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Cadmium-Containing Nanoparticles: Perspectives on Pharmacology & Toxicology of Quantum Dots

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Abstract

The field of nanotechnology is rapidly expanding with the development of novel nanopharmaceuticals that have potential for revolutionizing medical treatment. The rapid pace of expansion in this field has exceeded the pace of pharmacological and toxicological research on the effects of nanoparticles in the biological environment. The development of cadmium-containing nanoparticles, known as quantum dots, show great promise for treatment and diagnosis of cancer and targeted drug delivery, due to their size-tunable fluorescence and ease of functionalization for tissue targeting. However information on pharmacology and toxicology of quantum dots needs much further development, making it difficult to assess the risks associated with this new nanotechnology. Further, nanotechnology poses yet another risk for toxic cadmium, which will now enter the biological realm in nano-form. In this review, we discuss cadmium-containing quantum dots and their physicochemical properties at the nano-scale. We summarize the existing work on pharmacology and toxicology of cadmium-containing quantum dots and discuss perspectives in their utility in disease treatment. Finally, we identify critical gaps in our knowledge of cadmium quantum dot toxicity, and how these gaps need to be assessed to enable quantum dot nanotechnology to transit safely from bench to bedside.

Introduction and Scope

The health risks posed by cadmium toxicity have been investigated for over 50 years. Yet knowledge in this area is still expanding, as evidenced by the excellent reviews appearing in this volume. At the level of the organism, cadmium toxicity is associated with liver and kidney injury, osteomalacia, osteoporosis, skeletal deformations, neurological, and other deficits. Cadmium is classified as a category 1 carcinogen, but is not directly genotoxic or mutagenic in bacteria. It is known to affect genome stability via inhibition of DNA repair and generation of free radical-induced DNA damage. At the cellular level, cadmium induces oxidative stress by depletion of endogenous antioxidants such as glutathione and is associated with mitochondrial damage, induction of apoptosis, and disruption of intracellular calcium signaling. Despite the extensive studies on cadmium toxicity, there continues to be much territory left to cover regarding its mechanism of action, intracellular damage, and environmental exposure.

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At present, the primary cadmium nanoparticles are those of CdSe or CdTe, encapsulated in various coatings in the form of semiconductor quantum dots (QDs). The evolution of nanotechnology poses the scientific community with yet another aspect of cadmium toxicity - the biological effects of cadmium nanoparticles. The nanotechnology industry has grown exponentially over the last few years, a trend which will likely continue into the near future. In a recent review, Hardman (2006) estimated that a mere 2 grams of 100 nm diameter particles would contain enough material to provide every human with about 300,000 particles each, out of which several are likely to be cadmium-containing nano-constructs. Unfortunately, our rapid progress in nanotechnology has exceeded the progress of research on the impact of nanoparticles on human health. In the biological realm, nanoscale materials act via mechanisms and reactions very different than their “bulk” or “macro” counterparts. In the realm of the very small, quantum effects predominate, and we are just beginning to comprehend these effects on the biological milieu. This review will discuss the current knowledge of cadmium nanoparticle pharmacology and toxicology, focusing on quantum dots and highlighting areas where new information is critical and suggest directions for future research. Focus will be placed on new strategies for pharmacological and toxicological research that need to be developed in order to comprehensively investigate the unique principles that apply to nanopharmaceuticals.

Cadmium Nanoparticles – What is a Quantum Dot?

In nanotechnology, cadmium is primarily utilized in the construction of particles known as quantum dots (QDs), which are semiconductor metalloid-crystal structures of approximately 2 – 100 nm, containing about 200-10,000 atoms (Smith et al., 2008; Juzenas, et al., 2008). Due to their small size, QDs have unique optical and electronic properties that impart the nanoparticle with a bright, highly stable, “size-tunable” fluorescence. The large surface area imparted by small size also makes QDs readily able to be functionalized with targeting ligands for site-directed activity. Based on these properties, QDs have the potential for revolutionizing biological imaging at the cellular level, cancer detection and treatment, radio- and chemo sensitizing agents, and targeted drug delivery; and are the subject of several excellent reviews (Juzenas, et al., 2008; Alivasatos, 2004; Smith et al., 2008; Hardman, 2006). However enthusiasm for QDs is somewhat diluted by the fact that QDs contain substantial amounts of cadmium in a highly reactive form, and we know little about the health risks of exposure to cadmium nanoparticles.

The synthesis methods for QDs have recently been reviewed (Biju, et al., 2008), and will be discussed here as they pertain to pharmacology and toxicology. The active center of the QD is known as the core, shown in Fig. 1. The core is composed of atoms from groups II-VI, with CdSe and CdTe being the most commonly used for biological applications (Smith, et al., 2008). The most important feature of QDs is their size-tunable fluorescence. In a “bulk” or macro-scale semiconductor, the bandgap energy, or minimum energy required to excite an electron to an energy level above its ground state, is a fixed entity; unique to the nature of the semiconductor material (Juzenas, et al., 2008). Relaxation of the excited electron back to its ground state causes fluorescent emission of a photon. However as the size of a particle is decreased to the nano-scale (less than the Bohr radius of the material), a quantum confinement effect occurs, which makes the bandgap energy dependent on particle size. Hence, optical properties such as fluorescence excitation and emission, can be “tuned” by altering the size. QDs are also significantly brighter than organic fluorophores and far more stable. Since fluorescence is dependent on size, a single light source can be used for excitation and emission, which is tuned via particle size to various wavelengths spanning the UV, visible, and near-mid infrared regions of the electromagnetic spectrum. In contrast to organic fluorophores, QDs are also much larger, permitting easy addition of targeting groups to the surface of the nanoparticle. CdSe and CdTe have been particularly attractive for optical, bioanalytic, and bioimaging

applications, with CdSe fluorescence spanning the visible light region of the spectrum and CdTe utilizing the infrared regions.

