions plus LA should have been cytoprotective as well. PC12 cells treated with LA were protected from $CdCl_2$-induced toxicity (Figure 2A; 1 µM: 51.9 ± 4.0% compared with LA+Cd: 84.2 ± 0.7%, $p<0.001$). Conversely, LA did not protect against $TeCl_4$-induced toxicity even at low concentrations of the metal ion. Interestingly, combination of low concentrations of $CdCl_2$ and $TeCl_4$ (300 nM) did not elicit enhanced toxicity in PC12 cells, and LA pretreatment could no longer protect the cells against this combinatory metal ion insult (Cd+Te 0.3µM compared with LA+Cd+Te 0.3µM, $p<0.001$). In dispersed DRG cultures, LA treatment did not protect the cells from metal ion-induced cell death under any of the circumstances presented here. In fact, LA enhanced the toxicity of these metal ions significantly (4 µM $CdCl_2$: 22.0 ± 8.0% compared with LA+Cd: 8.0 ± 4.0%, $p<0.05$; 500 nM $TeCl_4$: 89.0 ± 0.4% compared with LA+Te: 22.0 ± 6.0%, $p<0.001$; Figure 2B). This discrepancy in the protection of LA in these two cell model systems suggests that i) the free metal ions may induce different cellular responses depending on the specific cellular environment, and ii) antioxidant drugs may elicit different protective cellular responses in cultures with homogenous cell types (PC12), versus mixed neuron-glia cultures (dispersed DRGs).

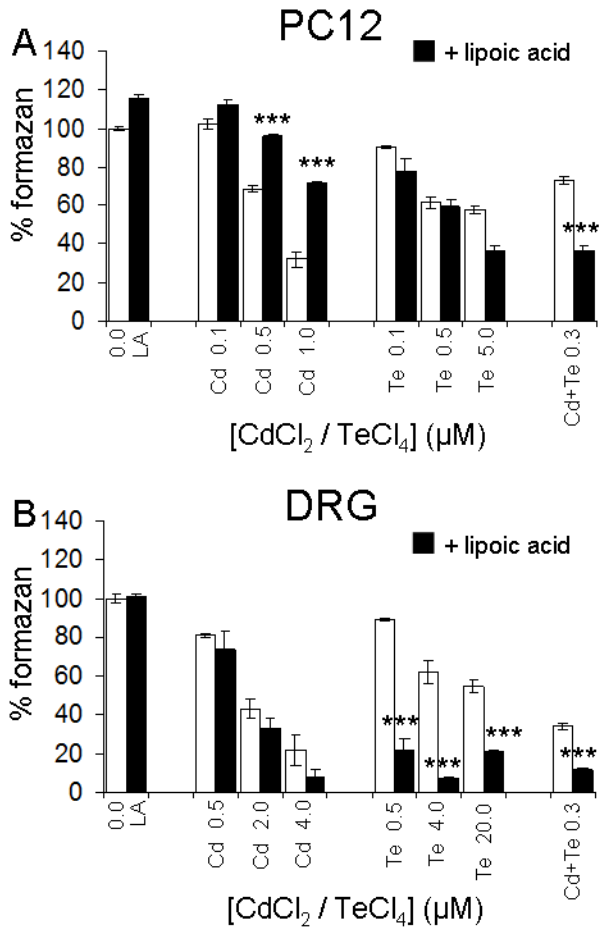

Figure 2. Lipoic acid protects PC12 cells from cytotoxic agents including the core metal components of QDs, but does not protect dispersed dorsal root ganglion neural cultures. PC12 cells (A) and dispersed DRG neural cultures (B) were treated for 24 hours with increasing concentrations of $CdCl_2$ and $TeCl_4$. Cultures pretreated with LA (200 μM for PC12 and 750 μM for DRG cultures) for 24 hours prior to treatment with $CdCl_2$ and $TeCl_4$ are indicated by the black bars. The condition denoted as Cd + Te 0.3 refers to an equimolar addition of both $CdCl_2$ and $TeCl_4$ (0.3μM of each). MTT and cell counting assays were used to assess the viability of the cells. Statistical significance was assessed intra-treatment based on the addition of LA to the treatments with metallic ions, indicating a significant change in viability due to co-treatment with LA; statistical significance is indicated by *** (p<0.001).

4.2 Lipoic acid exerts partial cytoprotection against QD-induced damage by upregulating intracellular glutathione

In order to discern the conflicting roles of LA as a cytoprotective agent in PC12 cells, and a cytodamaging agent in dispersed DRG cultures, we assessed the antioxidant potential of LA by measuring the levels of a potent and lipoic acid-responsive antioxidant, glutathione (GSH), in these culture systems respectively. We found that the intracellular glutathione level was significantly increased in PC12 cells pretreated with LA, even in the presence of the cyto-damaging QDs or Cd^{2+} and Te^{4+} metal ions (Figure 3B, $^{**}p<0.01$, $^{***}p<0.001$). This significant enhancement of intracellular glutathione by LA was not observed in dispersed DRG cultures (Figure 3C). However, endogenous GSH levels in untreated dispersed DRG cultures (MFI = 42415 a.u.) were more than double the basal levels in PC12 cells (MFI = 18448 a.u.), suggesting that LA, as an antioxidant, is perhaps only protective in cell systems with low basal GSH. \

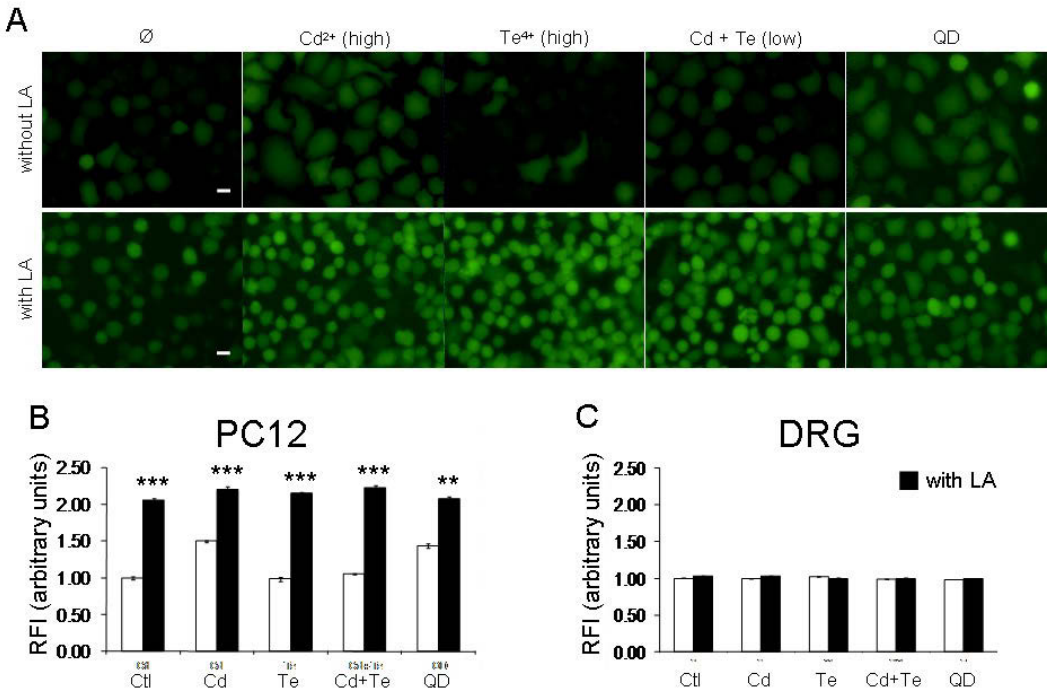

Figure 3. Lipoic acid exerts partial cytoprotection against QD-induced damage by upregulating intracellular glutathione. PC12 cells were treated for 24 hours with $CdCl_2$ (1 μM), $TeCl_4$ (1 μM), $CdCl_2$ + $TeCl_4$ (300 nM) or CdTe QDs (5 μg/ml) in the absence or presence of 24 hour lipoic acid pretreatment (LA, 200 μM) (A).

Intracellular glutathione (GSH) was labeled with monochlorobimane (mCBI, 50 μM). Phase contrast images are provided in control conditions for comparison of cell morphology and number. Representative pictures were selected from at least 3 independent experiments of triplicates (n = 9) with 40x objective (Leica DMI4000B microscope). Relative fluorescent intensity of the mCB-GS adduct in PC12 cells (B). DRG cultures (C) were treated for 24 hours with $CdCl_2$ (1 μM), $TeCl_4$ (5 μM), $CdCl_2$ + $TeCl_4$ (300 nM) or CdTe QDs (10 μg/ml) in the absence or presence of 24 hour lipoic acid pretreatment (LA, 200 μM). Relative fluorescent intensity of the mCB-GS adduct in DRG cultures. Relative fluorescence intensity is presented as the means ± SEM from three independent experiments of quintuplets (n = 15). Statistically significant differences were assessed intra-treatment, based on the difference measured due to the addition of LA to a specific metal or QD treatment; and are indicated by $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bars represent 10 μm.

4.3 Lipoic acid exerts cytoprotection when utilized as a QD-capping agent, metal-chelating agent or antioxidant agent

To better understand the impact of QD stability on QD-induced cytotoxicity, we used LA as a CdTe QD surface modifying agent (Figure 4). LA was tested as a protective agent in both dispersed DRG cultures and PC12 cells in three different ways: (i) as an inducer of protective endogenous antioxidant enzymes via pretreatment of the cells with the antioxidant, (ii) as a metal-chelator, and (iii) as a QD surface capping agent in its reduced form, dihydrolipoic acid (DHLA). In PC12 cells, the three modes of LA action: pretreatment (LA+QD), chelation of free Cd^{2+} ions in QD solution, *ex vivo* (LA-QD) and surface capping (DHLA-cap), significantly reduced the toxicity of QDs (Figure 4A, $p < 0.001$). The protective effects exerted by the antioxidant in PC12 cells may have been primarily due to the significant reduction of free cadmium ions (Figure 4B). Interestingly, in dispersed DRG cultures, only LA-QD (108.4 ± 7.1%) and DHLA-cap (110.6 ± 9.6%) conditions yielded significantly higher cellular viability compared to QD treatment alone (58.1 ± 2.7%, $p < 0.001$). Pretreatment of

DRG cultures with LA did not protect against QD toxicity despite the significant reduction of intracellular free Cd^{2+} ions (Figure 4D; 25.2 ± 20.1%, $p<0.01$). The difference in the cellular response to LA between the two model systems is further reinforced, thus cautioning against the generalization of toxic and protective responses from only one model system.

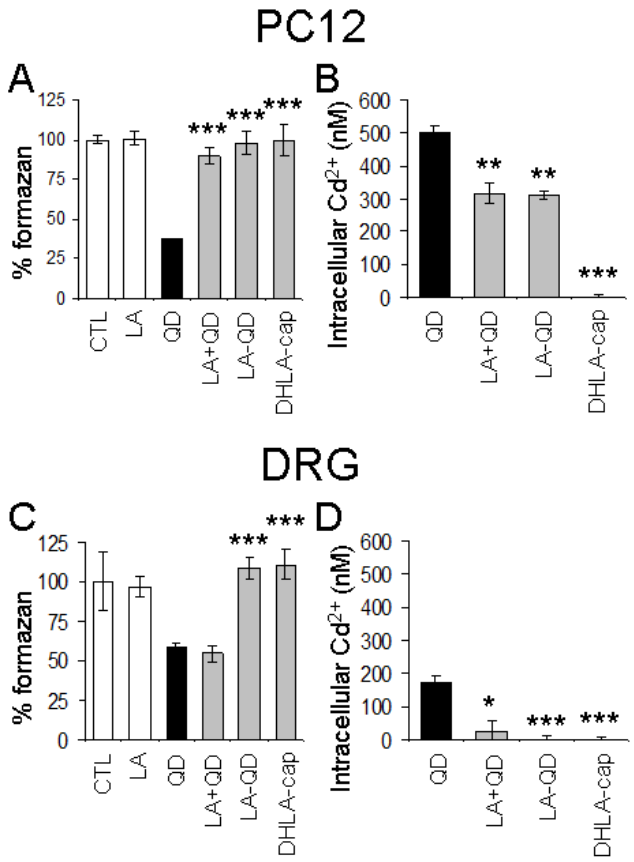

Figure 4. Lipoic acid is cytoprotective as a QD-capping agent, a QD-chelating agent or as an antioxidant agent. The antioxidant, lipoic acid (LA) and its reduced form (dihydrolipoic acid, DHLA) was used (i) to treat PC12 and DRG cultures (200 µM and 750 µM respectively) prior to QD treatment (**LA+QD**), (ii) to chelate the free Cd^{2+} ions in the QD solution *ex vivo*, prior to cell treatment (**LA-QD**), and (iii) to cap the surfaces of QDs (**DHLA-cap**). MTT assay was used to assess cell viability and the Measure-iT assay was used to measure the amount of intracellular free cadmium ions. Data represent the means ± SEM from three independent experiments. Statistically significant differences are based on

difference relative to QD treatment and indicated by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

4.4 Decreased cell viability is reflected by the disruption of intracellular lipid droplet formation in PC12 and dispersed DRG cultures.

Cytotoxicity induced by QDs and by the individual core metals has thus far only been assessed via biochemical assays including cell counting and MTT (Figures 1-4). We, therefore, examined the formation of lipid droplets, an organelle critically associated with triglyceride and cholesterol metabolism [24]. Early *in vivo* studies with $TeCl_4$ suggest formation of intracellular lipid droplets as an early indication of tellurium-induced cell death in the PNS [25]. PC12 cells treated with QDs and toxic concentrations of $CdCl_2$ and $TeCl_4$ (300 nM) all contained significantly fewer lipid droplets compared to the untreated control when stained by the BODIPY dye (green) (Figure 5A, top panel). Of note is the formation of distinct lipid droplets in the QD-treated cells (indicated by white arrows). LA normalized and maintained neutral lipid distribution similar to the lipid homeostasis in naïve control cells (Figure 5A). Dispersed DRG cultures contained less endogenous lipid droplets compared to PC12 cells (Figures 5A, 5B; Ctl), but DRG cultures treated with QDs and metal ions show an increase in lipid droplets formation (stained by Nile Red, and indicated by white arrows). LDs appear localized primarily in the non-neuronal (glial) cells in the DRG cultures, and are generally organized into distinct droplets, except in $TeCl_4$-treated cells (Figure 5B, top panel). LA pretreatment was only effective in reducing lipid droplet formation in $TeCl_4$-treated cells, but did not alter the neutral lipid distribution in other conditions in PC12 cells or dispersed DRGs.

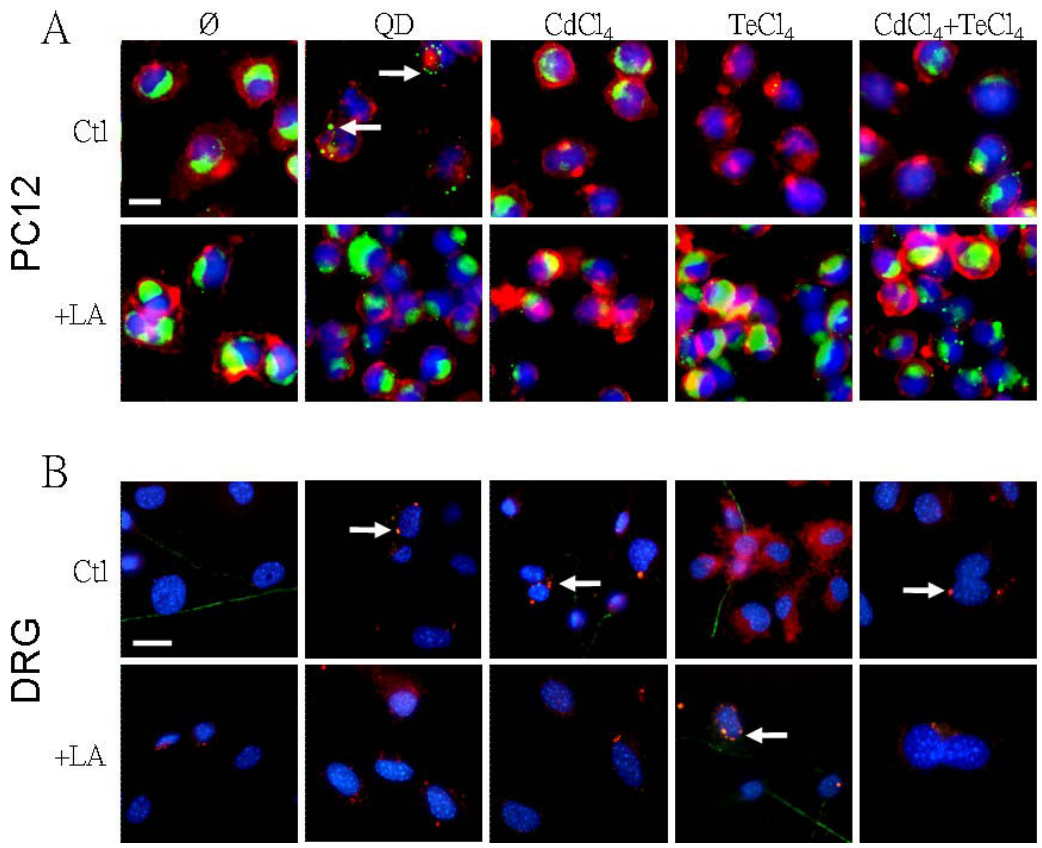

Figure 5. Decreased cell viability is reflected by the disruption of intracellular lipid droplet formation in PC12 and dispersed DRG cultures. PC12 and dispersed DRG cultures were pretreated with LA for 24 hours and treated with $CdCl_2$ (300 nM) or $TeCl_4$ (300 nM) or CdTe QDs (10 µg/ml) for a subsequent 48 hours. Immunostaining of lipid droplets were performed in fixed cells using BODIPY® 493/503 (green) or Nile Red (red) for PC12 and DRG cultures respectively. PKH26 dye (red) was used to stained for cell membranes in PC12 cells (A), and antibodies against β-tubulin (green) was used to detect neurons in the DRG cultures (B). Cell nuclei were stained with Hoechst 33342 (blue). Pictures were taken in triplicates at 40X and 63X. Scale bars represent 10 µm.

4.5 Lipoic acid protects dorsal root ganglia explants from quantum dot- and metal ion-induced neurite damage

Although, the protective effects of LA against QD- and cadmium or tellurium ion-induced neural damage were not evident in dispersed DRG cultures, we tested the cytoprotectivity of LA in whole DRG explants against QD and QD-core components using both qualitative and semi-quantitative assessment (Figure 6). Cultures treated with CdTe QDs (10 μg/mL), and a high concentrations of cadmium chloride (2 μM) and tellurium chloride (5 μM) resulted in significant degeneration of neurites compared to untreated controls (Figure 6). However, low concentrations of QDs (< 5 μg/mL) that are more relevant from the biological standpoint, did not reduce neurite outgrowth (data not shown), preserving the morphologies observed in the untreated and LA (750 μM) treated samples (Figures 6A, 6B). In cultures pretreated with LA, the extent and density of neurite growth was preserved in the presence of Cd^{2+} and Te^{4+} ions, and even in the QD-treated samples, suggesting neurite protection by the antioxidant.

