ACCOUNTS
of chemical research

Are Quantum Dots Toxic? Exploring the Discrepancy Between Cell Culture and Animal Studies

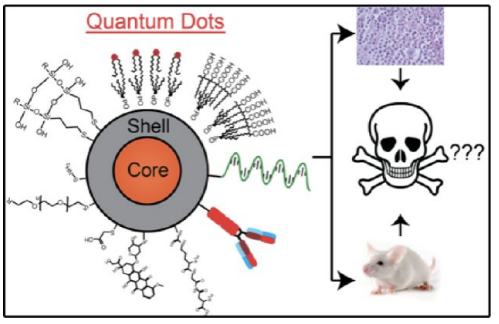
KIM M. TSOI,[†,‡,▽] QIN DAI,[†,‡] BENJAMIN A. ALMAN,[#,▽] AND WARREN C. W. CHAN*[,†,‡,§,∥,⊥]

[†]*Institute of Biomaterials and Biomedical Engineering,* [‡]*Donnelly Centre for Cellular and Biomolecular Research,* [§]*Department of Chemical Engineering,* [∥]*Department of Chemistry, and* [⊥]*Department of Materials Science and Engineering, University of Toronto, Toronto, ON, M5S 3G9, Canada,* [#]*Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, University of Toronto, Toronto, ON, M5G 1X8, Canada, and* [▽]*Division of Orthopaedic Surgery, Department of Surgery, University of Toronto, Toronto, ON, Canada*

RECEIVED ON FEBRUARY 2, 2012

CONSPECTUS

Despite significant interest in developing quantum dots (QDs) for biomedical applications, many researchers are convinced that QDs will never be used for treating patients because of their potential toxicity. The perception that QDs are toxic is rooted in two assumptions. Cadmium-containing QDs can kill cells in culture. Many researchers then assume that because QDs are toxic to cells, they must be toxic to humans. In addition, many researchers classify QDs as a homogeneous group of materials. Therefore, if CdSe QDs are harmful, they extrapolate this result to all QDs. Though unsubstantiated, these assumptions continue to drive QD research. When dosing is physiologically appropriate, QD toxicity has not been demonstrated in animal models. In addition, QDs are not uniform: each design is a unique combination of physicochemical properties that influence biological activity and toxicity. In this Account, we summarize key findings from in vitro and in vivo studies, explore the causes of the discrepancy in QD toxicological data, and provide our view of the future direction of the field.



In vitro and in vivo QD studies have advanced our knowledge of cellular transport kinetics, mechanisms of QD toxicity, and biodistribution following animal injection. Cell culture experiments have shown that QDs undergo design-dependent intracellular localization and they can cause cytotoxicity by releasing free cadmium into solution and by generating free radical species. In animal experiments, QDs preferentially enter the liver and spleen following intravascular injection, undergo minimal excretion if larger than 6 nm, and appear to be safe to the animal.

In vitro and in vivo studies show an apparent discrepancy with regard to toxicity. Dosing provides one explanation for these findings. Under culture conditions, a cell experiences a constant QD dose, but the in vivo QD concentration can vary, and the organ-specific dose may not be high enough to induce detectable toxicity. Because QDs are retained within animals, long-term toxicity may be a problem but has not been established.

Future QD toxicity studies should be standardized and systematized because methodological variability in the current body of literature makes it difficult to compare and contrast results. We advocate the following steps for consistent, comparable toxicology data: (a) standardize dose metrics, (b) characterize QD uptake concentration, (c) identify in vitro models that reflect the cells QDs interact with in vivo, and (d) use multiple assays to determine sublethal toxicity and biocompatibility.

Finally, we should ask more specific toxicological questions. For example: "At what dose are 5 nm CdSe QDs that are stabilized with mercaptoacetic acid and conjugated to the antibody herceptin toxic to HeLa cells?" rather than "Are QDs toxic?" QDs are still a long way from realizing their potential as a medical technology. Modifying the current QD toxicological research paradigm, investigating toxicity in a case-by-case manner, and improving study quality are important steps in identifying a QD formulation that is safe for human use.

Published on the Web 08/01/2012 www.pubs.acs.org/accounts
10.1021/ar300040z

1. Introduction

There has been significant interest in developing quantum dots (QDs) for biomedical applications that include sentinel lymph node mapping, multifunctional drug delivery, and photodynamic therapy. However, many researchers are convinced that QDs will never be used for diagnosing and treating patients because of their potential toxicity. The perception that QDs are toxic originates from in vitro studies where cadmium-containing particles killed cells in culture. Despite a lack of substantiating evidence, many then assumed that since QDs are toxic to cells, they must also be toxic to humans. This extrapolation is inappropriate as results from cell-based toxicity studies are rarely transferable to more complex biological systems. Notably, QD toxicity has not been demonstrated in animal models after single and physiologically relevant dosing.