Due to the high surface area of CdSe or CdTe QD, a large number of atoms are exposed at the surface. Many of these have molecular orbitals which lack the full complement of electrons necessary for stability. These are known as “defect” sites, which can be highly reactive in the biological milieu. Therefore, another semiconductor with a wider bandgap is grown over the CdSe core (Biju, et al., 2008). ZnS is commonly used for this purpose, and it enhances fluorescence efficiency (Hines and Guyot-Sionnest, 1996) and reduces toxicity imparted by the highly reactive core. This encapsulating layer is known as the “shell”, and is shown diagrammatically in Fig. 1. The ZnS shell also makes the QD less prone to oxidation and photobleaching, and increases chemical stability. For the sake of nomenclature then, a CdSe/ZnS-QD is a cadmium selenide QD core with a zinc sulfide shell. Additional shell materials have been utilized, and are the subject of recent reviews (Hardman, 2006; Juzenas, et al., 2008, Smith, et al., 2008)

As synthesized, QDs are hydrophobic, a limitation to biological applications. Functionalization with secondary coatings or “capping” materials such as mercaptopropionic acid and polyethylene glycol (PEG) are used to improve solubility and maintain QDs in a non-aggregated state. Such coatings can be further conjugated with targeting molecules such as antibodies or receptor ligands (see Fig. 1), which target the QD to a specific tissue or organ (Medintz, et al., 2005, Smith et al., 2008). The vast utility of QD are exemplified in an early report by Akerman et al. (2002), who demonstrated in vivo imaging of breast cancer in mice using CdSe/ZnS-QDs coated with targeting peptides. QDs were able to locate the tumors in mice and track metastases with imaging techniques. Gao et al. (2004) used triblock copolymer QDs with prostate tumor targeting ligands to locate tumors and tag systemically injected cancer cells in mice, combining a whole body illumination system with spectral QD imaging. Dubertret et al. (2002) utilized PEG-phosphatidylethanolamine and PEG-phosphatidylcholine CdSe/ZnS-QDs to image *Xenopus* embryo development. Since these experiments, in vivo work using QDs has dramatically expanded to potentially revolutionize cancer detection, follow temporal metastatic development, and develop image guided surgery and drug delivery. Additionally, QDs have energy levels in the range of 1-5 eV and can act as photosensitizers (Juzenas, et al., 2008) which absorb high energy photons from x-rays or gamma radiation, improving and focusing radiation therapy. From the standpoint of cancer detection and treatment, QDs have the potential to dramatically improve medical therapy. In contrast, the presence of highly toxic cadmium nanoparticles in an electronic state with high activity imparted by the nanoscale, suggest the potential to cause an equal amount of harm unless pharmacological and toxicological parameters are carefully examined. Medical research is utilizing QDs (and other nanoparticles) to target disease-modifying therapies to the “right place at the right time”. From a toxicological standpoint, we need to consider the consequences of cadmium-containing nanoparticles being in the wrong place at the wrong time.

QD Pharmacology

Pharmacological parameters of QD behavior in biological systems still require much investigation before they can be effectively utilized in human treatment paradigms. Dosing parameters, absorption, distribution, metabolism and excretion require considerable further study, since we have little information on these parameters to date. Further, when utilizing a nanopharmaceutical, it is important to realize that in contrast to delivering a drug, which is an organic molecule, we are delivering somewhat of a discrete entity in a nanoparticle - comprised of atomic scale parts. Due to the quantum effects and electronic interactions that predominate at the nanoscale, we need to alter the way in which we think about pharmacological parameters, to adapt to nanoscience.

Probably one of the most important parameters to be considered in pharmacological studies is physicochemical characterization of the particles used. To date, QD, as well as other nanoparticle literature, is difficult to compare due to lack of particle consistency across experiments. Characteristics such as size, coating, atomic composition, and purity need to be determined for all nanoparticle preparations utilized. Synthesis methods vary from lab to lab, resulting in different tailing contaminants, different coatings, and different particle activity. Hence complete materials characterization needs to be performed on all nanoconstructs prior to pharmacological or toxicological studies. To a nanoparticle, these characteristics are as important as chemical structure is to an organic molecule.

Dose is another parameter that requires consideration. First, what should the dose be? Adequate estimates of the QD exposure during treatments such as cancer therapy, need to be estimated so that both pharmacological and toxicological studies can be conducted within a physiologically relevant dose range. Importantly, how does cadmium content relate to dose? To date, these parameters have not been rigorously addressed.