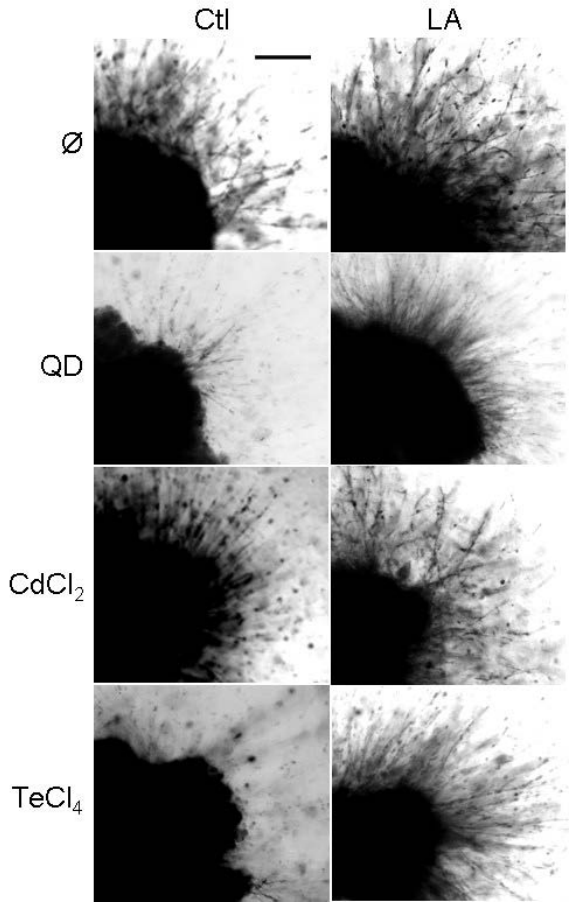

Figure 6. Lipoic acid protects dorsal root ganglia explants from quantum dot- and metal ion-induced neurite damage. Whole DRG tissues were treated with 750 μM of LA for 24 hours prior to treatment with QD (10 μg/mL), $CdCl_2$ (2 μM) and $TeCl_4$ (5 μM) for a subsequent 24 hours. DRG cultures were stained with MTT and light micrographs were taken in triplicates at 20X. Scale bar represents 500 μm.

5. Discussion

Many studies examining the cytotoxicity of QDs were reported over the past few years; however, none have examined potential toxicity in the context of the peripheral nervous system (PNS). Thus far the only study to investigate QD application in DRGs was by Cui *et al.* to examine whether NGF-conjugated QDs can enter DRG neurons and be utilized for tracking the internalization of NGF [26]. While their study highlights the potential of functionalized QDs as imaging

agents, the long term consequences of different concentrations of these QDs were not reported. Our current study demonstrates that even non-functionalized QDs can be taken up by dispersed DRG cultures quite readily (supplementary material). This non-specific QD-uptake could pose a threat to the PNS if the QDs remained within the target tissue (i.e. skin) for a long period of time. Clearly, one must keep in mind the complexity caused by the size, charge, stability, capping materials, conjugations, and treatment durations of functionalized and non-functionalized nanoparticles, as QDs could be transported both anterogradely and retrogradely in the PNS, and possibly contribute to neurodegeneration [26]. We, therefore, investigated QDs and their individual core-constituting elements, Cd^{2+} and Te^{4+} in DRG cultures, both dispersed and in 3D.

Thus far, QD induced-cytotoxicity has been often ascribed to free metal release, primarily cadmium; but also nanoparticles with partly or fully "naked" surfaces [18]. We investigated both core-constituting metals, Cd^{2+} and Te^{4+} in concentrations relevant to QD treatments, alone and in combination. Interestingly, the cytotoxicity induced by the metals correlated with QD-induced cytotoxicity in PC12 cells, however, in dispersed DRG cultures QD-toxicity was far greater; possibly due to the enhanced susceptibility of primary dispersed DRG cultures to oxidative stress. Further, QDs may pose a greater oxidative threat than their core constituting metals alone, due to the unique physical-chemical properties and surface chemistry of the QDs. Taken together, the enhanced oxidative stress posed by the QDs and the increased susceptibility of the dispersed DRG cultures to oxidative stress may explain the discrepancy observed between the two models.

We utilized LA, both an antioxidant and metal ion chelator, as a cytoprotective agent in cultures treated with QDs, Cd^{2+} and Te^{4+}. LA is considered a therapeutic drug for conditions of diabetic neuropathy [27]. Our findings with LA in PC12 cells demonstrate nearly complete cytoprotection against QD- and Cd^{2+}-insult, however, in dispersed DRG cultures, LA seems to increase the overall toxicity of Cd^{2+}, Te^{4+} and QDs. A possible explanation for this phenomenon is that LA forms a complex with the QD and the net charge of this complex facilitates QD entry into the primary cultures, more so than QDs alone (without LA). Subsequently,

the LA and QD complex likely disintegrates intracellularly, and free metals (Cd^{2+} and Te^{4+}) are gradually liberated from the QD-core. This QD degradation and LA-facilitated entry of metals and nanoparticles may allow Cd^{2+} and/or Te^{4+} concentrations intracellularly to exceed the capacity DRG cells can cope with, thus reaching lethal cellular concentrations. Enhanced Cd^{2+} concentrations may be of particular concern in neural cultures compromised by aging or disease, with reduced tolerance to different stressors. Another explanation for these opposing effects by LA in different cell systems is that LA may elicit different antioxidant responses in different cell types. Measurement of intracellular glutathione (GSH) in PC12 cells showed a significant increase in GSH levels following treatment with LA, a known source of cysteine to replenish intracellular glutathione stores [28]. However, such an increase in GSH was not observed to the same extent in the dispersed DRG cultures, thereby suggesting that pretreatment with LA in primary cultures may not be sufficient to bolster GSH levels, and that other protective mechanisms may be implicated in damaged cultures.

Every molecule of LA has the ability to chelate two Cd^{2+} ions. Chelating the free Cd^{2+} in QD solutions, *ex vivo,* with LA, prior to cell treatment (denoted as LA-QD in figures) renders the QDs innocuous (Figure 4). This method was successful as free intracellular Cd^{2+} was close to or below the detectable limit. DHLA coating of CdSe/ZnS QDs was also found to be effective. However, the combination of LA and Te^{4+} markedly enhances the overall cell death, in all cell types examined, likely due to enhanced internalization of Te^{4+}. Though, intracellular Te^{4+} measurements were not done, previous reports show that Te^{4+} in the PNS causes demyelination preceded by DNA fragmentation, chromatin condensation, and vesicular degeneration, indicative of necrotic cell death and autophagy [29]. Another study showed Te^{4+}-induced autophagic cell death in association with cholesterol depletion. The authors suggest that the intracellular stress response involves the FOS family of genes, disorganization of the rough endoplasmic reticulum leading to disruption of the protein synthesis machinery and multiple organelle damage [30]. Interestingly though, this group also suggested that "cytoplasmic remodeling" may be a mode of initial protection against Te^{4+}.

Intracellular Te^{4+} concentrations are difficult to measure and there are currently no commercial assays. We thus investigated changes in lipid droplets (LD), an organelle known to be responsive to Te^{4+}. Goodrum *et al.* showed previously that Te^{4+} can induce lipid droplet (LD) formation in the peripheral nervous system after $TeCl_4$ administration *in vivo* [31]. Examination of LDs in our cell models showed that intracellular LD formation can be decreased, as in PC12 cells, and increased, as observed in dispersed DRG cultures, in response to extracellular stressors, including QDs and metal ions (Figure 5). We found that LA may play a role in regulating lipid homeostasis, by increasing the number of lipid droplets, thus exerting its cytoprotective effects in PC12 cells. Consequently, the composition, dynamics, formation, and localization of LDs are expected to present differently in different cell model systems, despite being induced by the same stressor. This discrepancy was observed in PC12 and dispersed DRG cultures. However, it is still unclear exactly how LD redistribution can affect cell survival or death. In addition, assessment of LD composition requires lipidomic and proteomic analysis, which is currently under way in our laboratory.

LA remains an attractive therapeutic option in preventing QD-induced cytotoxicity, as it does not quench QD-fluorescence and when utilized either as a free Cd^{2+} ion chelator *ex vivo* or as a capping agent, the biocompatibility of QDs can greatly be enhanced, without requiring high pharmacological concentrations of the drug.

However, based on our analysis LA may be more cytoprotective in certain cell types and model systems than in others. A potent antioxidant, LA can scavenge hydroxyl radicals, hyperchlorous and singlet oxygen; DHLA on the other hand, is a potent scavenger of hydroxyl radicals, and hyperchlorous acid, but not of singlet oxygen, and neither LA nor DHLA have been found to scavenge superoxide radicals [20]. Previous studies from by our group have shown that cellular treatment with QDs leads to the production of superoxide, singlet oxygen reactive oxygen species and peroxyl radicals [14, 15, 32]. This could explain why LA does not ensure complete protection against QD-induced cytotoxicity. Furthermore, cellular susceptibility to superoxide and singlet oxygen may also vary between

cells types and model systems, thereby contributing to the discrepancy between the cytoprotection provided by LA.

Cellular internalization of QDs correlates with QD-induced cytotoxicity [16]; small, cationic QDs are more cytotoxic because they are most readily internalized by cells. In addition, most coating and capping materials used to protect the surface of the QD and allow QD-dispersion in aqueous solutions, are not sufficient to maintain the integrity of the QD intracellularly and in the extracellular environment. CdSe QDs are the other commonly discussed and utilized QDs, and though studies do show these QDs to be somewhat toxic, adding a layer of ZnS renders these QDs non-toxic. Other reasons why CdSe QDs are a better alternative to CdTe QDs is that aside from being a cadmium chelator [33], selenium in low concentrations may exert beneficial effects in living cells and *in vivo* [34]. Further, a recent study found selenium to be cytoprotective against cadmium-induced toxicity [35]. Selenium reduced cadmium-induced JNK phosphorylation, and decreased caspase activation. The antioxidant properties of selenium were shown to be comparable with N-acetylcysteine (NAC); an antioxidant previously shown by our group, to completely protect against QD-induced cytotoxicity [14]. This effect would also imply that any toxic threat posed by QD degradation and subsequent release of the core metals would be nullified due to the cytoprotection of selenium against cadmium; rendering CdSe QDs increasingly biocompatible. However, recent studies show that these CdSe QDs are cytotoxic in kidney cells and suggests that QD core material cannot solely explain nanotoxicity [36].

In terms of CdTe QDs and nanoparticle toxicity we present the differences between three different model systems: (i) a stable monolayer cell line (PC12), (ii) primary dispersed DRG cells, (iii) a three-dimensional (3D) DRG explant tissue. Results from this study show that conclusions drawn from monolayers and 3D cultures with heterogeneous cell populations are dissimilar.

In general, cell lines can be utilized to provide accurate, reproducible results, facilitating high throughput screening and extensive statistical analysis. In addition, with PC12-like cells studies examining both neurite outgrowth and cell

proliferation can be conducted depending on whether differentiated or undifferentiated PC12 cells are employed, respectively. Though PC12 cells have been used as an invaluable cell model, particularly in neuroscience, they are inadequate for predicting effects in mixed cell populations or *in vivo.* Discrepancies in QD-toxicity between various cells lines have already been demonstrated [37] and reviewed [1, 38].

Utilizing mixed cultures for evaluating NP-toxicity, allows this inter-cell type discrepancy to be better examined. Upon treatment of mixed cultures, all cell types are simultaneously exposed to the molecule or nanoparticles discussed here. Neural cultures enable the study of glia-neuron interactions and provides insight into normal physiological responses [39, 40]. It is, therefore, important to conduct studies in primary animal cell models, particularly mice models, since prior to any clinical testing all nanoparticles undergo preclinical screening *in vivo*. Transgenic and non-transgenic mice models are particularly useful for exploring the mechanisms involved in cell responses to NPs. While primary, neural cultures are a versatile model for screening NP toxicity, they have not been extensively used in nanomedicine, mainly because they are more difficult to work with than cell lines and because they still lack the connectivity and the actual physical tissue structure.

This lack of connectivity can at least in part be overcome by utilizing DRG explants, representing 3D, multicellular systems. To date there have been no studies examining NP toxicity in such systems, and the present study provides some data in comparison with the same PNS model system in monolayers. The disadvantage of 3D DRG model system is that imaging single cells is presently difficult. Further, DRG explants are representative of axotomized neurons [23]. Therefore, the DRGs are preconditioned as injured peripheral nerves, thereby allowing the evaluation of the regenerative abilities of the DRG [23, 41]. Screening for nanoparticle toxicity in such 3D cell systems enables the visualization of neurite extension or retraction in response to nanoparticle treatment. Conversely, this cell system can also help predict whether nanoparticles may compromise the regenerative abilities of the peripheral nerves.

6. Conclusion

Our study shows that considerable differences exist within the same system depending on the architecture of the model; whether the cells are in monolayers or 3Ds. In heterogeneous, 3D DRG explants, communication between the cells more accurately resembles the *in vivo* autocrine and paracrine loops, and basic functioning of the cells. LA was ineffective at preventing QD-induced cytotoxicity in dispersed DRG cultures, however, effective in protecting DRG explants; as seen by evaluating the functional properties of the explant to maintain and extend neurite outgrowth. The complex structure of the DRG explant combined with the potent antioxidant and metal-chelating effect of LA provided synergistic protection to maintain the viability of the explants under metal ion- or QD-induced stress. This highlights the limitations of cell lines, and stresses the importance of evaluating nanoparticle-induced toxicity in various models to understand the biochemical changes that may be occurring due to treatment. Thus, while cell lines remain a valuable tool and first step, the results obtained must be complemented with either *in vivo* studies or at least 3D model systems prior to deeming a nanostructure or nanomaterial toxic or non-toxic.

7. Future Perspectives

Nanotechnology holds much promise for the field of medicine. However, prior to any therapeutic introduction, sufficient consistent data regarding the true toxic threat of nanoparticles needs to be irrefutably ascertained. To date this has been limited mainly due to the lack of standardization in, and sensitivity of screening techniques. One factor that would lend heavily to facilitate thorough assessment of nanoparticle toxicity is the use of appropriate model systems. This study illustrates the diverse effects of nanoparticles (i.e. CdTe QD) in increasingly complex cell systems and points toward the need for assessment of nanomaterials in more than monolayered cell lines. Three dimensional, multicellular, explants together with *in vivo* studies in real time [5] will likely afford reliable assessment and standardization of new nanomaterials for medical applications. Combining

several model systems for evaluating toxicity from acute changes in cell metabolism and lipidomics, to functional impairments, and finally cell death, will increase the sensitivity of our evaluation of nanoparticles. Such rigorous testing of toxicity is not limited to the field of nanotechnology, however, it is particularly important as these unique particles possess properties (both toxic and other) that are not simply a sum of their molecular components, and therefore, must be investigated as new entities. In exploring the various mechanisms and manifestations of NP toxicity, the goal is to find *in vitro* toxicity assays that could, most reliably, predict *in vivo* toxicity. Further, such screening techniques will provide not only a means by which to delineate toxic and non-toxic nanoparticles, but rather form a spectrum from which specific nanoparticles can be selected for specific purposes and targeted use. These carefully selected nanoparticles will likely prove more effective for biomedical applications, and be better understood candidates for rigorous pre-clinical testing.

8. Executive summary

- In this study, we emphasize the importance of utilizing various cell systems for thorough assessment of QD-toxicity. Monolayered, homogenous cell lines facilitate critical screening, however, monolayered, heterogeneous cell systems should also be examined. More saliently, we highlight the need for testing nanoparticles in 3D, multicellular systems, where functional impairments of the system induced by NPs can be detected.
- Analysis of the individual core constituting metals of cadmium telluride QDs revealed different degrees of toxicity in different cell types. The sum of these toxicities was comparable to equimolar concentrations of QDs in some cell types but not all.
- Mechanisms of LA action involve several modes of activity, including acting as a direct and indirect antioxidant, as well as a metal chelator, and QD-surface modifier. LA remains an attractive tool for preventing QD-

induced cytotoxicity and a useful tool for improving our understanding of nanoparticle toxicity.

- Further investigation of early cytotoxic events incurred post nanoparticle treatment, such as the examination of biochemical markers, and cell lipidomics may enhance the sensitivity by which nanoparticle toxicity will be evaluated.
- We also stress the need to examine the potential effects of nanoparticles on the peripheral nervous system, as ultimately post any exposure, NPs may be able to travel through the nerves and impact the PNS, potentially compromising this delicate system.

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Connecting Text

In chapter 2, we demonstrated that neither the physic-chemical properties of QDs nor their toxicity is a simple sum of constitutive parts. Further, we demonstrated that the toxicity of each core element, cadmium and tellurium, was different in different model systems. We also showed that QDs are capable of inducing oxidative stress accompanied with glutathione (GSH) depletion. We, therefore, investigated several antioxidant therapeutics to prevent this QD-induced oxidative stress. Lipoic acid was able to increase intracellular GSH in QD-treated cells. Prevention of QD-induced cytotoxicity was achieved through several means, including direct and indirect scavenging of ROS, upregulation of intracellular antioxidants, chelation of cadmium and the protection of the QD surface by the reduced form of lipoic acid. The results of this study led to interesting findings: (1) small amounts of cadmium released from QDs were not toxic to cells, (2) the QD-toxicity and side effects observed were unique in each cell type. We therefore, asked the question: if small amounts of cadmium released from QD does not induce cytotoxicity, what other cellular effect is it capable of inducing? Previous studies have indicated that cadmium is an endocrine disrupter capable of exerting metalloestrogenic effects and estrogenic signalling in estrogen receptor expressing cells. Based on the fact that QDs release cadmium ions from their core, in chapter 3 we evaluated whether QDs may induce metalloestrogenic effects both *in vitro* and *in vivo*.

Chapter 3

Metalloestrogenic effects of quantum dots

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1. Abstract

Aim: To investigate the metalloestrogenic effects of cadmium telluride quantum dots (QDs) in both human breast cancer cells and *in vivo* in mice. **Materials and methods:** Human breast cancer cells (MCF-7 cells) were utilized to study QDs, cadmium and 17β-estradiol induced estrogen-related genomic and non-genomic signalling. Female prebupescent and ovariectomized adult mice were treated with CdTe QDs to assess whether QD-induced estrogenicity would lead to uterine changes. **Results and Discussion:** Our findings demonstrate that *in vitro* cadmium-containing QDs induce cellular proliferation, estrogen receptor (ER) α activation, and biphasic phosphorylation of AKT and ERK1/2, comparable with 17β-estradiol. Green QDs elicited a more robust estrogenic response than orange QDs. Addition of the selective estrogen receptor antagonist, ICI 182 780 completely abolished all QD-induced estrogenic effects, suggesting that QD-induced estrogenic signalling is mediated via the estrogen receptor. *In vivo*, chronic treatment of mice with QDs led to a 2-3 fold increase in uterine weight, comparable or greater than 17β-estradiol. **Conclusion:** These findings suggest that certain cadmium-containing nanocrystals are endocrine disruptors, whose effects can exceed those induced by ionic cadmium or 17β-estradiol.

2. Introduction

Different classes of compounds have been found to exert estrogenic effects [1]. Phytoestrogens, plant derived estrogens are found in abundance in nature [2]. Their estrogenic activity has been ascribed to the presence of aromatic groups in their structure; thus phytoestrogens share structural semblance with steroid hormones. Xenoestrogens, or “strange” estrogens such as bisphenol A, also share the aromatic groups, lending structural similarity to endogenous estrogens [3]. Phenolic groups in both phytoestrogenic and xenoestrogenic compounds are proposed to interact with the ligand binding domain (LBD of the estrogen receptor (ER), displace endogenous estrogens and activate the receptor [4]. Metalloestrogens are a relatively less understood class of estrogens with no

structural semblance to endogenous estrogens [5]. This category of estrogens include metals such as lead (Pb), mercury (Hg), nickel (Ni), copper (Cu), cobalt (Co), chromium (Cr), and most commonly cadmium (Cd) [6, 7] . Of these metalloestrgens cadmium has been best studied and shown to interact with the ERα LBD and inhibit the binding of radioactively labelled 17β-estradiol [8-10]. Cadmium, a heavy metal widely present in the environment, has been deemed a toxic pollutant [11]. Cadmium pollution can largely be attributed to industrial waste, factory emissions and mining. Its high concentration in water and soil has resulted in the presence of detectable amounts in aquatic life and fresh produce [12, 13]. Human exposure to high levels of cadmium has also been ascribed to cigarette smoke and tobacco use [14]. Much of the caution surrounding cadmium is due to its long half life (~20 - 30 years), and slow clearance from the body [15]. Bioaccumulation of cadmium is the cause of numerous negative side effects, including cytotoxicity in the lungs, liver and kidneys [11].

In this report, we provide evidence for a new class of metalloetrogens, cadmium containing, bioluminescent nanocrystals (i.e. cadmium telluride quantum dots, QDs). Nanoparticles such as QDs, are popular fluorophores due to their size, tunability and superior optical properties [16]. QDs consist of a heavy metal, semi-conducting core that lends unique physico-chemical properties, rendering the QD a highly efficient fluorophore. Initially introduced in medicine for the real time bioimaging of tumours, QDs also have the potential to be exploited as both diagnostic and therapeutic tools via surface modifications permitting the conjugation of various drugs [17]. Recent studies have proposed the use of QDs in cancer as novel theragnostics [18, 19] (combination of a therapeutic agent and diagnostic tool). Alternatively, QDs are also considered potential agents for photodynamic therapy of cancers [20].

Other studies, however, have shown that QDs with unstable cores, bearing surface molecules (e.g. cysteamine, mercaptopropionic acid) are cytotoxic [21] largely due to the gradual degradation of the metal core and the release of cadmium ions [22]. QDs have been found to induce various modes of cell death [23, 24]. However, in cells expressing ERs we hypothesized, that at low (picomolar)

concentrations, cadmium-containing QDs may exert estrogenic effects, rendering these QDs novel nanometalloestrogens. As such, nanoparticles capable of releasing cadmium ions and exerting estrogenic effects may prove detrimental should QDs be utilized as theragnostics in estrogen responsive cancers, such as many breast cancers [25, 26].

To investigate whether QDs may elicit estrogen-like activity, we utilized human breast cancer cells (MCF-7 cells) known to express ERα, ERβ and the plasma membrane ER, GPR30 [27]. We treated these cells with QDs in various concentrations for different lengths of time. QD treatments initiated both rapid and long lasting activation of signal transduction pathways leading to genomic activation. We examined both genomic effects such as cell proliferation and nuclear-ER receptor activation, and estrogen associated rapid (non-genomic) signalling events, such as AKT/PKB (protein kinase B) and ERK (extracellular regulating kinase) phosphorylation. We further employed the ER antagonist, ICI 182 780, to verify that these effects were mediated via the ERs. Finally, to prove that nanoparticle-induced metalloestrogenicity is not exclusive to *in vitro* cultures, we determined changes in female uteri in mice treated with QDs *in vivo*. Results from both experimental approaches suggest that cadmium-containing nanocrystals are much more potent endocrine disruptors than ionic cadmium.

3. Materials and Methods

3.1 Materials

Charcoal treated serum (CTS) was purchased from Wisent (Montreal). Cell media, RPMI 1640, fetal bovine serum, antibiotics were purchased from Gibco (Montreal). All plates for cell culture were purchased from Sarstedt (Montreal). Estradiol and ICI 182780 were purchased from Sigma (Montreal).

3.2 CdTe QD-synthesis

Cysteamine-capped CdTe QDs were synthesized by a modified procedure [28]. Modification details reported in previous studies [22, 23, 29]. Green cationic CdTe QDs had a diameter of 2.81nm and the larger, orange QDs had a diameter 3.10nm, with emission maxima of 525nm and 543nm respectively. The zeta potential of the green QDs was +14.2 and +15 mV for the orange QDs.

3.3 Cell culture

Human breast cancer (MCF-7) cells (ATCC, USA) were grown in phenol-free RPMI 1640, supplemented with 10% FBS and 1% penicillin/streptomycin and maintained at 37˚C (5% CO_2) in a humidified atmosphere. For seeding, cells were trypsinized (Gibco, Montreal) and centrifuged for 3min. Cells were re-suspended in 5% charcoal treated serum, counted and plated as required for experimentation (24well or 96well plates or Petri dishes). Prior to cell treatments, existing media was removed and fresh media was added. Cells were treated with 10nM 17β-estradiol, 500nM of cadmium chloride, 0.5ug/ml of orange cysteamine capped QDs and 10ug/ml of green cysteamine capped QDs (unless otherwise specified). For cell proliferation assays, cell media was changed every 48 hours, and cells were retreated. For use of the estrogen receptor inhibitor, ICI 182780, cells were pre-treated with 1uM for 5min prior to estrogenic treatments.

Rat pheochromocytoma (PC12) cells were maintained in phenol-free RPMI 1640, supplemented with 5% FBS and 1% penicillin and streptomycin. For experimentation, PC12 cells were also seeded in 5% CTS similar to MCF-7 cells.

3.4 Alamar blue assay

Cells seeded at a density of 30 000 cells/well, in 500µl of media, in a 24 well plate, were treated after 24hours. 48hours post initial treatment, existing media was aspirated and fresh media added. Cells were retreated. After 96hours of treatment, cell media was aspirated. 225µl were replenished, along with 25µl of the fluorescent Alamar blue reagent (10X, Invitrogen). The cells were returned to the incubators, and maintained at 37˚C for 60minutes. Post incubation, media

from each well was transferred in triplicate to a black, half area, 96 well plate (Costar, 3694), and fluorescence was measured spectrofluorometrically (FLUOstar Optima, BMG Labtech, with an excitation/emission wavelengths of 530-560/590.

3.5 BrdU Cell proliferation assay

A colorimetric Bromodeoxyuridine ELISA kit (Roche) was utilized to assess estrogen-induced cell proliferation. Approximately 5000 cells were seeded per well of a cell culture treated 96well plate. Media was changed and cells were treated 24hours after seeding, and again 48hours later. 24hours post the second treatment the BrdU labeling agent was added to a final concentration of 10uM, and incubated with the cells overnight. The following day all media was aspirated from wells and cell fixation solution (provided in kit) was added and incubated at room temperature for 30minutes. After the fixation solution was removed, the anti-BRdU-POD antibody was added to each well and incubated for 90minutes. Post this step all wells were washed and the substrate was added and incubated in the dark for approximately 30minutes. Photometric measurement was done at a wavelength of 492nm. Data was analyzes based on proliferation as per control, CTS, untreated cells.

3.6 Assessment of Phosphorylated AKT and ERK1/2

AKT and ERK1/2 phosphorylation was assessed by employing ELISA assay kits purchased from Calbiochem (AKT-P – CBA005; ERK1/2-P – CBA006). Samples were prepared and assays were executed as per kit instructions. Approximately 5million cells were seeded in Petri dishes. The following day each dish was treated for varying times with different treatments. At the end of the treatment incubation media was aspirated and cold PBS was added to the dishes. Cells were scraped and transferred to 15ml conical tubes. Post a second washing with PBS cells were transferred to Eppendorfs to be lysed. The kit specified lysis buffer, containing a cocktail of phosphatase inhibitors, was made, and the cells were incubated on ice with the lysis buffer for 30min, with periodic vortexing at

intervals of 10minutes. Next, the samples were centrifuged for 10 minutes at 4°C at 13000 rpm, and the supernatant was aliquoted. Only for the phosphorylated ERK1/2 ELISA, in order to ensure protein denaturation of the samples, the samples were boiled for 5minutes. An aliquot of the supernatant (100µl) was pipetted in duplicate on the equilibrated monoclonal antibody coated, 96 well plate strips provided with the kit. Using the standard diluent buffer, a standard curve the substrate provided was constructed using serial dilutions. The standard curve was also pippetted on the micro well strips in duplicate. The plate was incubated at 4°C over night. The following day the plate was washed three times using the wash buffer provided. Subsequently a detector antibody was applied, and the strips were left to incubate for 60 minutes at room temperature. The plate was again washed 3 times. Following this second washing the horseradish peroxidase bound polyclonal antibody was pipetted onto the strips and incubated for 30 minutes. Post the incubation; the wells were again washed three times prior to the addition of the tetramethylbenzidine substrate. The strips were kept in the dark for 30 minutes, after which a stop solution was added and the strips were read with a microplate reader at a wavelength of 350nm. Regression curves were drawn using the standard curve concentrations and optical density values, and utilized to assess the phosphorylated protein content in samples.

3.7 Assessing nuclear estrogen receptor activation by metalloestrogens

Nuclear ERα activation via metalloestrogenic particles was evaluated via the Human Estrogen Receptor 1 Reporter Assay (INDIGO Biosciences, Inc IB00401-32. This assay consists of a reporter cell system engineered to express ERα, and following ligand binding or activation of this receptor, induces the expression of the luciferase gene. Samples of various metalloestrogens used in parallel with a standard curve of estrogen concentrations allows for the evaluation of the estrogenic activity of estrogens as compared to 17β-estrdiol. In order to commence the assay, reporter cells were thawed and combined with the provided cell recovery media (CRM) and 100µl of the cells were plated on the white, sterile, snap in 8-well strips. To this cell suspension an additional 100µl of

compound screening media were added. Finally, the cells were treated with either various concentrations of 17β-estradiol, in order to construct a standard curve, or the cells were treated with cadmium, orange CdTe QDs, green CdTe QDs, or green CdSeZnS capped QDs. Post treatment the cells were place in a 37°C, humidified, 5% CO_2 incubator for 24hours. At the end of the incubation period, all media was aspirated from the cells and 100µl of the Luc-detection reagent (LDR) was added to each well of cells. The plate containing the strips of wells was next shaken for 10 minutes prior to being place in a Luminometer for reading. A logarithmic transformation of the data and was required prior to graphing the readings obtained, with 17β-estradiol concentrations comprising the x-axis and the relative fluorescent units (RLU) measured on the ordinate. As such a regression curve was formed, allowing for the analysis of the estrogenicity of the metalloestrogens in terms of nuclear ERα activation (supplemental materials figure 2) as compared to and expressed as 17-β-estradiol equivalence measured in pM.

3.8 Assessment of QD-estrogenicity in vivo

To evaluate the estrogen-like effect of QDs, BABL/C pre-pubescent and virgin ovariectomized female mice (~15grams, ~30grams, respectively), purchased from Charles River Canada (St Constant, Canada) were maintained on a 14:10-hour light: dark cycle in the Animal Resources Centre of McGill University. Control and treated mice were provided with food and water ad libitum; there were no differences in food intake between groups of animals. All animal studies were conducted in accordance with the procedures and principles outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill Animal Research Centre protocol 4687).

After the 10-day recovery, post ovariectomization, the animals were randomly divided into four groups, each composed of 5 females: vehicle control (sterile dd H2O), positive control (0.56mg/kg 17β-estradiol), cadmium-treatment groups (0.25mg/kg), and QDs (2.5mg/kg or 25mg/kg). For calculation of QD doses, please see Supplementary Methods 1. All injections were administered

intraperitonealy every other day for two weeks. Post all treatments, animals were euthanized with CO_2, followed by cervical dislocation. Uteri were removed, weighed, and frozen in liquid nitrogen for cadmium evaluation.

3.9 Statistical Analysis

All experiments were done at least three times, with replicates of three or more in each experiment. Data were analyzed using SYSTAT 10 (SPSS, Chicago, IL, USA). Statistical significance was determined by Student's *t*-tests with Bonferroni correction. Differences were considered significant where $*p<0.05$, $**p<0.01$, $***p<0.001$.

4. Results

4.1 CdTe QDs induce estradiol-like proliferation in estrogen receptor expressing cells

In order to evaluate the estrogenic action of QDs, MCF-7 cells were cultured in media containing charcoal-treated serum (CTS), devoid of estrogens. Control treatments included both 17β-estradiol and ionic cadmium. QD-induced cellular proliferation was evaluated via two different methods: the Alamar blue assay (an overall measure of cellular metabolic activity), and more directly by measuring the incorporation of bromodeoxyuridine (BrdU). After 48hours of treatment with 17β-estradiol, the relative fluorescence intensity (RFI) increased 139.7% ± 4.5%, indicating cell proliferation that was comparable with the effects of orange QDs (138.29% ± 2.64%) (Figure 1). Green QDs induced significantly more cell proliferation at 48h (161.8% ± 4.58%, $p<0.01$), whereas cadmium induced moderate cytotoxicity, or a slight decrease in the cellular activity. At 72hours both green and orange QDs exceeded the cell proliferation induced by 17β-estradiol (173.3% ± 10.06%, 162% ± 3.91%, and 148% ± 8.97% respectively) and post 96hours, green QDs had induced the maximum amount of cell proliferation (219% ± 10.1%), followed by orange QDs (198% ± 6.14%) and 17β-estradiol (191% ± 8.13%); cadmium induced less, though still comparable cell proliferation

(175.2% ± 19.87%) (Figure 1a). QD treatments for 96hours were examined within a relatively narrow concentration range (0.1-10µg/ml). Maximal QD-induced cell proliferations were: 281% ± 18.37% *($p<0.001$)* with green QDs (10µg/ml) and 198% ± 6.14% *($p<0.001$)* with orange QD (0.5µg/ml). Orange QDs between 2.5µg/ml - 10ug/mL induced cytotoxicity.

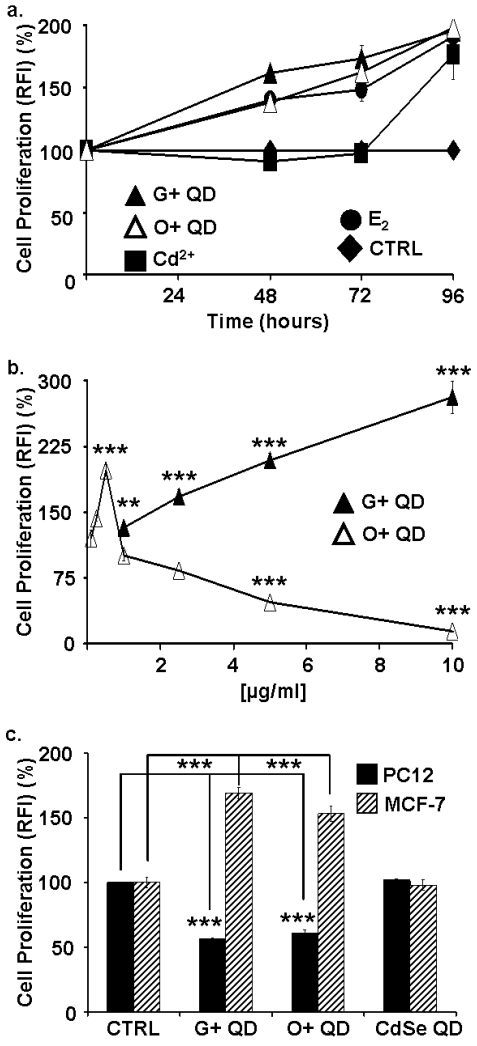

Figure 1: Estrogen receptor expressing cells proliferate in response to 17β-estradiol, cadmium, and QD treatment. a. MCF-7 cells, treated with E_2, Cd^{2+} and orange and green CdTe cationic QDs, induced cellular proliferation over the course of 96hours, as measured by the fluorescent Alamar blue assay. Post 48hours of treatment, G+ QDs (10µg/ml) induced the maximum cellular proliferation of (*$p<0.01$*), followed (10nM) E_2 and (0.5µg/ml) O+ QDs; whereas,

Cd^{2+} (500nM) induced a slight decrease. After 72hours of treatment, G+ QDs continued to induce maximal cell proliferation, followed by O+ QDs and E_2; Cd^{2+} again appeared to have no detectable proliferative effect. After 96hours G+ QDs and O+ QDs induced comparable, significant cell proliferation, followed closely by E_2. Cd^{2+}, post 96hours induced less, but still significant cellular proliferation (*p<0.001*). b. Cell proliferation induced by QDs is concentration specific. Several O+ QD concentrations were examined; low concentrations ranging between 0.1µg/ml to 1µg/ml induced cell proliferation, and 0.5µg/ml induced the maximal cell proliferation (*p<0.001*) post 96hours of treatment. Concentrations of O+ QDs greater than 1µg/ml induced cytotoxicity. After 96hour of treatment, of G+ QDs examined between the concentration range of 1-10µg/ml, maximal cell proliferation was observed at 10µg/ml (*p<0.001*). c. QDs only induce cellular proliferation in estrogen-receptor expressing cells such as MCF-7 cells, but not in non-ER expressing cells such as rat, pheochromocytoma cells. G+ QDs induced significant cell proliferation (*p<0.001*) in MCF-7 cells, whereas in PC12 cells the same treatment led to a marked decline in cell viability (*p<0.001*). Similar results were obtained with O+ QD. CdSe/ZnS quantum dots had no affect on cell viability or cell proliferation in either cell type.

4.2 Cellular expression of estrogen receptors is critical for mediating QD-induced estrogenicity

The Alamar blue assay was performed on both MCF-7 and PC12 cells. PC12, pheochromocytoma cells do not express ERs, and should not respond to estrogenic treatments like MCF-7 cells (Figure 1c). QDs (green and orange) that induced cell proliferation in MCF-7 cells, actually induced cytotoxicity in PC12 cells. Green QDs at a concentration of 10µg/ml after 48hours induced 169% ± 4.42 (*p<0.001*) proliferation in MCF-7 cells but reduced the number of PC12 cells to 57% ± 4.42% (*p<0.001*). Orange QDs induced significant proliferation (153% ± 6.14%, *p<0.001*) in MCF-7 cells, but reduced cellular viability to 61% ± 2.37% (*p<0.001*) in PC12 cells. CdSe/ZnS capped QDs were innocuous in both MCF-7 and PC12 cells, and induced neither cytotoxicity nor cell proliferation.

4.3 Estrogenic CdTe QDs elicit non-genomic signalling, including AKT and ERK1/2 phosphorylation after both acute and prolonged treatment in MCF-7 cells

MCF-7 cells were treated with 17β-estradiol, cadmium and green and orange QDs for periods of time ranging from 5mins to 6hours. The samples of these cell treatments were then analyzed via sandwich ELISA for AKT and ERK1/2 phosphorylation (AKT-P, ERK1/2-P) (Figure 2). The data obtained for both AKT-P and ERK 1/2-P can be divided into responses from acute estrogen treatments, and longer treatments.