Dose metrics of QDs have been reported in terms of mg/kg (or mg/ml for in vitro studies) or on a molar basis, and this will no doubt need refinement. Given that a QD, or any other nanoparticle, is an engineered entity or “mini-reactor”, dose via mass or molar number may be an inappropriate descriptor. Since surface area is critical to nanoparticle function, Oberdorster and colleagues (2005) proposed that dose be described in terms of surface area, rather than mass. In a study on CdSe-QD with various coatings, Kirchner et al. (2005) converted the concentration of CdSe to the number of cadmium atoms exposed on the QD surface, and found that smaller particles with greater surface cadmium atoms, were more cytotoxic. Other groups have utilized the parameter of number of particles delivered per cell or organism, or have based molar values on a mole being equivalent to Avogadro's number of particles (Zhang et al., 007)

This works well for particles of similar size and shape or when catalytic activity is not related to particle size, but falls short when size and catalytic activity are closely linked. Possibly, a combination of parameters, such as mass/cm² or reactivity/unit mass may provide a more relevant dosing parameter (Rzizgalinski, et al., 2006). For QDs, cadmium content should also be specified. Clearly, more applicable dosing parameters need to be defined and kept constant for experimental comparisons. However until further research is completed, it makes good experimental sense to define dose according to several mathematically interrelated parameters, using nanoparticles that have been fully characterized both physically and chemically.

ADME

Nanopharmacology is further complicated by the need to establish the behavior of nanoparticles such as QDs within the traditional pharmacological parameters of absorption, distribution, metabolism and excretion (ADME). Nanoconstructs, in many cases, have limited metabolism and excretion and persist in biological systems, which gains importance when containing toxic atoms such as cadmium. This poses a need to carefully examine our common ADME parameters and revise them if necessary.

Absorption into the system is generally the first hurdle to be met, and is of course dependent on route of delivery. To date, research has established that various nanoparticles can enter an organism through skin absorption, inhalation, oral delivery, and parenteral administration. For QDs, the most important route of delivery at present appears to be systemic distribution through parenteral delivery, although occupational and environmental exposures via dermal and inhalation routes are also possible. What few studies are available on QD absorption at the organism level primarily utilize parenteral iv delivery. QD targeting studies have shown that QDs with targeting functional groups can be accumulated in selected target tissues upon iv

administration. However distribution to non-target tissues in an organism has not been examined and is an area where information is critically needed. Due to the high fluorescence of QDs and the metallic cores, particle deposition within an organism should be readily measureable.

Regarding occupational exposure during manufacture and handling of QD preparations, several groups have investigated dermal exposure to QDs using topical delivery models (Zhang et al. 2008, Ryman-Rasmussen et al., 2007). Zhang et al found little skin penetration of PEG-coated QD621 beyond the uppermost layers of the dermis in intact, undamaged skin, yet other studies using differentially coated QDs of varying shape found dermal absorption to be dependent on shape, size, and surface coating, underscoring the need for complete particle characterization prior to biological experimentation (Ryman-Rasmussen et al., 2007). Particle toxicity, particularly after light exposure, in dermal areas was not evaluated in these studies, and is yet another area requiring future investigation

At the cellular level, numerous absorption studies are available, but these are often difficult to compare due to varied dosing parameters and lack of physicochemical particle characterization. In general, it appears that most QDs examined are absorbed readily at the cellular level, primarily via endocytic mechanisms (Jaiswal, et al., 2003; Derfus, et al., 2004; Hoshino, et al., 2004, Smith, et al., 2008). Several reports indicate selective incorporation of ligand-targeted QDs, such as those directed to the EGF receptor. EGF-conjugated CdSe/ZnS-QDs were found to be highly specific for the EGF receptor erbB1 in CHO cells, and entered the endocytic pathway via this receptor system (Lidke, et al., 2004). Several studies observed perinuclear localization within the cell for CdSe/ZnS particles (Parak, et al., 2002). Others (Lovric, et al., 2005) found that subcellular localization appeared to be dependent on size, with 5.2 nm CdTe-QDs localized to the cytoplasm while 2.2 nm particles were found within the nuclear compartment, suggesting that nuclear pores may permit passage of QDs below a certain size range. Passage of QDs to the nucleus will be critical to assessing genomic effects and the correlation with size requires further study. Thus although cellular absorption appears to readily occur with most QDs tested, the additional parameters of size and coating or functionalization groups may further affect how QDs are absorbed within the cell.

Regarding distribution, one of the first elements that parenterally delivered QD will encounter is the environment of the blood. Here, we have little to no information about blood/QD interactions. Plasma half lives are no doubt related to surface coating and addition of biological targeting ligands, if any. For example, PEG coating was reported to increase the plasma half life (Ballou, et al., 2004). In the case of oxide nanoparticles, many are coated with endogenous plasma proteins immediately upon entry into the circulatory system (Rzizgalinski, et al., 2006) and this appears to enhance tissue delivery. However the interactions between QDs and plasma proteins are unknown. Immune responses may also be triggered at this level, as reviewed by Dobrovolskaia and Mcneils (2007), but the precise interactions of QDs at this level have not been examined. Such studies in the future will be necessary to assess immunotoxicological effects of QDs.

Important parameters for distribution of QDs include size and agglomeration tendencies. However few studies are published to date that address these parameters in a consistent manner. Ballou et al. (2004) used QD coatings with varying molecular weights and composition and found differential tissue and organ distribution in mice, which was both time and size dependent. For example, smaller PEG-QD conjugates were cleared from the circulation faster than larger conjugates. Effective tissue distribution after iv administration of functionalized, targeted, QDs has been shown for lung, vascular system, and lymph (Akerman, et al., 2002, Dahan, et al., 2003; Chen et al., 2004). However distribution throughout other non-target tissues at low concentrations has not been determined. Twenty four hrs post injection of PEG-QD

conjugates, particles were distributed in lymph nodes, liver, and bone marrow and persisted, in some cases, through 133 days post administration (Ballou, et al., 2004). Studies by Dubertret and colleagues (2002) found that CdSe/ZnS QD conjugated to PEG-phosphatidylethanolamine and phosphatidylcholine could be transferred to daughter cells upon cell division in *Xenopus* embryos, and persisted for several days. The fact that QDs are, in general, able to enter most cells suggests that unless specifically targeted, distribution to a broad range of tissues is possible. Given the inherent fluorescence emission, delivery and accumulation in tissues should be readily detectable by either fluorescence assay or inductively coupled plasma mass-spectrometry to detect the metal moiety.