Post 15mins of treatment, 17β-estradiol elicited maximal AKT phosphorylation, (308.51% ± 21.06% $p<0.001$), which was significantly higher than that induced by ionic cadmium or QDs (Figure 2a). However, at 30mins, AKT-P induced by 17β-estradiol decreased to 191.49% ± 4.51%, whereas all three other treatments (green QDs, orange QDs and cadmium) significantly increased AKT-P, 324.47% ± 5.27%, 319.15% ± 13.54, 318.51 ± 6.27, respectively ($p<0.001$). At the 1hour time point the AKT-P induced by treatments had somewhat decreased. Further at this time point 17β-estradiol induced AKT-P began to rise again (230% ± 9.03%) and continued to steadily increase at the 4hour treatment point (Figure 2b). In addition, both green and orange QDs, as well as cadmium also appeared to instigate a second wave of AKT-P, as the value of AKT-P increased for all three treatments, 398.94% ± 18.81%, 225.31% ± 17.04% and 395.94% ± 13.54% respectively. At the last time point of 6hours, 17β-estradiol induced AKT-P continued to increase to 367.02% ± 29.34%, and AKT-P induced by all treatments remained significantly greater than control ($p<0.001$). Two interesting observations from these studies were: (i) – estrogenic treatments resulted in a biphasic AKT-P curve within 6hours of treatment, and (ii) - a 15min right shift of the curve was noted with all cadmium-containing treatments.

ERK1/2 phosphorylation was evaluated much like AKT-P; similar results were also obtained. At the first time point of 5mins, all treatments induced a significant ($p<0.001$) increase in ERK1/2-P; the maximal being induced by 17β-estradiol

(293.65% ± 4.23%), followed by green QDs (285.23% ± 2.74%), orange QDs (277.05% ± 1.99%) and cadmium (268.16% ± 5.98%) (Figure 2c). At the subsequent time point of 15mins, 17β-estradiol induced slightly less ERK1/2-P (281.31% ± 2.12%) compared with all other treatments. A significant decline leading to near basal values of ERK1/2-P levels was measured after 30mins (Figure 2c). However, post 4hour treatments a dramatic and significant ($p<0.001$) increase in ERK1/2-P was again observed; estradiol induced ERK1/2-P of 382.82% ± 5.86%, green QDs let to an increase of 370.66% ± 3.68, followed by orange QDs (328.82 ± 2.52%) and Cd (324.98% ± 2.21%) (figure 2d). Maximal increase in ERK1/2-P was observed post 6hours treatments: 17β-estradiol induced ERK1/2-P (682.07% ± 4.81), followed again by green QDs (613.92% ± 7.21%), cadmium (518.37% ± 7.4%) and orange QDs (504.27% ± 0.01%). The general trend for all treatments was again a biphasic increase in ERK1/2-phosphorylation. However, 17β-estradiol elicited ERK1/2-P more rapidly and consistently to a higher degree than all other treatments. Green QDs mimicked 17β-estradiol ERK1/2-P profile following an initial 5min lag.

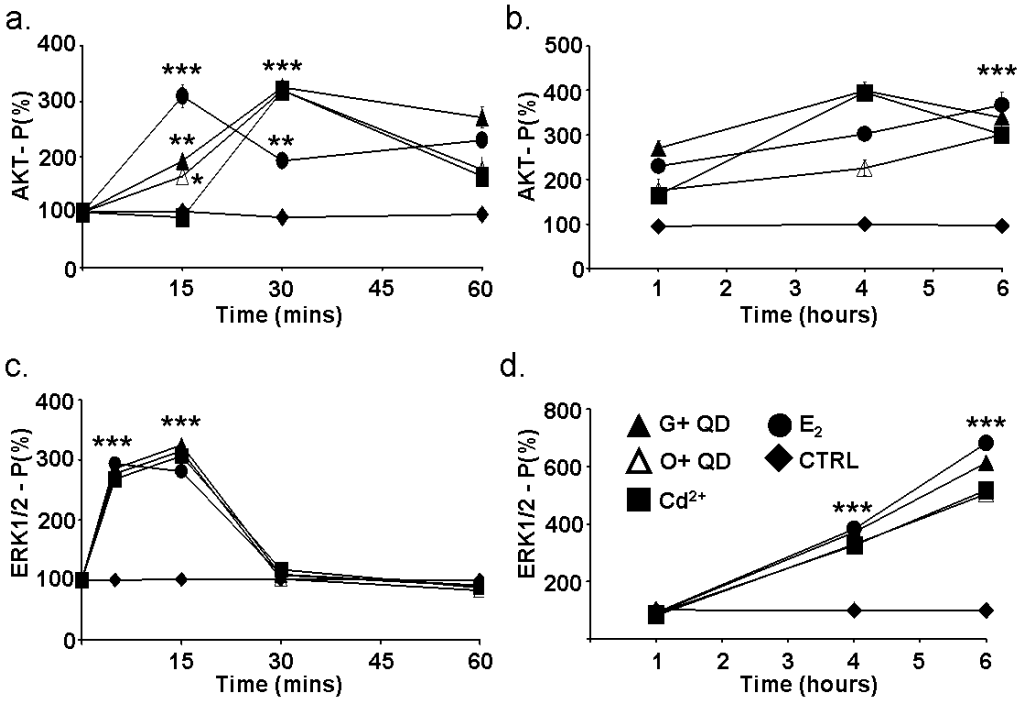

Figure 2: Estrogens, including, 17β-estradiol, cadmium and QDs induced biphasic AKT and ERK1/2 phosphorylation. a. Acute AKT-P induced by treatments was measured via sandwich Elisa, at several time points within 1hour. Post 15mins E_2 induced a rapid significant spike in AKT-P ($p<0.001$), while all other treatments lagged behind. Post 30mins of treatment, Cd^{2+}, O+ QD and G+ QD treatments induced AKT-P peaks (all $p<0.001$). At 30mins a dramatic decrease was noted with E_2 treatment followed by a similar drop with all other treatments at the 1hour mark. Post 1hour E_2 treatment began gradually inducing a second increase in AKT-P. b. Post 4hours of treatment E_2 induced a 3-fold increase in AKT-P ($p<0.001$); and metalloestrogenic treatments also began to induce a second wave of AKT-P. Post 6hours of treatment E_2 induced the maximum AKT-P (~3.5-fold increase), followed by G+ QDs, O+ QDs, and Cd^{2+} (all increases $p<0.001$). c. Estrogens induced acute ERK1/2-P within 1hour, as evaluated via sandwich Elisa. Post 5mins of treatment, E_2 induced a marked initial peak of ERK1/2-P ($p<0.001$); other treatments also induced ERK1/2-P, though not to peak values. Post 15mins of treatment, the metal compounds induced maximal early ERK1/2-P (~3-fold increase; $p<0.001$). A dramatic decline in ERK1/2-P was observed after 30mins by all treatments that persisted past the 1hour time point. d. During the longer treatment times of 4-6hours a second more robust increase in ERK1/2-P was induced by all estrogens. Post 6hours of treatment, E_2 induced the greatest increase of ERK1/2-P (~7-fold), followed closely by G+ QDs (over 6-fold); Cd^{2+} and O+ QDs induced about a 5-fold increase in ERK1/2-P, (all $p<0.001$).

4.4 Use of the specific ER inhibitor, ICI 182 780 abolished both genomic and non-genomic effects induced by estrogens; including CdTe QDs.

The estrogen receptor inhibitor, ICI 182 780, is a specific ER antagonist demonstrating no agonist activity. To confirm whether the non-genomic estrogen signalling depicted in Figure 2, is in fact mediated via the ER, the previous experiments showing AKT-P and ERK1/2-P were repeated in the presence of the ER antagonist. The results obtained demonstrate that all increases in AKT-P,

induced by treatments with 17β-estradiol, ionic cadmium or QDs, at all time points were completely abolished (*p<0.01 or p<0.001*) in the presence of the ER antagonist. Previous values of AKT-P signal ranging as high as 398.94% ± 18.81% were reduced to between 86.53% ± 1.52% to 117.27% ± 0.34% in the presence of the inhibitor (Figure 3a).

Similarly regardless of the values previously obtained with 17β-estradiol, ionic cadmium or QDs, in the presence of the ER antagonist no peaks or biphasic trends were noticeable in the ERK1/2-P curves. While the values of ERK1/2-P from different treatments previously ranged between 82.34% ± 1.52% to 682.07% ± 4.81%, with the addition of the inhibitor they were all significantly (*p<0.01 or p<0.001*) reduced to between 87.98% ± 0.01 and 146.77% ± 2.63% (Figure 3b). As such the ER antagonist blocked non-genomic estrogenic signalling, further indicating that the AKT-P and ERK1/2-P observed post treatment with 17β-estradiol, cadmium and both orange and green QDs is mediated via the ER.

The BrdU assay, validating the indirect cell proliferative effects obtained via the Alamar blue assay and estrogen treatments (as shown in Figure 1), is based on measuring cell proliferation via the incorporation of uridine in new cells. The results obtained with the BrdU assay corroborated the findings of the Alamar blue assay; 17β-estradiol, cadmium and both orange and green QDs induce significant (*p<0.001*) cell proliferation post 96hours of treatments. However, the magnitude of cell proliferation was more accurately measured using the BrdU assay and found to be greater than estimated by the Alamar blue assay; results showed that after green QDs induced the maximum cell proliferation (372.10% ± 12.69%) followed by 17β-estradiol (347.36% ± 2.01%), orange QDs (323.9 ± 12.7) and cadmium (266.7 ± 6.53%) (Figure 3c). Addition of the ER antagonist, ICI 182 780, reduced this cell proliferation slightly below control, charcoal treated serum (CTS) conditions, likely due to the inhibition of basally present intracellular estrogens. The ER inhibitor in conjunction with 17β-estradiol, cadmium and QD treatments significantly (*p<0.001*) reduced cell proliferation between 77.95% ± 1.27% to 91.96% ± 1.71% as compared to control, CTS maintained, untreated cells. Thus estrogen receptor inhibition prevents ER-mediated genomic events

leading to cell proliferation, further affirming that QDs and ionic cadmium behave like estrogens and interact with the ER.

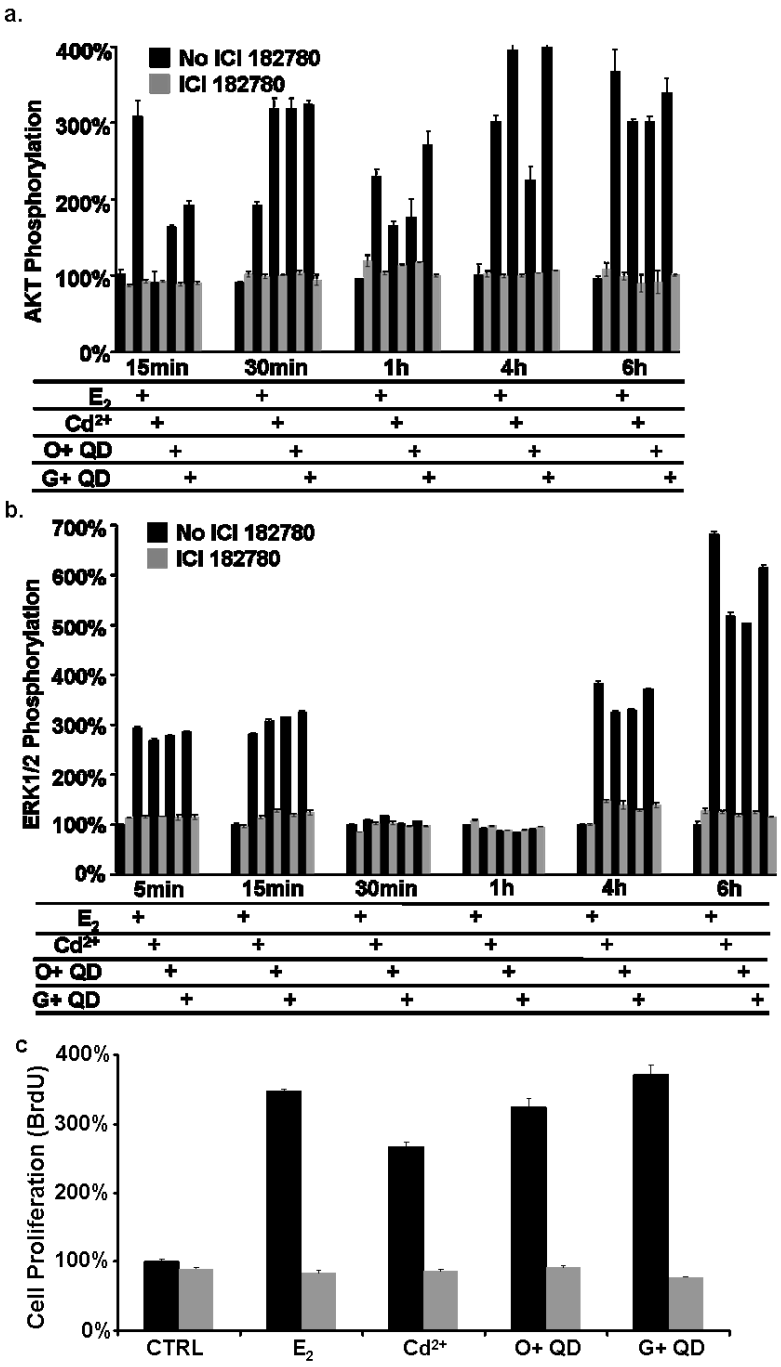

Figure 3: The selective estrogen receptor inhibitor, ICI 182780, prevents all estradiol, cadmium and QD-induced effects. a. Utilizing the AKT-P Elisa again, ICI 182 780 pre-treatment prevented E_2, Cd^{2+}, and both G+ QD and O+ QD

induced AKT-P at all time points examined in figure 2a &b. AKT-P increases induced by treatments were completely and significantly abolished upon pre-treatment of cells with the ER inhibitor; including both acute and secondary AKT-P peaks (at least $p<0.01$). b. ICI 182 780 pre treatment prevents all increases in ERK1/2-P observed post E_2, Cd^{2+} and QD treatment at all time points examined in figure 2c & d. Significant increases in ERK1/2-P induced by estrogenic treatments (using the ERK1/2 Elisa) were significantly reduced in the presence of the ER inhibitor (at least $p<0.01$) c. Estrogen induced cellular proliferation, evaluated utilizing the BrdU assay after 96hours of treatment, was inhibited in the presence of ICI 182 780. E_2 induced a ~3.5-fold increase in cell proliferation; which was completely abolished by the addition of the ER inhibitor. Cd^{2+} induced a ~2.5-fold increase in cell proliferation; again reduced post ER inhibitor employment. O+ QDs induced a ~3-fold increase in cellular proliferation; that was completely prevented by ER inhibitor application. Finally, maximal cell proliferation was obtained post treatment with G+ QDs (~4-fold) but entirely blocked by inhibition of the ER, affirming the integral role of the ER in mediating these responses. All treatment-induced cell proliferation was significantly different from results obtained in the presence of the inhibitor ($p<0.001$).

4.5 CdTe QD treatment leads to nuclear estrogen receptor activation

A nuclear receptor assay was employed to examine the nuclear ER activation by metalloestrogens. This commercial assay is based on reporter cells transfected with nuclear ERs and an ERα-responsive luciferase reporter gene. The standard curve of 17β-estradiol concentrations was plotted against the relative luminescence values (RLU); representing the 17β-estradiol concentration-dependent nuclear ER activation. This regression allowed for comparisons between metalloestrogens and the associated nuclear ER activation. Analysis of our 17β-estradiol control (10nM) via the assay (reportedly 1.315nM) ranked similarly to that obtained by 1nM of 17β-estradiol (detection limit of the assay) in the standard curve; likely indicating a saturation of the nuclear ERs. The RLU obtained from green QD treatment exceeded the maximal RLU obtained with

1nM of 17β-estradiol, and from regression analysis indicated a 17β-estradiol equivalence of 1.471nM. Both orange QDs and cadmium seemed to activate significantly fewer nuclear ERs, with a 17β-estradiol equivalence of 0.177nM and 0.008nM, respectively. However, both green and orange QDs induced significantly ($p<0.001$) higher receptor activation than ionic cadmium. Green QDs consisting of a CdSe core only demonstrated 0.0028nM 17β-estradiol equivalence (Figure 4).

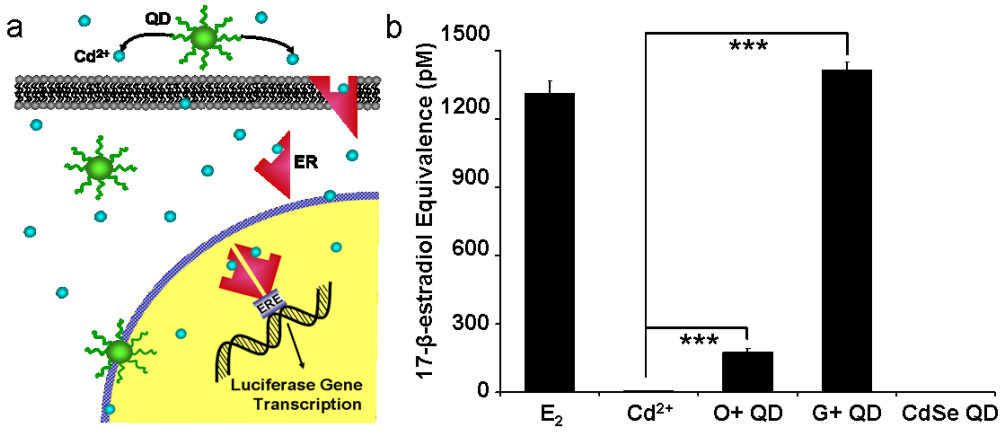

Figure 4: Metalloestrogens, including QDs can activate the traditional estrogen pathway involving ligand binding to nuclear receptors, receptor translocation into the nucleus, gene transcription and estrogen associated protein expression. a. The schematic represents QD decomposition and cellular internalization, as well as Cd^{2+} binding to the LBD of ER nuclear receptors, comparable with estrogen binding. Subsequent dimerization of the ER and nuclear binding of the dimer to the estrogen response element leads to downstream genomic, estrogenic effects. b. A reporter assay system was utilized to evaluate the estradiol equivalency of Cd^{2+} and QDs. Reporter cell treatment with estrogens for 24hours leading to ER activation, and the transcription of the luciferase reporter gene and subsequent luciferase luminescence was quantified and compared to an estradiol – luciferase luminescence standard curve. 10nM of E_2 induced similar luminescence as the 5000pM concentration maximum of the standard curve (supplementary figures). Cd^{2+} incubation led to a slight luminescence and depicted an estradiol equivalence of 83.4pM. O+ QDs demonstrated a somewhat greater estradiol equivalency of

177pM. However, G+ QDs produced a luminescence and estradiol equivalence of 1417pM; comparable with results obtained by 10nM of E_2. Both G+ and O+ QD-induced receptor activation was significantly different from Cd^{2+} ($p<0.001$).

4.6 Quantum dots demonstrate estrogen-like effects in vivo by increasing the uterine weight of ovariectomized mice

Young, five week old, prepubescent and adult ovariectomized BALB-C female were treated with 17β-estradiol, cadmium and two doses of green QDs for a period of two weeks, with alternate day dosing, prior to being sacrificed and their uteri being removed and weighed.

In the first group of animals both the dose of 17β-estradiol, cadmium and the low dose of QDs failed to induce any real change in uterine weight. The high dose of QDs, however, was able to induce a potent, two fold increase in uterine wet weight (198.91% ± 4.95%, $p<0.001$) (Figure 5a). Due to the greater magnitude of estrogenic effects induced by green QDs *in vitro*, green QDs were selected over the larger orange QDs to investigate whether QDs can illicit estrogenic effects *in vivo*, and how these effects compare in magnitude to those induced by 17β-estradiol.