One consistent theme in the existing literature is that QDs are eventually taken up by the reticuloendothelial system, including the liver, spleen, and lymphatic system (Smith, et al., 2008; Hardman, 2006). Ballou et al. (2004) reported rapid removal of QD from the blood to the reticuloendothelial system, where they persisted for several months. Fisher et al. (2006) demonstrated that albumin coated QD were removed from the circulation within hrs of injection, and were found in Kupffer cells of the liver. This suggests that the reticuloendothelial system may be a toxicological target for QDs that requires further investigation, given the known effects of cadmium in this system.

Another general theme that appears across the literature is that QDs, along with their associated cadmium, persist in the system. At the cellular level, Jaiswal et al. (2003) showed that CdSe/ZnS-QDs were retained by HeLa cells for over 10 days. In other studies, fluorescence was retained for over 52 days, even after passaging (Selverstov, et al., 2006). In vivo reports also suggest retention of fluorescence for weeks to months after intravenous administration (Ballou, et al. 2004; Dubertret, et al., 2002). The length and extent of QD tissue retention is an important parameter for dosing considerations, since repeated doses may induce systemic accumulation, contributing to potential toxicity. Furthermore, persistence of QDs in tissues suggest the distinct need for long term studies to accurately assess risk.

Metabolism of QDs is yet another understudied aspect of cadmium QD. QD cores do not appear to be subject to extensive enzymatic metabolism, but shells and coatings are. The extent of metabolism of the shell and coating becomes critical for toxicity, since they shield the more toxic CdSe or CdTe cores from the intracellular environment, as shown in Fig. 1. Rather than metabolism per se, degradation of the shell and coatings within the biological environment appears to be more important. QD shells and coatings appear to degrade under photolytic and oxidative conditions, yet we know little about the degradation products or their biological effects, which may regulate release of toxic cadmium cores.

Excretion poses yet another pharmacological hurdle for QD research in that we have no comprehensive studies of QD removal via this route. Given the cadmium content of QDs, and the known renal toxicity of cadmium, the kidneys may be an important site for toxicological effects. Excretion will undoubtedly be regulated by size, coatings and physicochemistry. Several studies suggest that QDs of size less than approximately 5 nm can be removed by the kidneys. After IV administration of CdSe/ZnS-QDs, the cadmium content of the liver and kidney were increased 29 days post injection. However only 10% and 40% of the injected dose was found in the kidney and liver respectively, suggesting that only a fraction of the total QD dose passed through this route. Whether this was in the form of free cadmium was not determined in this study, but fluorescent QD were found in liver and kidney suggesting that at least some remained in their original form (Yang, et al., 2007). Yet many reports propose that a portion of the administered QD dose may not be excreted, and persists in the tissues. The extent of excretion and the extent of persistence in tissues takes on added importance when one considers the potential delivery of QD as a cancer-targeting drug. Since QDs are photoactive, persistence in an organ such as the skin may have unknown consequences when

the skin is exposed to light. More comprehensive studies of potential excretion will therefore be critical to QD development as a nanopharmaceutical.

In summary, we have considerable ground to cover with regard to cadmium QD pharmacology, particularly in the area of ADME. There is a need for a comprehensive study of characterized QD of a single type administered via oral or IV routes. Tissue distribution, particularly outside of targeted areas, needs to be assessed and persistence of QDs needs to be quantified to assess adverse effects.

Toxicity of Quantum Dots

QD toxicity, as with other nanoparticles, depends on multiple parameters associated with physicochemistry, as discussed above. Parameters of size, shape, concentration, charge, redox activity, surface coatings and mechanical stability all need to be considered for toxicological assessment, as shown in Fig. 1. The wide variation in these parameters in different experimental paradigms has been the nemesis of toxicological research in this area. To date, the literature on toxicity of QDs is an olio of reports of numerous types of QDs with widely varying physicochemical parameters, making comparisons quite difficult. To assess toxicity at our current level of understanding, we will first discuss general concepts regarding core, core shell, and coating toxicities in cellular studies, followed by a recap of our current knowledge from animal studies.

Toxicity of Core QDs

A primary source of QD toxicity results from cadmium residing in the QD core. Toxicity of uncoated core CdSe or CdTe-QD have been discussed in several reports and is associated, in part, with free cadmium present in the particle suspensions or released from the particle core intracellularly (Samia, et al., 2003; Kirchner, et al., 2005; Derfus et al., 2004). Lovric et al. (2005) found that CdTe-QDs were cytotoxic in rat pheochromocytoma cells (PC12) at 1 µg/ml concentration and induced apoptotic-like cell death including chromatin condensation and membrane fragmentation. The cytotoxicity observed in these studies was found consistent with cadmium toxicity from the QD core. Derfus et al. (2004) found that CdSe-QDs incubated with rat hepatocytes released cadmium via surface oxidation of the uncoated particles, suggesting that the particle cores could degrade in biological environment. Therefore cadmium toxicity from QD cores is likely to be a significant contribution to QD toxicity.

However QDs of CdSe and CdTe are also highly reactive electronically, and are subject to photo- and air oxidation. Therefore, free radical formation is considered another primary mechanism for QD cytotoxicity (Ipe, et al., 2005; Green, et al., 2005). Whereas free cadmium itself does not directly generate free radicals (although it does increase oxidative stress), the active QD core does participate in radical formation. Using a cadmium reactive dye, Cho et al. (2007) found that CdTe-QD cytotoxicity in cell culture was not related to cadmium release from the QD, but was due to free radical generation. Additional studies have recently shown that CdSe generates free radicals that “nick” DNA, both in the absence and presence of light activation (Green, et al., 2005). Free radical generation by CdSe-QDs was increased when photoactivated via visible or UV light, or via air oxidation (Ipe, et al., 2005; Green, et al., 2005; Lovric, et al., 2006). Under these conditions, a photon of light excites the QD generating an excited electron which transfers to molecular oxygen, producing singlet oxygen. Singlet oxygen in turn initiates free radical formation upon reaction with water or other biological molecules. This is the basis for photodynamic therapy in cancer applications of QDs, but must also be considered when assessing toxicity of QDs, particularly if QDs distribute to normal tissue. Should the normal tissue containing QDs then be exposed to light or other oxidative stress, radical production by the resident QDs could significantly contribute to cellular demise.

Alternatively, tissue culture studies in which cells are exposed to light may overestimate actual *in vivo* toxicity.

Uncoated QDs have also been associated with other cytotoxic effects, similar to those resulting from cadmium toxicity and/or oxidative stress. In SH-SY5Y neuroblastoma cells, CdTe-QD induced cell death was associated with upregulation of Fas expression, possibly through an increase in oxidative stress (Choi, et al., 2007). Activation of Jun-N-terminal kinase (JNK) was induced by CdSe-QD, along with increased Bax, decreased Bcl-2, and activation of caspases, all suggestive of apoptosis. In human breast cancer cells, CdTe-QD treatment induced hypoacetylation and activation of p53 (Choi, et al., 2008). Tang et al. (2008) examined neurotoxicity of CdSe-QDs in hippocampal neurons, finding a dose dependent increase in neuronal death. However at low doses (1-20 nM) perturbations in intracellular free calcium signaling were identified, including increased influx of extracellular calcium and release of calcium from intracellular stores. Alterations in N-type calcium channels, Na⁺ channels, and mitochondrial calcium were also noted. These results imply that low level chronic exposure to CdSe or other QDs may induce perturbations in calcium signaling that promote delayed neuronal (or other cell) dysfunction, suggesting a need for more chronic and long term studies of the effects of QDs on cell signaling. This becomes particularly important given the apparent persistence of QDs in tissue and cells.

Given that many of the studies discussed here utilize fluorescent dyes for determination of intracellular biomolecules, a word of caution is in order. Many nanoparticles, by virtue of their active electronic structure, react with commonly utilized organic dyes, including many of the dichlorofluorescein derivatives. Therefore it is critical to perform adequate controls to ensure that nanoparticle activity does not skew results by electronic interactions with organic assay dyes. Careful attention to such controls should be a primary consideration in cellular studies of toxicity, since dye/nanoparticle interactions can result in over or underestimation of toxicity.

Toxicity of Core/Shell and capped QDs

Encapsulation of the CdSe-QD with a ZnS shell or other capping material has been shown to reduce toxicity, although much work remains to be completed in this arena. In human breast cancer MCF-7 cells, CdTe caused cellular alterations resembling cadmium toxicity, while CdTe/ZnS core/shells or CdTe capped with mercaptopropionic acid, cysteamine, and *n*-acetylcysteine did not, demonstrating that ZnS shells and capping materials could reduce toxicity for the time periods studied (Bakalova, et al., 2004). In these studies, free cadmium in the intracellular compartment was also reduced by ZnS and capping. ZnS core/shell particles also reduced apoptosis, JNK activation, and loss of mitochondrial membrane potential induced by CdSe-QDs (Chan, et al., 2006). Derfus et al (2004) demonstrated that free cadmium released by CdSe-QDs dissolved in aqueous solution could be dramatically reduced by ZnS shell encapsulation. Further, ion channel perturbation was blunted in RBL and CHO cells treated with CdSe/ZnS-QDs for incubation times of up to 2 days (Kirchner, et al, 2005). In addition to reduction in release of free cadmium, ZnS shells were also found to reduce free radical generation due to air oxidation of QDs (Dermus, et al., 2004). Therefore, encapsulation of QDs with a ZnS shell or other capping material appears to abrogate, at least in part, toxicity associated with release of cadmium or generation of free radicals.

However to accurately assess safety of shell or capped particles, the degradation of the shell or capping material, along with its cytotoxicity must also be considered. One group found that fluorescence intensity of CdSe/ZnS decreased with time along with a shift to blue spectra within living cells over time, suggesting that the ZnS core shell deteriorates intracellularly (Zhang, et al., 2006). Other studies found that the ZnS shell did not completely eliminate cytotoxicity due to air or photooxidation (Dermus, et al., 2004) and that CdSe/ZnS-QDs could also generate free radical species (Green and Howman, 2005). These authors hypothesize that although the ZnS

shell protected the CdSe core from oxidation, it could not inhibit electron-induced radical generation in the surrounding water and suggest that the ZnS slowly oxidized in the presence of air or water, generating the SO^{2-} radical.