In the case of the older, ovariectomized mice, the treatments induced increases in uterine wet weights, though to different degrees: 17β-estradiol caused an increase of 227.4% (± 19.87%, $p<0.001$)) and cadmium of 189.49% (± 10.56%, $p<0.001$). The high dose of green QDs, however, was able to induce the largest increase in uterine wet weight (250.03% ± 9.40%, $p<0.001$), thus acting as a potent estrogen capable of inducing a 2 to 2.5 fold increase in the uterine wet weight of mice, regardless of whether the animals were pre-pubescent or post-pubescent and ovariectomized (Figure 5b). Sample images (Figure 5c, 5d) depict the ovariectomized animals opened ventrally so as to display the uterus. The uterus of the green QD-treated animal is distinctly larger, with markedly more visible vascularisation as compared to the control animal; a typical estrogenic effect.

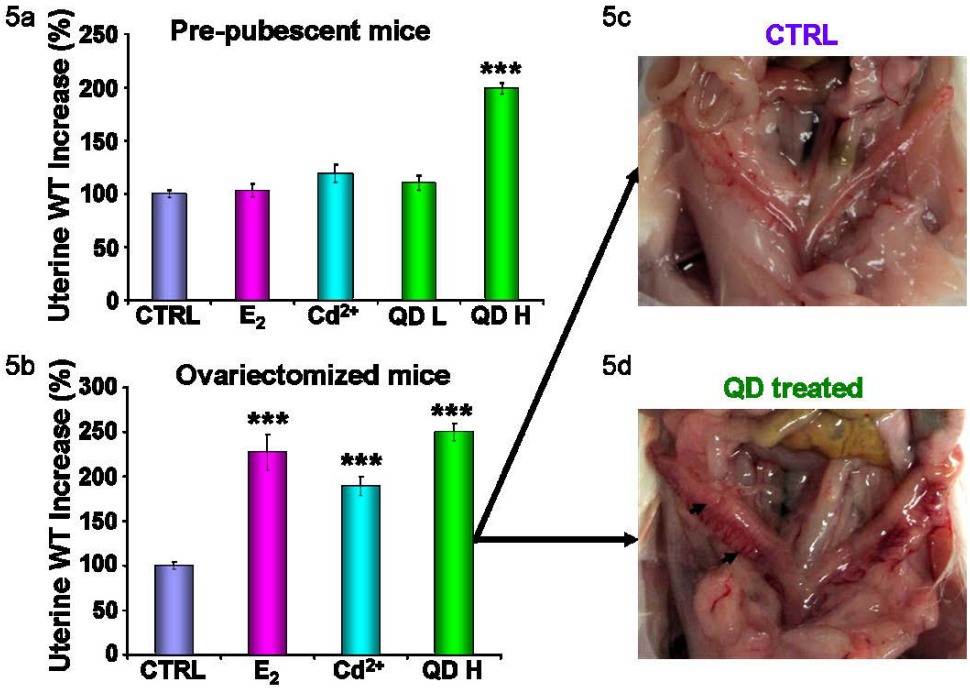

Figure 5: *In vivo* results show that the estrogenic effects of E_2, Cd^{2+} and QDs led to an increase in female mouse uterine wet weights post chronic intraperitoneal injections, administered every second day for two weeks. a. In prepubescent mice no significant change in uterine weight was observed post sacrificing the animals, in the E_2 (0.56mg/kg), Cd^{2+} (0.25mg/kg) and the low G+ QD (2.5mg/kg) group. Animals treated with the high dose (25mg/kg) of G+ QDs demonstrated a significant two-fold increase in uterine wet weight ($p<0.001$). b. In adult ovariectomized mice, uterine wet weights increased post all treatments; Cd^{2+} induced the least increase, followed by E_2, and a 2.5-fold increase induced by G+ QDs (all increases $p<0.001$). c. Image pictorially represents a sample uterus from a control, ovariectomized mouse. d. The image pictorially represents the marked increase in uterus size and vascularizatioon in an ovariectomized mouse treated with chronic, high doses of G+ QDs for two weeks.

5. Discussion

Here we show that CdTe QDs can induce estrogenic signalling and estrogenic effects in ER expressing cells both *in vitro* and *in vivo*, in mice. Previous studies

demonstrated that cationic green and red QDs are taken up by various cells, including MCF-7 cells [30-32]. Internalized CdTe QDs lacking shells are unstable and gradually release cadmium ions [21, 22]. Ionic cadmium can bind to the ligand binding domain (LBD) of the estrogen receptors and elicit estrogenic signalling, including the rapid phosphorylation of AKT and ERK1/2 in MCF-7 cells [27], and nuclear ER meditated cellular proliferation (Figure 6) [6, 33]. Estrogenic effects instigated via QD-treatment were comparable to 17β-estradiol treatment, and occasionally exceeded the effects induced by the latter. Selective ER antagonist, ICI 182780, prevented all QD-induced estrogenic effects. *In vivo*, chronic QD treatment in pre-pubescent female mice and ovariectomized mice, led to an increase in uterine wet weight, suggesting that QDs exert metalloestrogenic effects both *in vitro* and *in vivo*.

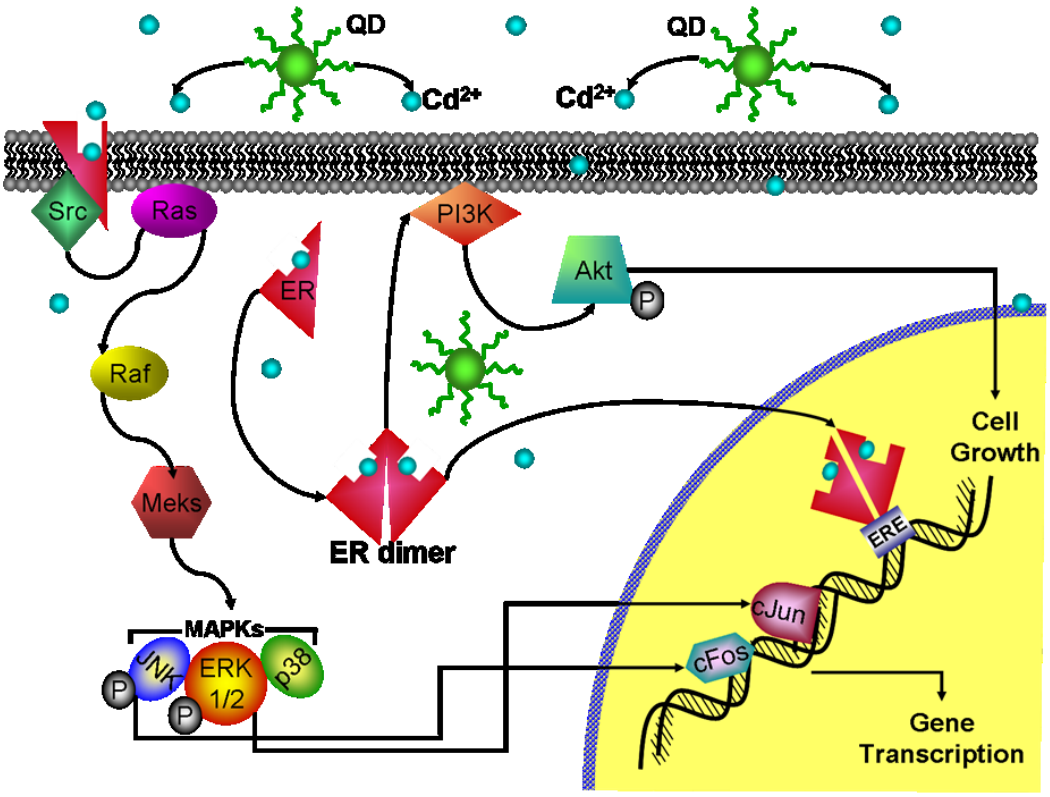

Figure 6: Schematic representation of QD-induced metalloestrogenicity. Surface compromised CdTe QDs can leach heavy metals such as Cd^{2+} from their core. This metal leaching may transpire extracellularly, leading to Cd^{2+} internalization via transporters; conversely, the cationic QDs may be passively internalized and

degraded within the cell, leading to the presence of intracellular free Cd^{2+} ions. Cd^{2+} may then bind to the LBD of the ER and trigger estrogenic effects, including: 1- the activation of the SRC, RAS, RAF, Meks and MAPK pathways, leading to ERK1/2-P, 2- activation of the PI3K, pathway, leading to AKT-P, and 3- activation of the traditional genomic estrogenic pathways involving ER dimerization and binding to the ERE preceding gene transcription and protein expression.

Human breast cancer (MCF-7) cells expressing ERs were employed to explore the potential metalloestrogenicity of QDs [6, 10, 27, 34, 35]. Results from the studies presented here differ from previous studies employing a single dose of cadmium [27, 33, 36]. This is likely due to (i) gradual and sustained release of ionic cadmium from QDs, and (ii) the presence of cadmium-containing nanocrystals which may interact with the ER localized in the plasma membrane. Cellular internalization of cadmium ions may occur through multiple modes, two of which are the divalent metal transporters (DMT1, more specifically) and calcium channels [37, 38]. It is suggested that "free" cadmium ions enter the nucleus via diffusion [39], where they bind to nuclear matter and gradually accumulate. However, as the cell detects this increase in cadmium concentration, it gradually increases the transcription and production of antioxidant proteins such as glutathione (GSH) and most importantly, metallothionein (MT). As the MT concentration in the cytosol increases cadmium slowly translocates from the nucleus to the cytosol, binds to MTs and becomes innocuous [40]. However, the QDs tested here, are likely not exported from the nucleus in a manner similar to cadmium; rather they may interact with chromatin (leading to the epigenetic regulation, [24]) and/or bind to the nuclear ERs (leading to gene regulation similar to 17β-estradiol [41, 42]. Studies done by Lovric et al [30], suggest that these small QDs can enter the nucleus, likely via pores in the nuclear envelope. Once present in various cellular compartments, QDs may continue to release cadmium ions, perhaps inducing a modest increase in MTs expression, and a greater

interaction with nuclear ERs. This may contribute to the more robust estrogenic response in QD-treated cells than those treated with cadmium.

Cell proliferation induced by estradiol was detectable within 48hours of incubation, however, cadmium induced proliferation was only detectable after 72hours of treatment. Post 96hours of treatment in MCF-7 cells, all estrogenic and metalloestrogenic agents tested here induced comparable cell proliferation. The delay in cadmium-induced cell proliferation may likely be ascribed to a plateau in MT induction [43] resulting in the enhanced cadmium interaction with and binding to ERs. If the cadmium released from the QDs was below the threshold required to elicit a robust MT response, more free metal ions may bind to ERs [21]. In the absence of ERs, however, QD-released cadmium can contribute to QD-induced cytotoxicity at concentrations lower than in cells expressing these receptors (as depicted in Figure 1c).

Early, non-nuclear, ER-mediated estrogenic events include ligand binding to cytoplasmic and cell surface membrane receptors, leading to the activation of two signal transduction pathways also involved in cell growth and proliferation; namely the PI3-K/AKT pathway, and the RAS-RAF-ERK pathway [44, 45]. Involvement of these two pathways in the metalloestrognic response was first reported by Lui *et al*. who demonstrated similarities in AKT and ERK phosphorylation induced by 17β-estradiol and cadmium[27]. Results from the experiments examining AKT and ERK1/2 phosphorylation presented here depict comparable trends between estrogenic and metalloestrogenic treatments. With respect to AKT-P, 17β-estradiol induced an initial peak and subsequent decrease within the first hour of treatment, followed by a more gradual sustained increase in AKT-P. In the case of cadmium and the two QDs, comparable patterns of AKT-P were observed, although the peaks and falls were slightly right shifted, indicating a delay in the AKT-P induced by metalloestrogens. This delay may be ascribed to the greater time required for the cellular internalization of cadmium and nanopaticles or the greater affinity of the ER for 17β-estradiol versus cadmium. Currently no consensus exists in the literature as to whether cadmium has comparable or less affinity than 17β-estradiol to the ER [10, 46]. While a

delay was noted in cadmium-induced cellular proliferation (relative to the other treatments), AKT-P induced by cadmium and all other metallic treatments was relatively comparable, perhaps due to the insufficient time for MT up-regulation. The biphasic ERK1/2-P induced by the various treatments was both comparable in terms of trend and phosphorylation magnitude. The overall magnitude of ERK1/2 phosphorylation induced by green QDs and 17β-estradiol, post 6hours of treatment was greater than induced by the other two treatments. However, while a robust estrogenic response was anticipated by17β-estradiol treatment, the equivalent if not greater estrogenic effect induced by green QDs in several experiments, was unexpected.

In the classical model of estrogen signalling, estrogens bind to the nuclear ER, dimerize and then along with numerous cofactors and co-activators form a complex which binds to the ERE on DNA and leads to transcription and translation of various genes [47]. To assess whether metalloestrogens, namely cadmium and QDs were also capable of activating this classical estrogenic signalling pathway, a nuclear ERα reporter assay was utilized. Results from this assay attested to the potent estrogenic activity of both green QDs and 17β-estradiol, and to a somewhat lesser extent, orange QDs; barely detectable activation of ERα by cadmium was noted. It is conceivable that the constitutive MT and glutathione levels of the reporter system were higher than those in MCF-7 cells, thereby preventing ionic cadmium from interacting with ERs. Additionally, relatively fewer calcium channels and/or DMT1 receptors (compared to MCF-7 cells) may have limited the cadmium internalization and subsequent nuclear ER activation. Indeed, several studies have shown that nanoparticle entry into the nucleus can vary dramatically between cell types and can depend on nanoparticles size [48]; smaller green QDs can be visualized within the nucleus, whereas, larger, red (and orange) QDs remain in the cytoplasm. Our data are in accord with this since robust estrogenic effects were observed with green (small, easily diffusible) QDs as opposed to orange QDs (larger, less likely to enter the nucleus and bind to ERs). Concomitant application of the selective ER inhibitor obliterates ERα activation, and as such verified that

the estrogenic effects observed here, and in other experiments, are in fact mediated via the ERs. These findings implicate green QDs as being highly estrogenic, and eliciting estrogenic responses comparable to 17β-estradiol in several cell systems.

Gagne *et al.* demonstrated in a recent study that treatment with toxic QDs led to an increase in ubiquitin protein, suggesting ubiquitin binding to the QD surface [49]. Given that ubiquitin is a protein necessary for the degradation of many intracellular proteins and receptors, including the ER, we speculate that sequestration of ubiquitin by QDs, may lead to a decrease in ER degradation. The extended life of the ER may facilitate a greater metalloestroginc response by QDs than cadmium or even 17β-estradiol. The same group also proposed that QDs may cause the sequestration of heat shock protein 90 (HSP90); a protein that under basal conditions is bound to the ER and maintains the ER in an inactive state [50]. Thus, HSP90 sequestration by QDs enables both ligand dependent and independent ER activation and potentially explains the enhanced estrogenic response induced by CdTe QDs in ER expressing cells.

Having ascertained the metalloestrogeinicity of QDs in two cell systems, it was imperative to examine whether similar QD-estrogenicity was detectable *in vivo*. BALB-C mice were treated with 17β-estradiol (0.56mg/kg), cadmium (0.25mg/kg) and green QDs (2.5mg/kg or 25mg/kg), via intraperitoneal injection every alternate day for two weeks. These doses were selected from the literature due to maximal estrogenic responses reported to be elicited; for explanations regarding QD-dose selection, please refer to the supplemental material. Results showed that in both pre-pubescent animals and the older, ovariectomized animals green QDs were able to exert potent estrogenic effects. While all the estrogenic treatments induced a significant increase in the uterine wet weight of the mice, green QDs induced the most significant increase. In the ovariectomized animals the increase in uterine weights was expected; though the extent of change obtained by QDs was surprising. The remarkable increase in uterine weight in the pre-pubescent animals was unique, because neither 17β-estradiol nor ionic cadmium treatment produced such an effect. Estrogen levels in the

ovariectomized animals are significantly reduced due to removal of the ovaries, whereas, in the pre-pubescent animals the levels are naturally low, as the reproductive system has not yet developed to rely and respond to subtle changes in estrogens. Thus, the results presented here point towards several possible untoward effects due to the cadmium-containing nanocrystals: (i) young females exposed to QDs may be at risk for endocrine disruptions and complications including an increased risk for developing breast cancer [51]. (ii) BrdU and Alamar blue assays following 17β-estradiol, cadmium and cadmium-containing nanocrystals QDs, induced several fold cell proliferation implying a further risk of carcinogenesis. According to the Environmental protection agency (EPA) cadmium is considered a class B1, probable carcinogen, associated with hyperplastic growth and cancers of various organs [52, 53].

Pregnant mothers and their offspring's may be susceptible to both the toxic and estrogenic effects of cadmium-containing QDs and other cadmium-releasing nanomaterials. A recent study affirmed that small QDs (1.6–3.2 nm) and cadmium ions were able to traverse the placenta of pregnant rats, and as such accumulate within the offspring *in utero* [54]. Therefore, the risk of developmental complications posed by cadmium can be numerous: from direct toxicity [55, 56] to metalloestrogenicity and oxidative stress [57]. Ionic cadmium was clearly detectable in offspring; however, the ratio of cadmium concentration in the mothers' livers (~6000ng/g tissue) versus the pups' tissue (~20ng/g) was far greater. This high concentration of cadmium in the livers, other vital organs and systemic circulation of mothers, not only poses a threat to the mother's health, but also an indirect threat to the pups' health and growth. These findings implicate these populations as being at the greatest risk, if exposed to excessive cadmium, either in its ionic form or even more so, in the form of QD nanocrystals.

Cadmium containing QDs and other nanostructures have been used as versatile research tools in cell biology and biochemistry, telecommunications, solar cells, light emitting diodes (LEDs) and photo inks [58-60]. The long-term effects of disposed nanomaterials on the environment and human health are currently unknown, particularly when present in barely detectable amounts. Cadmium

exposure from pollution: industrial emissions and cigarette smoke are already a health concern [61] and cadmium contamination of water supplies can lead to deleterious side effects, both toxic and estrogenic in the aquatic life forms [13, 46], which poses a potential threat to humans upon consumption. Thus potential effects of metallic nanostructures, including QDs, warrants thorough investigation to not only better understand these effects but institute adequate prevention and protection strategies from any undesirable side effects. Already, there have been many advances in the production of novel cadmium free QDs (CFQDs) [62].

6. Conclusion

In summary, results from the present studies show that: (i) certain cadmium-containing QDs can elicit estrogenic effects. (ii) These QDs can initiate both non-genomic and genomic estrogenic signalling involving ERs. (iii) Cadmium-containing nanocrystals are more potent metalloestrogens than cadmium ions *in vitro* and *in vivo*. **This study introduces the concept of metalloestrogenicity induced by cadmium-containing nanostructures and suggests further mechanistic studies to reveal possible unexplored long-term (undesirable) effects. The ultimate goal of such and similar research is to aid the development of adequate means and measures for human and environmental protection against nanometalloestrogens**

7. Future Perspectives

Quantum dots are highly versatile tools with the potential for wide application in various industries, including the biosciences. However, due to CdTe QD-induced cytotoxicity, the use of these nanocrystals will likely be restricted to electronics and research tools in scientific laboratories. Cadmium exposure and prevalence is quite high due to industrial use, cigarette smoking and cadmium pollution of water supplies and thus food. What is characteristic about nanoparticles is their ability to self-assemble; the spontaneous, unintentional assembly of nanoparticles and subsequent human and environmental exposure may be a cause for concern.