In addition to a ZnS shell, other capping materials may also be utilized to insulate the QD core, which reduce toxicity in many cases. Lovric et al. (2005) found that CdTe-QDs coated with mercaptopropionic acid and cysteamine required higher concentrations to produce toxicity in PC12 cells, as compared to uncoated QDs. *N*-acetylcysteine coating was shown to reduce CdTe-induced Fas upregulation and apoptosis (Lovric, et al., 2005, Choi, et al., 2007) and decrease cytotoxicity in neuroblastoma cells (Choi, et al., 2007). Voura et al. (2004) utilized dihydroxyloipoic acid (DHLLA) coated CdSe/ZnS to reduce toxicity of QDs in several cell lines, finding that the label was stable and retained for over 1 week with no adverse effects. Bovine serum albumin has also been used to coat QD for reduction in toxicity (Derfus, et al., 2004). It is interesting to note that these effective capping materials all appear to be good antioxidants, further supporting a role for oxidative stress in cadmium QD toxicity.

Toxicity of capping materials must also be considered, since several groups have found increased toxicity associated capping materials such as mercaptoacetic acid and Topo-tri-*n*-octylphosphine oxide (TOPO) (Smith, 2006). Taken together, these reports suggest that the integrity of shell and capping materials, as well as toxicity, needs to also be more thoroughly assessed and that shell/capping materials must be assessed for different QD preparations. Further, based the potential for degradation of the shell or capping materials and the persistence of QDs in tissue, long term studies of effects on both cell viability and signal transduction are also needed.

Toxicity in QDs with functionalized coatings

In addition to shell and capping materials, QD constructs may also be created with functionalization groups to target specific cells or tissues. Toxicological investigations need to consider the physicochemical parameters of these functionalized constructs, which may reduce toxicity by targeting QDs to specific locations. However the fidelity of targeting material needs to be determined, since distribution to non-targeted tissue may produce toxicity, particularly when the QD is used as a photosensitizing agent or drug carrier. Although several studies detail the use of functionalized QDs both in vitro and in vivo, no studies directly assess the toxicity of these nanoparticle constructs or their distribution to non-target tissues. Questions to be answered include how stable are the coatings? How long do they last within the organism? Are they degraded? Redistributed to other tissues? These questions need to be thoroughly addressed before utilization of coated QDs for human applications.

Targeted Toxicity

Toxicity can also be used to the biomedical advantage, in specifically directing cellular demise to tumors or metastases, as reviewed by Juzenas, et al. (2008). For example, a QD may be constructed with a photoactivatable coating and a targeting ligand. Photoactivation toxicity can then be directed to a specific location (such as a tumor), followed by application of light to impart toxicity. The light photon excites the QD, and an excited electron can transfer to nearby molecular oxygen initiating a chain of radical generation ultimately inducing cell death. Whereas this is the principle for photodynamic therapy in oncology, aberrant distribution of the QD to non-cancerous tissue such as skin or retina, which is normally subjected to light, must be evaluation.

Animal Toxicity Studies

Animal studies are decidedly lacking for both pharmacology and toxicology of QDs. Zhang et al. injected uncoated CdTe-QDs into rats and reported little morbid toxicity and lack of overt organ damage (Zhang, et al., 2007), but comprehensive histological analyses were not performed. This group did report alterations in motor function after injection, suggesting potential effects on neural function which need to be considered in future studies. Additionally, long term effects of exposures remain to be determined. Ballou et al. injected amphiphilic polyacrylic acid polymer and PEG-coated QD into mice at a concentration of 20 pMols per gram animal weight (Ballou, et al., 2004). Mice were viable until necropsy (133 days) and showed no signs of necrosis or damage at the sites of tissue injection and no signs of QD breakdown in vivo. Larson et al (2003) reported no noticeable adverse effects in mice injected with 20 nM and 1 μ M CdSe/ZnS, however lack of overt adverse effects does not imply lack of toxicity. Gao et al. (2004) injected QDs with polymer and amphiphilic coatings and found slow degradation of coatings over time in vivo, leading to alterations in QD fluorescence over time, suggesting that toxicological parameters may indeed change with residence time of the QD in tissue. Fischer et al (2006) injected mercaptoundecanoic acid, lysine, and BSA coated CdSe/ZnS-QDs into rats and found differential distribution based on the coatings. Interestingly, no QDs were found in the urine or feces over the course of 10 days, suggesting persistence in the body. The particles were reported to be non toxic, but they were not cleared from the system either. These few studies dramatically underscore the need for short and long term toxicity evaluation that examines multiple organ systems before QD risk can be adequately assessed.

Size, Dose, and Exposure Considerations

As with pharmacological studies, toxicity studies face the same difficulties in terms of size, dose, and exposure – underscoring the need for rigorous physicochemical characterization of QDs. Particle size is critical to biological actions of nanoparticles (Rzizgalinski, et al., 2006). For QDs, several studies have demonstrated that particle size affects toxicity at the intracellular and animal level. In cellular studies, 2.2 nm CdTe-QD had greater toxicity as compared to larger, 5.2 nm particles (Lovric, et al., 2005). Additionally, smaller particles were found localize in and around the nucleus of the cell, while larger 5.2 nm particles were distributed within the cytoplasm. Similar results were reported by Zhang et al. (2007) in HepG2 hepatoma cells, in which size substantially altered the EC₅₀ for cell death.

At the nano-scale, size is intricately connected to dose, since surface area is critical to nanoparticle actions. Furthermore, studies by Zhang (2008) and Ryman-Rasmussen (2007) suggest that shape is also a key factor in nanoparticle activity, particularly with respect to dermal delivery. Critical questions to be answered relevant to toxicological studies are related to what the estimated human exposure will likely be, and how to effectively represent dose. Cellular studies using milligram or microgram/ml media concentrations may impart too high a concentration to be physiologically relevant, given that QDs are likely to be utilized at low concentrations due to specificity if targeted. Until such dosing parameters are effectively estimated, toxicological studies should employ a wide range of concentrations, from high to low, and include estimations of surface area and number of particles given.

Lastly, exposure issues deserve further consideration. QDs, as with most nanoparticles, appear to widely distribute in tissues unless targeted, and most have little to no metabolism or excretion. Based on tissue persistence, long term studies are critical to assessment of toxicological risk. In the case of QDs, an electronically active cadmium nanoconstruct may be retained in tissues in excess of years. Given that QDs impart toxicity via the effects of cadmium and free radical generation, effects on transcription, DNA synthesis, and signal transduction may be significantly altered over the long term. Recent studies (Tonks, et al., 2005) have shown

that low levels of free radical production are integral to signal transduction pathways, hence low level radical generation from QDs may substantially interfere with these pathways over time, particularly as the various layers coating the toxic cadmium core are degraded or altered, as shown in Fig. 1.

Cadmium Nanoparticles – The Cancer Perspective

Nanotechnologies offer some of the most innovative approaches to cancer diagnosis and treatment, but also present plausible and incompletely assessed risks. In this section, we briefly review a few ways cadmium nanoparticles are being developed to tackle cancer problems. The current level of understanding of the carcinogenic risks posed by their use is summarized. Evaluation of these risks requires urgent attention in light of the rapid pace of research in this field.

Cancer Applications

The selective accumulation of QDs in solid tumors enabling their use as fluorescent tumor imaging agents was a seminal observation (Akerman et al, 2002; Gao et al, 2004). As a result of imperfections in vascular structures which form in and around tumors in response to tumor angiogenic factors, QD exit the blood and concentrate in tumors. By this mechanism, QD achieve a valuable objective, tumor selective activity, and this enables the acute cytotoxicity of various nanoparticle constructs to be focused upon tumor cells (Cho et al 2007; Hardman 2006; Kirchner et al., 2005; Lewinski et al., 2008). Several investigations have confirmed that QD induce apoptotic death in cancer cell lines in cell culture (Chan et al., 2006; Choi et al, 2007). The tumor selectivity of QD can be further augmented using biochemical surface tags to hone in on cancer cells, and with the addition of conjugated drug molecules, effectively deliver treatments to tumor cells directly (Biju et al., 2008; Smith et al., 2008). In addition, QD undergo photoactivation and have potential applications in photodynamic therapies and as radiosensitizing agents for cancer treatments (Anas et al, 2008; Juzenas et al., 2008).

Carcinogenic Risks

Known human carcinogens are classified under two broad categories, genotoxic and epigenetic agents (Smart et al., 2008). Genotoxic agents damage DNA and result in loss of DNA integrity, mutagenesis and chromosomal aberrations. Genotoxicity assays constitute a well-accepted, standardized approach to carcinogenic risk assessment. Epigenetic carcinogens modify the expression of genetic information without altering the primary DNA sequence. Changes in DNA methylation, histone acetylation and histone methylation are important epigenetic carcinogenic events. Cadmium is a human carcinogen (Huff et al, 2007; Waalkes, 2003) and this raises concern about the carcinogenic potential of cadmium QD. Our purpose here is to review data relevant to evaluation of the carcinogenic risk of cadmium QD nanoparticles.

A limited number of publications address the carcinogenic risks of cadmium QD, yet data accumulating from these studies and investigations of other nanoparticles (Bhattacharya, et al., 2008; Kang, et al., 2008; Maenosono, et al., 2007; Mroz, et al., 2008; Reeves, et al., 2008; Vevers, et al, 2008) suggest that a more systematic approach to the cancer risk posed by cadmium QD is necessary. Comet assays detect DNA damage which occurs within live mammalian cells. Hashino and colleagues (2004) were among the first to describe acute genotoxicity of CdSe/ZnS QD in mammalian cells using the Comet assay. The genotoxicity of CdSe/ZnS QD was shown to vary with the QD coating material, 11-mercaptoundecanoic acid coated QD being significantly more damaging than other particle coatings tested. The ability of CdSe/ZnS QD to directly cleave DNA was demonstrated by Green & Howman (2005) and Anas and coworkers (2008) using supercoiled plasmid DNA as the template. After incubating supercoiled DNA with CdSe/ZnD QD, nicked circular and linear forms of the

plasmid DNA were observed, indicative of single-strand and double-strand DNA breakage events, respectively. Photoactivation of QD by UV-irradiation increased DNA damage and radical formation (Anas et al, 2008); however the work by Green & Howman is notable for the detection of CdSe/ZnS QD-induced DNA damage in the dark. The demonstration of DNA damage by CdSe/ZnS QD is consistent with but does not constitute proof of carcinogenic risk. The risk, of course, be dependent on the stability of capping and coating materials, as described in Fig. 1.