Knowledge of the toxicity and estrogenic threat of ionic cadmium is relatively well documented, but there is currently limited information regarding cadmium-containing nanocrystals. Our studies are the first to examine and characterize the estrogenic threat of cadmium-containing nanocrystals *in vitro* and *in vivo*. Based on our findings we propose that cadmium-containing QDs may induce far more potent estrogenic effects than ionic cadmium alone. Further, given the long half-life of cadmium and how harmful cadmium toxicity and estrogenicity may be, particularly in pregnant females, appropriate measures are needed to protect and prevent this cohort and the general population from the deleterious effects of ionic cadmium and cadmium nanocrystals. Already numerous studies have reported the feminizing effects of various xenoestrogens in aquatic life; and though yet to be ascribed to the estrogenicity of QDs, a study has reported the upregulation of the vitellogenin gene, an estrogen responsive gene, in rainbow trout livers post CdTe QD exposure [63]. **Thus, this study introduces CdTe QDs as potent metalloestrogens, and aims to raise awareness about this potentially dangerous side effect of QDs. Further, understanding the mechanisms of QD-metalloestrogenicity is the first step towards implementing appropriate measures to both protect and prevent the side effects associated with these nanocrystals.** Lastly, this study also points towards the need to move away from cadmium-containing QDs and instead increase the use of cadmium-free QDs (CFQDs) in the future.

8. Executive Summary

- In this study we demonstrate that CdTe nanocrystals induce estrogenic effects by signalling through estrogen receptors.
- Cellular responses to QDs are remarkably different in cells expressing ERs from those devoid of them.
- Nanometalloestrogenicity involves both genomic and early, non genomic signalling pathways, including the activation of Ras-Raf-MEK-ERK1/2 and PI3-K –AKT.

- The selective estrogen receptor inhibitor, ICI 182 780 abolishes all estrogenic effects induced by 17β-estradiol, ionic cadmium and QDs, thus indicating that QD-induced estrogenic effects are mediated through the ER.
- Our *in vivo* studies show QD-metalloestrogenicity can be observed both *in vitro* and *in vivo.* Chronic administration of green QDs can induce a two to three fold increase in the uterine wet weight, comparable or greater than 17β-estradiol, in both pre-pubescent ovariectomized mice and adult mice.
- Taken together out results demonstrate that CdTe nanocrystals are more potent metalloestrogens than ionic cadmium.
- **The results from this study suggest that nanotoxicological assessments should include assays for more subtle effects such as estrogenicity and endocrine disruption, that may be associated with carcinogenesis and other deleterious effects and may be induced by low picomolar concentrations of nanomaterials**
- Understanding the mechanisms of QD-metalloestrogenicity is the first step towards implementing appropriate measures to both protect and prevent the side effects associated with these nanocrystals.

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Chapter 4

Discussion

General Discussion

The application of nanostructured materials in medicine, including nanoparticles, is rapidly expanding. Although, they hold tremendous potential, their long-term effects, especially in minute quantities, are unknown. This thesis addressed several questions related to the effects of cadmium containing nanocrystals by employing various biological models, starting with immortalized cells, primary cultures, three dimensional explants and finally whole animals.

Certain nanoparticles, particularly polymeric and non-metallic nanoparticles are already in clinical trials and a select few have even been FDA approved for patient use [1]. However, much ambiguity remains regarding the toxic potential of these nanoparticles as there are currently no strict guidelines regarding how toxicity should be assessed. Numerous assumptions regarding nanotoxicity have been made, but few thorough studies have thus far been conducted to adequately identify and understand the factors mediating nanoparticle-induced toxicity. Therefore, my thesis work strived to uncover what mediates CdTe QD toxicity, and better understand how to evaluate nanotoxicity.

1. A brief summary of results

Our preliminary studies were intended to ascertain whether QD-toxicity was simply a consequence of cadmium ions leaching from the QD-core. If this were the case we expected that the concentrations of cadmium released from the QDs and present intracellularly should correlate with the induced cytotoxic effects. We, therefore, adapted a fluorescent assay capable of quantifying cadmium ions in solution, to measure cadmium ions both in the cellular media, and intracellularly [2]. First, human breast cancer cells (MCF-7) cells were treated with various concentrations of cadmium chloride and various QDs (green, red, cationic, anionic), after which the free cadmium present in the cell media, and intracellularly in the cells was measured. Next, the cytotoxicity of the various concentrations of cadmium chloride and various QDs was measured. Finally, the two sets of data were graphed; a high concentration-dependant correlation ($R=0.86$) was apparent between the intracellular cadmium, from cadmium

chloride treatments and the degree of cytotoxicity induced by each. However, the intracellular cadmium released by QDs showed no correlation with QD-induced cytotoxicity. Confocal microscopy with lysosome specific fluorescent dyes demonstrated that QD treatment lead to lysosomal damage; likely caused by the free cadmium, reactive oxygen species formation or photoxidative processes. These preliminary studies indicated that QD-induced cytotoxicity was more complex than the simple leaching of cadmium ions, and further, that QD-toxicity varied based on the nature and composition of the QD. Cadmium selenide, zinc-sulfate capped QDs neither induced cytotoxicity nor released a detectable amount of cadmium intracellulary.

Having observed that cadmium toxicity does not solely explain QD-toxicity, we next examined whether QD-toxicity could be explained as a sum of parts of the core constituents' toxicity. The results showed that QD-toxicity is not simply an additive effect induced by the combination of cadmium and tellurium ions [3]. The study also evaluated QD-induced toxicity in three increasingly complex models of the peripheral nervous system (a stable cell line – PC12 cells, primary dispersed dorsal root ganglia cell cultures - consisting of neurons and Schwann cells, and 3D dorsal root ganglia tissue explants). CdTe QDs induced maximal toxicity in the PC12 cells, but less in the dispersed DRG cultures. Even the LD_{50} of cadmium ions in the DRGs was significantly higher than in the PC12 cells. These results highlighted the importance of using both simple and complex cell systems to better understand and assess nanoparticle toxicity. Lipoic acid, a cadmium chelator and both potent direct and indirect antioxidant was utilized in three ways to prevent QD-induced cytotoxicity. The cells were pretreated with LA before QD-treatment (utilizing LA as an antioxidant), LA and the QDs were incubated *ex-vivo*, prior to cell treatment (utilizing LA as a cadmium chelator), or the QDs were capped with the reduced form of LA, DHLA (utilizing LA to stabilize the QDs). All three modes of LA application prevented significant QD-toxicity, and decreased the amount of free cadmium present in PC12 cells; however, in dispersed DRGs only LA pretreatment was unable to prevent QD-toxicity, though it did reduce the intracellular cadmium. DRG explants

demonstrated functional impairments upon QD-treatment and neurite degeneration. However, explants pretreated with LA maintained healthy neurite morphologies, thus stressing the need to rigorously screen for nanoparticle toxicity.

Having ascertained in the previous two studies that the cadmium released from the QD-core was not the mediating factor in QD-toxicity, we also concluded that the cadmium, once liberated from the QD, is deposited in the cell. However, cadmium, known to have an extremely long half-life, is also poorly cleared from cells. We were thus interested in examining the potential effects of QD-released-cadmium in cells, particularly the long term implications of low doses of this cadmium. In preliminary studies, we incubated both human breast cancer, MCF-7 cells and rat PC12 cells with low doses of QDs, for a prolonged period. Our findings in the two cell lines were paradoxical; in PC12 cells, the QDs continued to induce low levels of toxicity, whereas in the MCF-7 cells the QDs appeared to induce a proliferative effect. From scientific literature it has been shown that in cells expressing estrogen receptors (ERα and GPR30), cadmium can induce estrogenic signalling via acting on the estrogen receptors [4, 5]. As such cadmium is not only an endocrine disruptor, but a metalloestrogen [6]. Therefore, given that QDs release cadmium, we next investigated whether, at lower concentrations, QDs may also exert metalloestrogenic effects. Our finding showed that in MCF-7 cells, expressing all three estrogen receptors (ERα, ERβ and GPR30) QDs were able to induce both genomic and non-genomic effects [7]. Treatment with the specific estrogen receptor inhibitor, fulverstrant, completely prevented all effects, thus suggesting metalloestrogenic effects were mediated via the ERs. This finding was strengthened by the additional studies in PC12 cells lacking estrogen receptors; as in PC12 cells, both estrogenic and metalloestrogenic effects were absent. Having demonstrated that QDs can exert metalloestrogenic effects *in vitro*, we next examined whether similar nanometalloestrogenicity could be exerted *in vivo* in both prepubescent and older female mice. We, therefore, treated female mice for a duration of two weeks, and injected either saline, 17β-estradiol, cadmium chloride or QDs every alternate day. After the two weeks, the mice were

sacrificed, the uteruses were removed and weighed. In the prepubescent animals, the QDs, but not 17β-estradiol, induced a two-fold increase in uterine wet weight, and in the adult animals 17β-estradiol and QDs induced a comparable 2.5-fold increases in uterine wet weight (more than cadmium chloride). Taken together this study shows for the first time that CdTe QDs exert metalloestrogenic effects, and as such are potent endocrine disruptors.

2. Current perspective of QDs in medicine

Despite their unique photophysical properties and advantageous qualities for biomedical applications, the actual promise of QDs in medicine is limited due to potential cytotoxic effects. It is, therefore, our suggestion to not consider cadmium-containing QDs for further *in vivo* studies, but instead improve the quality of non-cadmium containing QDs which might be more useful as diagnostic tools in medicine. Nonetheless, over the last decade numerous studies have emerged demonstrating the potential of QDs for biomedical applications. These studies have sustained the efforts and investigations to develop improved cadmium-free QDs. Discussed below is the current status of biomedical QD applications.

2.1 QDs for biomedical imaging and diagnosis

There are several optical properties which make QDs especially attractive for imaging and diagnostics. Aside from being resistant to photobleaching, QDs have narrow, symmetrical emission bands that are both long and stable [8]. This is due to the exciton excitation also being long; with respect to fluorescence lifetime, molecules that take a long time to become excited also remain excited and fluoresce stronger for greater periods of time [9]. Additionally, the shell added to QDs (ie. zinc sulphate) adds much stability to the QDs and raises the quantum yield from 10% to between 40-90% [10]. Many QDs can be excited from a single source, but emit a specific wavelength, allowing for multiple labelling of a sample. Overall these superior optical properties remain unaltered post

conjugation to biomolecules, and QDs are resistant to metabolic degradation (facilitating long term, repeat imaging).

Diagnostic and laboratory applications of QDs include, immunolabeling, cell motility assays, in situ hybridization, live cell labelling/markers and confocal microscopy [11-17]. The recent popularization of using QD-probes for fluorescence immunostaining is due to their diagnostic sensitivity and specificity. Currently, many Western blot kits and other kits employing QD-conjugated anti-rabbit and anti-mouse antibodies are available [18]. The ability to excite several QDs by the same wavelength allows for the multiplexing potential of QDs [19], such as high throughput screening of biological samples [20], which facilitate information acquisition regarding the location, abundance and distribution of specific proteins. QD staining employing multiplexing permits a correlation between molecular and morphological data.

Studies done by our group have demonstrated that naked, surface unmodified QDs can be internalized by cells and based on their size, imaged in different organelles [2, 21]. In one of the initial studies, Wu *et al*. in 2003, demonstrated that multiple QDs (CdSe, polymer encapsulated and Streptavidin functionalized, conjugated to immunoglobulin proteins) targeted to specific subcellular proteins could be used for live imaging and labelling of the HER2 (Human Epidermal Growth Factor Receptor 2) protein and organelle specific proteins [22]. The ability for QDs to cross cellular barriers, localize subcellularly, and facilitate imaging of particular targets is a promising diagnostic application, highlighting how QDs can be used for labelling and potentially quantifying cellular cancer markers and visualizing their specific cellular location.

In our study using tissue explants of the dorsal root ganglia, we showed the potential toxicity associated with high concentrations of QDs. However, in a more recent study, Cui *et al*., demonstrated that QDs conjugated to nerve growth factor (NGF) could be used to elucidate the mechanisms by which the NGF signal is propagated during retrograde axonal transport back to the cell body [23]. The fluorescence of the QDs conjugated to NGF, permitted the visualization of the complex in rat dorsal root ganglia cultures. The findings of this study

demonstrated the primarily retrograde movements of NGF. Additional analysis of the samples using electron microscopy, also revealed that endosomes and vesicles contained only a dimer of NGF, suggesting that this dimer may be sufficient to sustain NGF signalling during retrograde transport. This study exemplifies the value of QDs as an imaging tool.

Fundamentally, the high signal to noise ratio render QDs attractive for *in vivo* imaging [24, 25]. An early study that demonstrated QD use for *in vivo* imaging, injected cultured human prostate cancer cells loaded with QDs conjugated to prostate specific membrane antigen (PSMA) into nude mice [26]. Tumors were allowed to grow until they reached sufficient sizes for imaging. Subsequently, the mice were injected with QD-conjugates either via tail vein (active targeting) or subcutaneously (passive targeting). Both means of targeting, active and passive (due to EPR), were successful in labelling the tumors; highly sensitive, multicoloured fluorescence imaging of the cancer cells was achieved.

In a more general imaging study, Larson *et al*., injected water soluble QDs into mice in order to image both skin and adipose tissue [27]. Despite both these tissues being able scatter light, QDs injected into vasculature of a 1μm diameter, were clearly visible when imaging with multiphoton microscopy. Further, fluorescence correlation spectroscopy indicated that the QDs were stable and visible for over nine months. In a preliminary study by Kim *et al*., it was demonstrated that near infrared (NIR) QDs could also be used for effective mapping and visualization of the sentinel lymph nodes, due to better photon penetration and the lack of autofluorescence issues in the NIR region [28]. Further studies indicate that NIR QDs may prove valuable even during surgery, providing guidance for mapping the lymph nodes.

2.2 Quantum dots for drug delivery

Quantum dots have also been considered for drug delivery purposes. In our study using lipoic acid as a multi-modal agent capable of preventing QD-induced cytotoxicity, we employed QDs as drug delivery agents by conjugating them to dihydrolipoic acid. Conjugation of such a drug to the QD-surface, not only

stabilized the QDs and prevented QD-toxicity, but allowed the delivery of this powerful antioxidant to the cells. However, the use of QDs for drug delivery is particularly attractive in situations where the optical properties can be combined with drug delivery, so as to visualize where the drug is being delivered. Additionally, functionalized QDs can deliver drugs to specific organelles and intracellular targets. One such example demonstrated by Bagalkot *et al*., utilized an elegantly designed QD-aptamer-doxorubicin theragnostic system to both specifically deliver the drug to prostate cancer cells while simultaneously imaging the cells (Figure 1) [29].

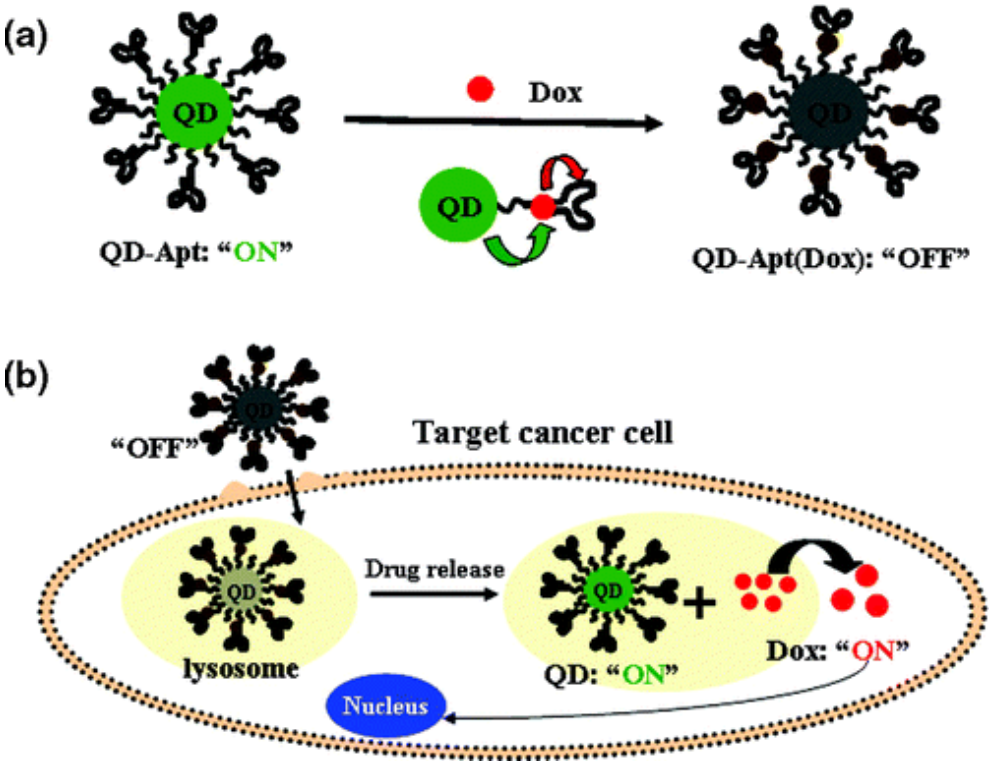

Figure 1: Schematic representation of QD-aptamer-doxorubicin drug delivery sensor [29]. a. Depicts that when doxorubicin is bound to the QD-aptamer system, neither the QD nor doxorubicin fluoresce. Whereas when doxorubicin is released from the system, both doxorubicin and the QD can fluoresce. b. Depicts the cellular internalization and co-localization of the OFF-system in the lysosomes.

Once in the lysosomes, doxorubicin is gradually released, and both doxorubicin and the QDs fluoresce.

The RNA-aptamer was employed as both a drug carrier and targeting moiety for the prostate specific membrane antigen, expressed on prostate cancer cells. When the three components of the system were conjugated, the system remained in a non-fluorescent "OFF" state. Given that both QDs and doxorubicin fluoresce when conjugated via the aptamer, a donor-acceptor model of fluorescence resonance energy transfer (FRET) took place, such that doxorubicin quenched the QD-fluorescence via absorption of the fluorescence. The fluorescence of doxorubicin was quenched by the presence of the double-stranded aptamer, thus maintaining the intact system in an "OFF" state. However, once the system was taken up by the prostate cancer cells, doxorubicin was gradually released from the conjugate, which led to the activation of the fluorescence of both doxorubicin and the QD. As such, the targeted delivery of the drug and the tumor could be monitored via imaging studies, and visualization of the cancer cells would verify drug delivery. In another study, QDs conjugated to amino acids and RNAi were employed *in vitro* to demonstrate effective and efficient QD-based silencing of certain genes for therapeutic purposes, coupled with simultaneous visualization of the RNAi delivery [30]. Similarly, Tan *et al*., utilized QDs conjugated with HER2-siRNA to track the delivery of the therapeutic siRNA, as well as monitor the effectiveness of HER2 down regulation in breast cancer cell, as mediated by the siRNA [31].

Lai *et al.,* demonstrated that QD- drug delivery can be controlled by surface modification of the QD. A mesoporous silica nanosphere based controlled-release delivery system utilized modified CdS QDs as chemically removable caps to retain drug molecules and neurotransmitters within the mesoporous nanosphere [32]. The cap ensured that the drugs inside the system were retained until released by a disulfide bond-reducing reagent. As such, QDs were able to prevent pharmaceutical agents of a defined size from prematurely leaking out of the delivery system.