Chemical free radical generation causes DNA damage as well as oxidative stress and cytotoxicity (Smart, et al., 2008). According to reports published by numerous laboratories, CdTe QD, CdS QD, CdSe QD and CdSe/ZnS QD generate radicals detectable by EPR (Green, et al., 2005; Anas, et al., 2008; Ipe, et al., 2005; Lovric, et al., 2005; Cho, et al., 2007). Newer particle designs which include the ZnS shell exhibit substantially reduced radical formation as reported by Ipe (2005). However according to other reports (Green et al., 2005) do not completely suppress radical generation. Cell death as a result of cytotoxicity mitigates, but does not eliminate, carcinogenic risks posed by free radical-induced DNA damage. The prevalence of radical formation and the induction of DNA damage by cadmium QD raises the level of concern with respect to cancer. Further tests of cadmium QD in one or more classic genotoxicity assays such as the Ames bacterial reverse mutation assay or the mammalian cell micronucleus formation assay are needed to better define their carcinogenic risk.

Epigenetic mechanisms control cell survival and differentiation and exert profound effects on gene expression and the cancer cell phenotype (Verdin, et al., 2006). It is important to consider the epigenetic effects of cadmium QD to fully evaluate their carcinogenic risk. Cadmium carcinogenesis is thought to involve epigenetic activation of oncogene expression (Kawata, et al., 2009). Histone proteins form complexes with DNA which direct higher order chromatin structure and modulate transcription factor binding to DNA (Lennartsson, et al., 2009). These processes are regulated by DNA methylation status and dynamic histone acetylation/methylation events and result in changes in gene expression patterns (Verdin, et al., 2006). Two recent studies showed that CdTe QD can act as histone modifiers. Conroy et al (2008) reported that CdTe QD entered cell nuclei rapidly and bound tightly to core histone proteins. Choi et al (2008) showed there was a loss of histone acetylation marks (hypoacetylation) in human cells exposed to CdTe QD and this effect was regulated by the histone deacetylase enzyme inhibitor, trichostatin A. These are the first investigations to explore cadmium QD influences on histone proteins and to identify a mechanistic basis for epigenetic effects of QD in human cells.

Cadmium contamination in the environment could increase the risk of new cancer development among the general public. Already, cadmium exposure is associated with tumors in the lung, prostate, liver, kidney, pancreas, urinary bladder and the breast (Huff et al., 2007). The shell encasing the cadmium-containing QD core may insulates against the release of cadmium in the body, but the protection afforded by the shell after the nanoparticle enters the environment is unlikely to persist indefinitely. Then, the cadmium released from the nanoparticle pharmaceutical or medical imaging agent becomes an environmental contaminant. There is a growing awareness of the adverse health effects of water contamination with steroid hormones and antimicrobial agents released from normal use or through drug manufacturing (Doerr-MacEwen, 2006; Nikolaou et al., 2007). Urinary excretion is the primary route of elimination of cadmium nanoparticle pharmaceuticals from the body, necessitating the development of strategies to restrict the introduction of cadmium nanoparticles into water supplies.

In this brief review, we have shown that on the basis of their genotoxic actions and epigenetic effects, cadmium QD might present a human carcinogenic risk. To date, there is no definitive evidence to include or exclude a role for cadmium QD in human cancer risk. A simplified

approach to risk assessment involves additional testing in standard genotoxicity assays, while evaluation of the epigenetic-mediated effects necessitates more complex molecular analyses.

Summary and Future Directions

Cadmium containing QDs have the potential to revolutionize medical therapy. As described in Jain and Stroh (2004), one can foresee a future routine clinical exam in which the patient receives a QD tracer, followed by a whole-body scan for potential cancer lesions. If found, an optical biopsy, and possibly even eradication of the lesion, can be accomplished. Nanotechnology is rapidly progressing toward this worthy goal. However we must realize that we are utilizing a potentially toxic, highly persistent nanoparticle to eradicate disease, as is the case with most cancer treatments. Yet the targeting ability of QDs holds potential for the ability to dramatically decrease, if not eliminate, toxic effects if properly investigated. To make such a goal feasible and relatively risk-free, more extensive pharmacological and toxicological investigations of QD are critical. Future directions need to include:

- Complete physicochemical characterization of QD constructs
- Improved and comprehensive ADME studies, that include the potential for long term persistence and redistribution in tissues
- Develop of adequate dosing parameters, taking surface area and size into account.
- Increased animal studies to assess biological persistence of QDs in tissues, particularly long term studies
- Consistent toxicological studies that utilize fully characterized particles.
- Increased animal toxicity studies
- Long term studies, focused on elaboration of potential chronic effects from persistence in tissues
- Environmental Considerations - With increased utilization of cadmium-containing QDs in biomedical research as well as their potential use in therapy, studies are needed to determine the environmental persistence of core/shell particles, and the level of breakdown of shell materials in the environment, due to the potential for release of nanoscale cadmium constructs.

Proceeding without careful evaluation of these critical areas will blunt the progress on nanomedicine and place human health at risk. However judicious further research into these areas will undoubtedly contribute to development of nanopharmaceuticals for cancer treatment and drug delivery that have minimal to low risk and high benefit to public health.

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Figure 1. Quantum Dots – Where do we go from here?

Shown above are the components of a representative QD, the core, shell, and targeting ligands. When considering the pharmacology & toxicology of QDs and the cadmium contained therein, one must consider first the aspects of the nanoparticle itself, the functionalized layers, the shell and capping material, and finally, the highly reactive nanoscale cadmium contained in the core. For each component, as well as for the nanoparticle as a whole, subjects where information is lacking and future research is necessary are bulleted. See text for further description