While QDs theoretically hold much potential for drug delivery, they have not, and cannot be widely embraced for *in vivo* use, as issues of toxicity prevail. Thus, in order to capitalized on the promising qualities of QDs, is it imperative that non-cadmium QDs be investigated for potential biomedical application. Nonetheless, *in vivo* QDs studies continue to be conducted, as certain groups have demonstrated a lack of QD-toxicity. In a study by Dubertret *et al*., phospholipid encapsulated QDs were microinjected into single xenopous embryonic cells; these QDs were followed via fluorescence imaging for several days through early stage development and cell division [33]. Findings showed that the QDs were confined to the progeny of the injected cells, they did not leak through cell membranes, and no deleterious or abnormal effects on growth and division were noted. In another study, the authors reported injecting mice with five melanoma cell populations via tail vein injection, each population labelled with a different size (color) of cationic QDs. The QDs were tracked by multiphoton fluorescence microscopy as they extravasated to the lung tissue [34]. No changes or measurable differences were noted in labelled and unlabelled cells. Taken together, these studies suggest that surface protected QDs are much less toxic *in vitro* and *in vivo*. However, cadmium-free QDs (CFDQDs) remain better candidates for QD applications, though extensive research is required to ensure that they induce neither acute toxicity, nor any long term undesirable effects.

2.3 QDs for therapeutic applications

As shown in many of our studies, QDs are capable of inducing cytotoxicity in several cancerous cell lines. This toxic nature of QDs has been exploited for anticancer therapy, specifically, photodynamic therapy (PDT) [35]. PDT is based on the generation of singlet oxygen via the activation of a non-cytotoxic photosensitizer by light. QDs can act as photosensitizers by interacting with molecular oxygen via a triple energy-transfer process. Alternatively, QDs can act as activators of other photosensitizers by serving as energy donors that, via FRET, excite molecular oxygen into the singlet state. These altered oxygen molecules in turn lead to the production of reactive oxygen intermediates that can induce

oxidative stress and cytotoxicity. Reactive oxygen intermediaries are capable of damaging both purine and pyrimidine bases.

Though QDs are interesting candidates for PDT, empirically the quantum yield of QD-generated singlet oxygen is only 5%, whereas classical sensitizers have a quantum yield of 40-60%. Thus, QDs may be considered photosensitizers, however, additional carriers would prove a valuable addition so as to increase singlet oxygen generation and the efficacy of photodynamic therapy [36].

2.4 Mechanism of QD-induced cytotoxicity

From the initial reports of QD-induced cytotoxicity, our understanding of this phenomenon has both greatly evolved and yet remains to be elucidated. The common misconception that QD-toxicity is synonymous with cadmium toxicity has been dispelled. Since our early reports demonstrating that QD-induced cytotoxicity does not correlate with cadmium-induced cytotoxicity, and intracellular cadmium accumulation, many additional reports have confirmed and extended these findings [37]. Several mechanisms involved in QD toxicity and QD-metalloestrogenicity, have been proposed by our group. A summary of these mechanisms is shown in Figure 2.

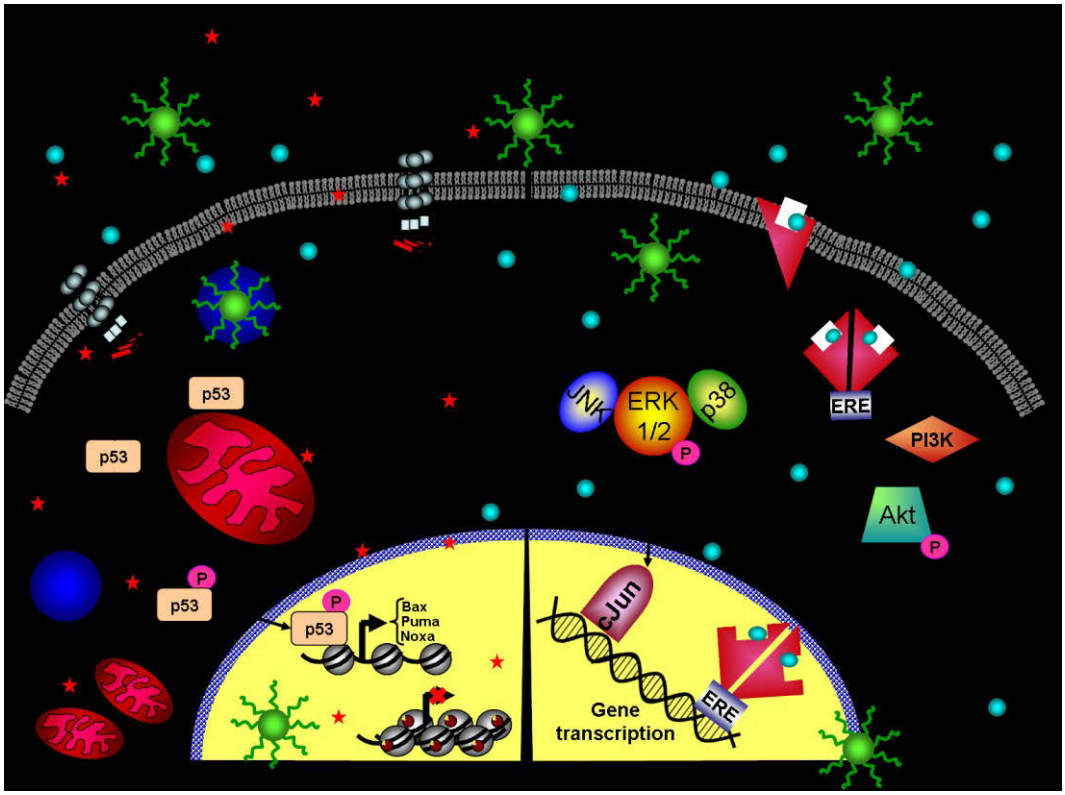

Figure 2. The paradoxical effects induced by QDs. In non-estrogen expressing cells, QDs induce cytotoxicity via ROS formation, cadmium liberation, upregulation of the Fas receptor, loss of mitochondrial membrane potential, and subsequent cytochrome c release and caspase activation. Conversely, in estrogen receptor expressing cells, QDs liberate cadmium that can interact with the estrogen receptor, and trigger non-genomic signaling, including AKT and ERK1/2 phosphorylation, followed by nuclear receptor activation of gene transcription related to cell proliferation.

Unpublished studies from my body of work established the inconsistency between QD and cadmium-induced cytotoxicity via the development of cadmium-resistant, human lung cancer (A549) cells [38]. These cells were maintained and passaged continually in the presence of increasing concentrations of cadmium, over four months. Experiments with QDs commenced once the cells became resistant to and were capable of growing in 2μM of cadmium, the former LD_{50} of these cells. We treated both cadmium resistant and non-resistant A549 cells equally, with 2μM of cadmium and both cationic and anionic CdTe QDs. Our findings showed that

cadmium resistant cells were far less susceptible to cadmium-induced toxicity (the previous LD_{50} underwent a right shift, such that 2µM of cadmium no longer induced any toxicity) but remained equally susceptible to QD-toxicity as compared to the control A549 cells. Further, the QD-toxicity endured in the resistant cells despite the significant reduction of intracellular free cadmium measured (Figure 3).

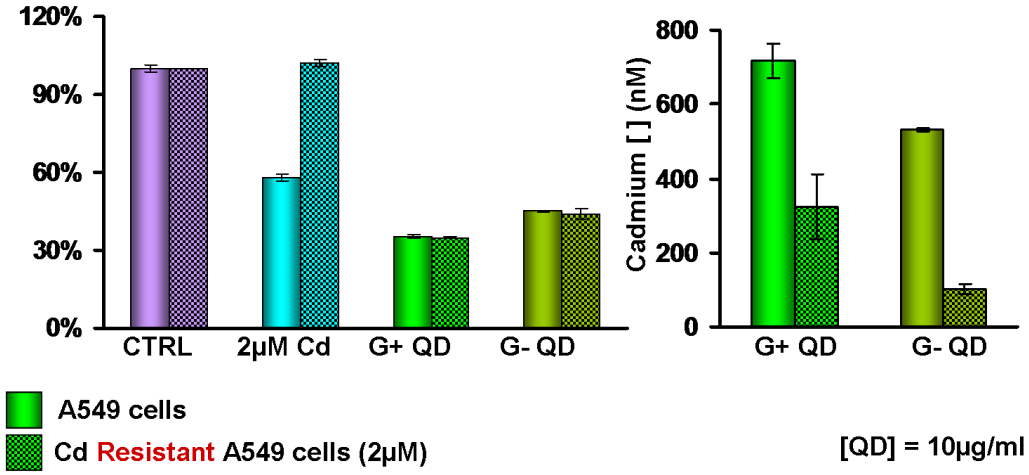

Figure 3. Cells continuously exposed to increasing concentrations of cadmium (from 10nM to 2µM) developed cadmium resistance. Cadmium resistant cells show a decrease in susceptibility to cell death from cadmium treatments, but respond equally to QD treatments (e.g. 10µg/ml of QDs, 24hours). Intracellular free cadmium, resulting from G+ (cationic) and G- (anionic) QDs is decreased in cadmium resistant cells as compared with regular A549 cells.

These results suggest that other mechanisms aside from free cadmium accumulation are involved in QD-induced cytotoxicity. Cadmium is a known inducer of oxidative stress [39], and studies by our group have shown that cellular treatments with antioxidants can prevent QD-induced cytotoxicity. Taken together, this implicates oxidative stress as a key factor involved in QD-induced cytotoxicity [2]. We thus, hypothesize that QD-induced oxidative stress occurs partially through a cadmium-independent mechanism, where QD-toxicity is initiated by the cellular photoxidation of QDs [40], involving electron transfers

from excited QDs to O_2 and resulting in the formation of superoxide (O_2^-), and an unpaired hole in the QD [41]. Subsequently, ligand oxidation, cleavage and additional oxidation and corrosion of the nanoparticle surface may be catalyzed [42], leading the excited QDs to potentially transfer energy to other molecules generating singlet oxygen [43], and other reactive oxygen species (ROS).

The induction of ROS and thus, oxidative stress, by nano-sized particles, has been well-established as an early indicator of nanoparticle toxicity [44, 45]. With respect to nanoparticle-induced ROS, Xie *et al.* suggested the use of a three tier system to evaluate the degree of oxidative stress. In the first tier, ROS generated is sufficient to instigate normal, protective cellular effects that are able to overcome the insult [46]. In fact, the naturally occurring intracellular antioxidants, such as glutathione are often sufficient to scavenge the ROS generated. In the second tier, the oxidative stress overwhelms the cellular antioxidant system, and the oxidative injury leads to the activation of proinflammatory transcription factors leading to an inflammatory response. In the third tier, the oxidative stress is so great that cytotoxicity and cell death are incurred. With respect to our findings in A549, lung cancer cells, we demonstrate the transformation of control lung cancer cells from intolerant to cadmium-induced tier 3 oxidative stress, to tolerant. Further, these cells depict an adaptive response, such that the same cadmium-induced stress in the altered cells barely induces tier 1 oxidative stress. This transformation or adaptation is ascribed to the upregulation of cellular antioxidant, glutathione and MT proteins [47, 48]; however, this adaptation was incapable of preventing QD-induced cytotoxicity, emphasizing key mechanistic differences between QD and cadmium-induced cellular stress.

To better elucidate the mechanisms involved in QD-induced cytotoxicity many studies have emerged examining the role of different cellular, morphological, biochemical and genomic markers involved. In a study by another member of our group, the involvement and upregulation of the Fas, death receptor on human neuroblastoma cells was reported, post QD-treatment, coupled with cellular lipid peroxidation [49]. The study also demonstrates that the nanocrystal's charge influences cellular internalization, such that more cationically charged QDs are

better internalized. Greater cellular uptake of QDs may correlate with a greater increase in cytotoxicity.

Early reports from our group and others, suggest that QD-induced cytotoxicity involves several modes of cell death that are not mutually exclusive [50]. Morphological and biochemical markers point towards the contributions of both apoptosis and necrosis with and without caspase activation. Investigations of QD-induced toxicity have generated much interest among QD experts and nanotoxicologists at large as it became evident that QDs were not equitable to cadmium ions, and nanoparticles were not simply miniaturized versions of their bulk products. This notion led to the undertaking of many nanotoxicological and mechanistic studies with the aim to improve QD-stability and reduce QD-toxicity. A study conducted by Chan *et al.,* claimed that in human neuroblastoma cells, un-capped or naked, CdSe QDs induce apoptosis via mitochondrial pathways [51]. The expression of several biochemical markers involved in apoptosis were examined, as induced by either naked CdSe QDs or ZnS capped CdSe QDs. The results showed that naked QDs increased ROS production, JNK activation, loss of the mitochondrial membrane potential, mitochondrial release of cytochrome c, and the activation of caspase 3 and 9. Further, naked QDs inhibited survival related events, including a decrease in Ras, Raf-1, pERK1/2, and HSP90. However, the study stressed the importance of QD-surface and core integrity protection, such that when QDs were capped with ZnS, no changes in JNK, caspase or Bax/bcl-2 were notable. These findings corroborated our results showing that CdSe ZnS capped QDs were neither cytotoxic, nor released detectable amount of cadmium intracelullarly [2, 3]. Similar findings were also reported by Yan *et al*. who demonstrated that the cytotoxic effects of CdTe QDs on human umbilical vein endothelial cells is both dose and time dependent [52]. Further, the authors showed that QDs induced oxidative stress and mitochondrial network fragmentation, leading to over a 400% increase in the expression of apoptotic proteins, including an increase in Bax and a decrease in bcl-2. The involvement of cytochrome c and caspase 9 and 3 were also shown, demonstrating common mechanistic events despite the different cell types.

Of studies investigating the toxic potential of QDs in non-human and non-mammalian cells, Gagne *et al*., were concerned with the implications of QD-toxicity on the environment and aquatic life [53]. They, therefore, examined the effects of CdTe/CdS QDs on the immunocompetence and hepatic gene expression changes in Rainbow trout after 48hours of exposure. With respect to immunocompetence, Gagne's group showed a significant decrease in leucocyte count and viability, coupled with a decrease in both resting and active phagocytic activity, in samples of kidney tissue. Harvested livers were used to prepare samples for a DNA microarray, the results of which showed that both cadmium and QD exposure led to an increase in MT and CP2K1 gene expression; both genes involved with metal detoxification and xenobiotic transformation/inflammation. Interestingly, the results of the microarray also indicated very different gene expression as induced by either cadmium or QD exposure. QDs, specifically, induced changes in the expression of 25 genes relating to inflammation, immunocompetence and endocrine changes; whereas, cadmium only precipitated changes in nine specific genes, pertaining to the binding and transport of solutes (sodium, potassium, urea). This study was the first of its type to compare genotoxic discrepancies between cadmium and QDs., The authors provide a mechanistic explanation for results highlighting the discrepancy between cadmium and QD-toxicity, first noted by our group. This study also demonstrates that QDs appear to be cytotoxic ubiquitously in all cells and cell types, regardless of their origin.

Several other studies have attempted to draw mechanistic comparisons between the toxicity induced by cadmium and QDs, and have also reported significant differences. Chen *et al*., evaluated the toxicity ZnS shell protected and non protected QDs in human embryonic kidney cells [37]. The study demonstrated that the ZnS capping of QDs can prevent QD-related cytotoxicity, as the shell stabilizes the metal core, preventing the leaching of toxic cadmium ions. Further, by examining genes that may be altered post QD treatment, the authors report the upregulation of stress responsive genes, involved in protective cellular processes (protein binding, metal ion binding, intracellular oxidoreductive reactions), and

seven genes from the MT family. Changes in gene expression induced by QD treatments were found not to be comparable with equivalent cellular cadmium treatments, only further affirming our findings, that QD-toxicity is not attributable to cadmium ion leaching. In addition, the authors hypothesize that the discrepancy in QD-mediated cytotoxicity and cadmium toxicity, as per imaging studies, is that while cadmium disperses throughout the cells, QDs congregate in the perinuclear area (near the nucleus and mitochondria), leading to spots of very high, local cadmium concentrations that can induce significant organelle damage precipitating QD-toxicity.

The most recent study probing the cytotoxicity of QDs by Ambrosome *et al*., examined the effects of QDs on invertebrates [54]. The authors exposed polyps to sub-lethal doses of QDs, and equivalent doses of cadmium, for a duration of 24 – 72 hours, during which time morphological and genotoxic changes in the polyps were observed. Firstly, it was noted that QDs cause significant morphological damage as compared to the equivalent cadmium dose, and low doses of QDs only resulted in the upregulation of stress-responsive genes and apoptotic genes. As compared to QD-treated cells, the cadmium treated cells internalized far greater amounts of cadmium; nonetheless, the toxicity was greater in QD-treated polyps. Analysis of polyp reproduction and regeneration showed that the polyps regenerate less quickly even post exposure to very low, sub-lethal concentrations of QDs, but this ability is rapidly lost with higher concentrations of QD. This study both highlighted the inability to equate QDs and cadmium and emphasized the need for appropriate functional studies, in order to understand and assess nanoparticle toxicity. The overall conclusions from this study were similar to our findings in the DRGs, based on which we too stress the need for three dimensional tissue analysis of QD-induced functional impairments.

As the field of nanotoxicology has evolved, the outcomes and implications of nanoparticle exposure examined have become more subtle. While initial studies sought to simply show cell death and viability, nanotoxicity studies have progressed towards evaluating biochemical, signal transduction and morphological changes at the organelle level. Currently even the genotoxicity

induced by nanoparticle exposure is being carefully investigated. The trend has been to move towards the evaluation of subtle changes induced by nanoparticles which could have longstanding implications. The field of epigenetics has thus far evaded most nanotoxicological studies. Epigenetic programming typically occurs during development, however, more studies now suggest that the adult's epigenome is sensitive to the surrounding environment, and that small epigenetic changes can accumulate to produce lasting alterations in the genome [55]. Gene transcription is controlled by subtle epigenetic processes occurring in the nucleus of the cell; these processes include DNA methylation and histone modifications (i.e. acetylation, methylation). In a pioneering study done by another member of our group, QD-induced epigenetic changes were evaluated. Even from earlier toxicity studies, it was evident that small QDs can enter the nucleus, and cause significant reorganization of the chromatin [21], as detected by electron microscopy and fluorescence labeling of the nucleus. These dramatic nuclear changes suggest that gene transcription was affected after long term QD exposure, and indeed we observed that p53, a well-established pro-apoptotic transcription factor, was upregulated and activated upon QD treatment, along with downstream targets, puma and noxa (both implicated in apoptosis induction) [56]. Additionally, global hypoacetylation was also noted, implying an epigenomic response to QD-treatment. This study, to date, remains the only study exploring cellular epigenetic changes as incurred post QD treatment.

In light of these genotoxic and epigenetic changes observed in the nucleus, there is a need to investigate and better understand the upstream signals that regulate these changes in gene expression. Such studies, exploring the subtle QD-induced changes will be increasingly necessary as nanomedicine and nanotoxicology eventually evolve to measure outcomes other than cell death and viability. Taken together, these studies still do not affirm the exact mode or mechanisms of QD-induced cytotoxicity, but do highlight the discrepancy between cadmium toxicity and QD-toxicity.

2.5 QDs and fetal development

Nanotoxicity is gradually moving towards examining subtle genetic, genotoxic and epigenetic changes that may be induced post nanoparticle exposure. With respect to epigenetic programming, nanoparticles that can initiate minute changes in gene expression, may not produce any immediate visible effects, but may have long term implications both for the individual and its progeny. Epigenetic changes have been shown to be passed down from parent to offspring via transgenerational epigenetic inheritance. In an early study, Anway *et al*., showed that the anti-androgenic fungicide, vinclozin, posed a transient threat during embryonic sex determination, resulting in spermatogenic cell defects and subfertility in males exposed [57]. These males at age one were found to develop a number of disease states, and further, this phenotype was transferred to the following three generations of male offspring via the male germ line. As such, seemingly harmless endocrine disruptors and agents that can alter the epigenome may have long standing effects that can be transferred through multiple generations. Nanocrystals, such as CdTe QDs, that have been shown to produce genotoxic and epigenetic changes in adult animals and cells, respectively. Additionally, as demonstrated by our studies, QDs are also metalloestrogenic endocrine disruptors. Such nanoparticles must also be investigated in gestating animals to ascertain whether the undesirable effects of these nanoparticles may be transferred to fetuses and lead to further trangenerational effects.

However, despite the numerous investigations surrounding QD-toxicity, relatively few studies have investigated the effects of QDs during gestation on fetal development. Oocyte development and viability is greatly influenced by the microenvironment; glucose, oxygen and heat stress can all play tremendous roles in determining the fate of the oocyte [58]. Additionally, during the embryogenesis many common chemicals may have magnified deleterious and teratogenic effects. Cadmium is considered to be a teratogen, as several reports have found abnormal morphological developmental occurrences resulting from high maternal concentrations of cadmium [59]. It has also been found that cadmium can traverse the placental barrier, and induce oxidative stress on the fetus [60]. Thus far,

relatively less is know regarding the implications of QDs on the fetus, though recently, a few preliminary, insightful studies have shown that QDs may be detrimental to the fetus *in utero*.

Chan *et al*., were the first to investigate the cytotoxic effects of QDs on embryonic development in mice [61]. They examined the implication of QD exposure both post-implantation and pre-implantation. Their results indicated that QDs induced dose-dependant apoptosis in mouse blastocytes. The authors showed that pre-treatment of the blastocytes with QDs inhibited cellular proliferation, development of blastocytes into morulas and post-implantation embryonic development. In general, fewer QD-pre-treated blastocytes reached the later stages of development, and those that did, had lower fetal weights, although many blastocytes exposed to the high dose of QDs (500nM) were associated with resorption. Overall, QD-exposed blastocytes remained capable of implantation *in vitro*, though post-implantation their development was retarded and eventually embryonic death was observed. However, the authors also demonstrate that QD capping with ZnS significantly reduces QD-toxicity on the blastocytes, but nonetheless conclude that QDs are potential teratogens.

A follow up study by the same group delved deeper into the cytotoxic potential of QDs. Using a similar model systems as in the previous study, the authors demonstrate a dose dependent decrease in the percent of fertilization and blastocyte development after QD-exposure [62]. Additionally, a significant decrease in total cell numbers and a marked increase in apoptotic cells were noted in QD exposed oocytes. Further, the authors report an increase in fetus resorption, and a decrease in implanation, survival and placental weight post QD treatment; all of which however, were prevented when the oocytes were exposed to QDs with a ZnS shell. The authors interpret this toxic potential of QDs on fetal development as being due to QD-surface oxidation, resulting in cadmium release and subsequent toxicity. They also imply that studies deeming QDs as non-cytotoxic may be due to brief or less prolonged QD exposure, where insufficient time has elapsed for the QD-shell to become compromised. The authors further suggest that QDs may remain in the system for days and months like ticking time

bombs, that may exert toxicity as and when their shell becomes compromised. Similar findings were observed by our group, that with time, QDs become increasingly toxic (unpublished data) regardless of the model system employed for evaluation.

While much attention has been directed towards the toxicity of nanoparticles in cells and even whole animals, the risk of nanoparticle toxicity was not explored in gestating animals and their offspring *in utero*. Therefore, Chu *et al*, investigated whether QDs can cross the placental barrier and be transferred from mother to pups, *in utero*, and whether the developing fetuses may be more sensitive to the risk of QD-toxicity [63]. In this study, pregnant mice were intravenously injected with QDs, 1-5 days prior to delivering their pups. Subsequently the pups underwent elemental analysis for cadmium deposits. The results of this study demonstrated that QDs can, in fact, traverse the placenta, as cadmium deposits were found in the pups. Further, it was found that smaller QDs were able to cross the placental barrier more easily and, therefore, accumulate in higher concentrations in the pups. In general, the higher the dose administered to the mother and the smaller the QD, the greater the accumulation in the babies. Capping of the QDs with either PEG or silica resulted in decreased placental crossing and pup exposure. Examining the distribution of QDs in the mother, showed that most cadmium was deposited in the liver, spleen, kidney, lungs and heart. Only a very minute (1 thousandth) amount of cadmium from QDs accumulated in the fetuses as compared to the maternal liver. No detectable fluorescent signal was visible in the pups, suggesting that the surface integrity of the QD may have been compromised, the QD may have degraded, or the QDs remained in too small a concentration to be detected. Finally, an inverse relationship between QD-dose and pup survival was reported.

The findings of our group showing that QDs are metalloestrogenic has yet to be factored into QD-toxicity in oocytes or on fetal development. Bisphenol A, is a well established estrogen mimetic [64] and endocrine disruptor [65]. Several papers have reported toxic effects *in utero* post bisphenol A exposure. Additional studies are required to ascertain whether QDs may be classified as teratogens.

Further, studies probing the root of QD-toxicity *in utero* are also needed to uncover whether such QD-toxicity is due to: 1. toxicity indirectly incurred post maternal QD-exposure, 2. QD-toxicity resulting from the nanocrystals crossing the placental barrier and directly exerting toxicity on the fetus, or 3. QDs acting as metalloestrogens and causing endocrine disruption. In humans these studies suggest that nanoparticle exposure both from direct contact and indirect environmental exposure may result in grave effects that could have implications for future generations. Further, these studies suggest that pregnant women and pubescent adolescents may be particularly vulnerable to the toxic potential of QDs, warranting specific precautionary measures to be implemented to protect this population.

2.6 QDs – Future perspectives

QDs are promising tools for a variety of industries, and in medicine, for applications including imaging, drug delivery and other therapeutic purposes. However, too many reports of QD-toxicity have been brought to our attention, and while surface modifications can prevent some of the potential toxicity, there is currently no way of predicting how enduring the protective shell may be. The risk of the ZnS or PEG shell becoming compromised, and the core of the QD being exposed *in vivo*, may lead to dangerous outcomes, including cadmium induced toxicity, carcinogenicity, metalloestrogenicity, genotoxicity and even epigenetic side effects with the possibility of transgenerational effects in offspring. Thus, the likelihood of cadmium-based QDs ever being used for medical purposes is minimal. Nonetheless, cadmium-based QDs continue to be popular and prevalent for many technology based applications, and, therefore, manufactured [66]. From an environmental standpoint, QD production and use has implications on air pollution, soil and water pollution, thereby affecting our food supply and aquatic life, and eventually us. Therefore, understanding the potential effects of QD-exposure, particularly the prolonged, repeat exposure to minute concentrations of QDs, and or QD-components is absolutely paramount to ensuring our safety.

The superior optical qualities of QDs render them highly attractive, lucrative and promising for use in the medical industry. Therefore, much research is being done to produce better, cadmium-free QDs [67]. These cadmium free quantum dots (CFQDs) consist mainly of rare earth elements, primarily the fifteen Lanthanides along with scandium and yttrium [68-70]. These CDQD retain the optical properties of cadmium-based QDs: they can be used for multiplexing, their synthesis is aqueous based, and their emissions depend on the specific rare metals used. One of the few reports investigating the potential cytotoxicity of CFQDs, by Chibli *et al*., examined indium phosphide (InP) QDs, and showed that though the toxicity associated with the constituent elements is very low, these QDs do still exhibit phototoxicity [71]. This phototoxicity relates to the generation of ROS by excited electrons and or electron holes interacting with water or oxygen. The authors report a considerable amount of superoxide and some hydroxyl radicals produced under QD illumination. However, the study reports that double ZnS shelled protection of the InP QD reduced the toxicity two fold, and survival assays in a variety of cell lines show a significant reduction in toxicity. Overall the toxicity seen with these CFQDs is significantly less than with cadmium based QDs.

Silver QDs, comprised of silver and selenium, are another option for replacing the cadmium-based QDs. These QDs also retain the attractive qualities of cadmium-QDs, but are associated with much less toxicity [72]. Further, these silver QDs possess size tunable fluorescence, avoid the use of organic solvents during synthesis and are water dispersible. They display good monodispersity and can be efficiently utilized for deep tissue NIR imaging.

Currently a potential application of QDs being investigated is in the development of sensors for biomedical testing and high throughput screening [73]. Such sensors would operate similarly to Bagalkot's system, where QDs would be attached to a fluorescent molecule [29]. In the OFF state FRET between the QDs and fluorophore would remain "OFF" or not fluorescent. Once the molecule/ protein/ hormone of interest binds to the sensor, the FRET reaction would cease and both the fluorophore and nanocrystal would begin to fluoresce brightly. This

may potentially be a highly useful, lucrative and a relatively safe application of nanocrystals.

Results showing undesirable effects of cadmium-containing QDs were disappointing, but necessary to learn how nanocrystals interact with cells and how to produce better and safer nanocrystals. The emergence of new cadmium-free QDs, points towards a brighter future for QDs in biology.

3. Why nanoparticles in medicine and future directions

Despite the findings that some nanoparticles are toxic, nanostructured materials are appealing for customizable devices that are in the same size range as a number of intracellular targets. Further, the enhanced stability and optimizable properties of nanoparticles render these devices especially attractive for use in drug delivery, and imaging. The therapeutic potential of nanoparticles is also under tremendous investigation, as the introduction of nanodevices in medicine present numerous non-conventional therapeutic strategies for a wide variety of pathologies. A particularly attractive aspect of utilizing nanoparticles for therapeutic purposes is the potential for combinatory therapy, and diagnosis, i.e. single nanoparticles that serve as a multi-therapeutic vehicle, capable of delivering more than one drug, and also being used as an imaging moiety. Such nanoparticles are termed, theranostics.

Another benefit of nanoparticle use in medicine is that nanoparticles can be readily functionalized with one or several targeting moieties; a highly attractive quality for designing molecular probes [74]. Several studies have reported much diagnostic success with the use of targeted nanoparticle probes for *in vivo* imaging. Nanoparticles can be effectively conjugated with antibodies, which can in turn be utilized for targeting [75, 76]. Radioactive copper labelled nanoparticles have proven efficient for PET scans [77]. Additionally, nanoparticles that were surface conjugated with folate were shown to be efficient at targeting and labelling tumors [78]. Finally, the ability to highly functionalize nanoparticles is

also being investigated to develop probes that allow both qualitative and quantitative analysis of the subject being imaged.

Most imaging technologies in current use are limited in terms of providing either high sensitivity or high resolution. The adjunct addition of nanoparticles to form novel multimodal imaging probes enables the use of multiple imaging technologies to provide both high sensitivity and high resolution images. These composite probes may consist of two or more components for imaging purposes, and include additional targeting moieties to improve imaging specificity. Such complex probes may not only facilitate several scans being done, but ultimately promote a greater quantity and quality of diagnostic information to be acquired.

A particularly attractive potential application of nanoparticles in biomedical imaging is the use of nanoparticles to image biological processes, and not solely outcomes. Nanoparticles may be introduced in order to non-invasively qualify and even quantify biological changes. Though a highly challenging undertaking, it is one that would allow for tremendous valuable information to be procured. Such an imaging probe would require not only a highly specific targeting moiety, but an efficient reporter system that can be imaged. Biological changes that such probes could assist in imaging include: enzyme activity, protein-protein interactions, gene expression, and ion-channel activity [79]. Even receptor density could be quantified in real time with the use of a nanoparticle probe with high receptor binding affinity. Such applications could prove especially attractive for cancer and tumor imaging, where the probes could be used to monitor and quantify tumor shrinkage with treatment.

The potential for nanoparticles in medicine is tremendous, however, the field of nanomedicine is still young, and before nanoparticles can be widely embraced for medical use, a much better understanding regarding the safety and possible toxicity of these particles is needed. Although, thousands of reports show both good and undesirable properties of different nanostructures, there are considerable discrepancies in findings; one of the reasons is that there are currently no standardized assays for biocompatibility data. One should at least consider three basic components of nanoparticles when conducting toxicological experiments: 1.

the nanomaterial composition, 2. the model biological system employed, and 3. the experimental procedure and assay chosen. Inconsistencies in data reported are often due to diversity of nanoparticles ranging from metallic to non-metallic. Synthetic procedures are often missing details regarding chemicals and solvents for fabrication; there are variations in constituents, and countless surface modifications and functionalizations. In addition, a range of *in vitro* models from cell lines to primary cultures, immortalized and cancer cells, have been used. These cells may come from any number of origins (species, tissue/organ). Alternatively, *in vivo* model systems have been employed, encompassing a variety of different species and strains of animals and animal ages. Finally, experimental procedures and assays were designed and detailed such that they differ in countless ways: nanoparticle incubation times, experiment duration, specific assays selected, outcomes evaluated (from cell death to subtle epigenetic changes). Taken together, these variations and inconsistencies in nanotoxicity evaluation lead to more confounding factors than actual conclusions. Therefore, the most imperative change that is needed in the field of nanotoxicology in the coming years is the standardization of nanoparticle evaluations.

Numerous reports thus far have been important to raise awareness of nanoparticle promise and limitations. Much of the data has been useful in the design of nanoparticles that are already entering the clinic and clinical trials. However, once proper standardizations for nanoparticle evaluation are in place, more accurate information will be available regarding nanomaterial safety.

4. Conclusion

The field of nanomedicine is rapidly expanding. Currently there are countless nanoparticles undergoing preliminary, pre-clinical and clinical trials, with much promise that some of these nanoparticles will eventually be adopted for clinical applications. Recently a few nanomedicines have already been FDA approved for patient use; for example, Vivagel®, a dendrimer based anti-microbial and anti-viral for the prevention of sexually transmitted diseases.

Despite their immense promise, the issues of nanomaterial toxicity and bioincompatibility have prevented certain classes of nanoparticles from reaching clinical trials. However, research focusing on biocompatible materials is warranted. Even from toxic nanoparticles, much has being learnt from understanding the mechanisms involved in their toxicity, pertaining to nanomedicine at large.

The bulk of our work has focused on CdTe QDs, fluorescent, semi-conducting functionalized and non-functionalized nanocrystals, and their effects *in vitro* and *in vivo*. Our studies have contributed to the understanding of the role of cadmium in mediating QD-induced effects.

Our preliminary studies were amongst the first to demonstrate that QD-toxicity did not correlate with cadmium liberation and cellular accumulation of cadmium. Numerous studies have since shown that mechanistically, genetically, and morphologically, the cadmium content in QDs is insufficient to induce the degree of toxicity observed with QDs, and further, that the cellular response to each is different. The role of ROS resulting from photoxidation of the QD surface coupled with cadmium leaching from the QD-core has now become the widely accepted cause of QD-toxicity.

In chapter two, we illustrated that QDs are unique entities and not simply a sum of parts (namely cadmium ions plus tellurium ions); a concept that is paramount for understanding nanoparticles. Bulk materials of the same components do not possess the same qualities and characteristics, nor do they react the same way as their nanoparticle equivalents. Similarly, the toxicity of nanoparticles cannot be interpreted as the sum of the toxicity of individual constituents.

Perhaps the most significant finding from our work is that QDs are potent endocrine disruptors that exert metalloestrogenic effects, mediated via the interaction with estrogen receptors, both *in vitro* and *in vivo*. This estrogenic burden suggests that cadmium containing QDs may exert both carcinogenic and teratogenic effects in animals and humans. Further, endocrine disruption may be a risk associated even with very low levels of prolonged QD exposure originating

from the environment; understanding this effect will allow for preventative and cautionary measures to be established.

The synthesis of cadmium-free QDs and silver QDs demonstrates the kind of development needed in the field of nanomedicine. These novel, safer QDs retain all the remarkable optical properties and advantages of QDs, with far fewer risks of toxicity. However, without the understanding of traditional cadmium QDs and their corresponding toxicity, these safer QDs may never have been discovered. As such, toxicological studies of nanomaterials are paving the road for the future of nanomedicine.

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List of original contributions

The novelty of this thesis encompasses both research findings as well as experimental approaches and assays. Overall the results presented in this thesis have contributed to our understanding of the mechanisms of interaction between nanocrystals and living cells.

A. Preliminary studies: Long-term exposure to CdTe quantum dots causes functional impairments in living cells
Langmuir. 2007 Feb 13;23(4):1974-80.

1. We show that intracellular and extracellular cadmium-content released from QDs is measurable by two complementary methods (adapted Measure-it Lead/Cadmium assay and atomic absorption).

2. We demonstrate for the first time that cellular toxicity induced by QDs does not correlate with the concentrations of cadmium liberated from the QD-core.

B. Chapter 2: Probing and preventing quantum dot-induced cytotoxicity with multi-modal lipoic acid in multiple dimensions of the peripheral nervous system
Nanomedicine (Lond). 2009 Apr;4(3):277-90

1. We demonstrate that increasingly complex biological systems should be used to assess and validate the cytotoxic effects of nanostructures and prevent them. These studies serve to exemplify the urgent need for standardization procedures to be used for comparing data from nanotoxicological studies across different laboratories.

2. We compared QDs to their individual core components and demonstrate that QD-toxicity does not result from the sum of parts of the toxicity associated with each QD-component.

3. We introduced lipoic acid as a multi-modal cytoprotective agent capable of preventing QD-induced cytotoxicity either i) directly, by acting as an antioxidant, ii) indirectly by upregulating the intracellular antioxidants, iii) by chelating cadmium, iv) by stabilizing and protecting the integrity of the QD-surface.

4. We employed three dimensional tissue explants of the DRG, to assess subtle morphological and functional impairments of the peripheral nervous system induced by QDs.

C. Chapter 3: Metalloestrogenic effects of QDs
Nanomedicine (Lond). 2012 Jan;7(1):23-37

1. We propose that QDs may be endocrine disruptors, and novel metalloestrogens, capable of inducing estrogenic signalling in estrogen receptor expressing cells.

2. We demonstrate that both the non-genomic and genomic estrogenic signalling are induced by metalloestrogenic QDs.

3. We show that the selective estrogen receptor inhibitor, ICI 182 780 fully abolishes QD-induced metalloestrogenicity.

4. We show for the first time, that QDs can act as metalloestrogens both *in vitro* and *in vivo*. *In vivo* findings show a 2.5 fold increase in uterine wet weight post 2 weeks of chronic treatment with QDs.

General and other findings

1. We established a cadmium resistant cell line to demonstrate that cells resistant to cadmium toxicity remain equally susceptible to QD-induced cytotoxicity. Further, we show that despite a decrease in free, intracellular cadmium in

cadmium resistant cells, post QD-treatment, QDs remain equally toxic as in the control cells.

2. We depict the paradoxical effects of QDs as being inducers of both QD-toxicity and QD-estrogenicity.

3. In another manuscript:
Multi-tasking with single platform dendrimers for targeting sub-cellular microenvironments.
Hourani R, Jain M, Maysinger D, Kakkar A.
Chemistry. 2010 Jun 1;16(21):6164-8.
We show that non-cadmium containing, organic nanomaterials (dendrimers) can easily enter cells, but do not induce any cytotoxicity. Dendrimer synthesized using "click" chemistry were shown to be effective drug carriers and theranostics.

4. In another manuscript:
Thiolated polyaspartamide is an effective biocompatible coating agent for quantum dots
B. Zorc, J. Lovrić, M.P. Jain, A. Moquin, S.J. Cho, J. Filipović-Grčić
Journal of drug delivery science and technology., 19 (2) 139-144 2009
We demonstrate the ability for thiolated polyapartamide polymers to effectively coat quantum dots and render them non-toxic in two different cell lines.