QDs are a heterogeneous group of materials and it is difficult to make general statements regarding their toxicity (Figure 1). They vary in their core and shell composition, in their size and shape, and in their surface chemistry. QD surfaces can have hydrophobic ligands that make them soluble in the organic phase. They can be surface-modified to render them water-soluble and biocompatible. Their surface can have bifunctional molecules such as mercaptoacetic acid, can be coated with amphiphillic polymers such as poly(acrylic acid)−octadecylamine polymers, or can be trapped within micelles. QDs can be further modified with targeting ligands such as oligonucleotides, peptides, or antibodies that will direct the QDs to specific sites within the body or to specific locations within a cell. A vast number of QD formulations are possible, each with a unique combination of physicochemical properties that dictate the QD's interaction with a biological entity. In this Account, we summarize key findings from in vitro and in vivo studies, explore causes of the discrepancy in QD toxicological data, and finally provide our view on the future direction of this field.

2. Mechanisms of Quantum Dot Toxicity Using In Vitro Cell Cultures

Mechanisms of QD-induced cytotoxicity have been elucidated by studying model cell culture systems exposed to group II−VI QDs (CdS, CdTe, CdSe, or ZnS-capped CdSe) (Figure 2). Derfus et al. demonstrated that, when oxidized in air or by ultraviolet light irradiation, mercaptoacetic acid-stabilized CdSe QDs released free cadmium ions into solution and caused primary liver cell (hepatocyte) death.[1] These results led researchers to believe that QDs would also be toxic to humans since cadmium is a known carcinogen and has been epidemiologically linked to renal, skeletal, pulmonary, and reproductive damage.[2] Intracellular QD degradation with cadmium release has also been suggested. Microscopy studies have shown that QDs localize within cellular endosomes and lysosomes[3] and are thereby exposed to an acidic or oxidative microenvironment. In a cell-free assay, Mancini et al. found that hypochlorous acid, present in phagocytic cells, oxidized polymer-encapsulated CdS/ZnS-capped CdSe QDs with solubilization of cadmium, zinc, sulfur, and selenium species.[4]

Group II−VI QDs can also induce cytotoxicity by generating reactive oxygen species (ROS), which in turn damage cellular proteins, lipids, and DNA. Ipe et al. irradiated mercaptoacetic acid-stabilized CdS, CdSe, and ZnS-capped CdSe QDs and identified photogenerated radical species with electron paramagnetic resonance spectroscopy and a radical-specific fluorometric assay. They found that redox potential was dependent on QD chemical composition. While CdS QDs had sufficient redox power to produce hydroxyl and superoxide radicals, CdSe QDs exclusively formed hydroxyl radicals. Additionally, the ZnS shell was an efficient inhibitor of QD reactivity as ZnS-capped CdSe QDs did not generate any ROS.[5] Lovric et al. then established a link between ROS formation and cytotoxicity. They first determined that mercaptopropionic-acid-stabilized CdTe QDs caused dose-dependent cytotoxicity in the human breast cancer cell line, MCF-7. In a follow-up experiment, the superoxide indicator dihydroethidium was used to demonstrate ROS generation in QD-treated cells. As further evidence, cotreatment with *N*-acetylcysteine, an ROS scavenger and strong antioxidant, partially reversed cytotoxicity.[6]

While the release of free cadmium and ROS production are often discussed in isolation, it is more likely that these mechanisms act in concert to produce QD toxicity. King-Heiden et al. demonstrated that free cadmium release could not solely explain why ZnS-capped CdSe QDs were toxic to the zebrafish embryo *Danio rerio*. The authors first determined that a 10-fold higher concentration of ionic cadmium (20 μM $CdCl_2$) was required to induce equivalent mortality (3−6%) as a solution of QDs (2 μM, based on cadmium content). They then compared sublethal toxic effects at these exposure doses. Ionic cadmium caused four toxic responses: altered axial curvature as well as pericardial, ocular, and submaxillary edema. Contrastingly, QDs induced these "cadmium-like" end-points in addition to a deformed tail, yolk sac malformation, and opaque tissue reflecting necrosis in the head, body, and yolk sac. Presence of the latter "non-cadmium-like" sequelae suggested that a second

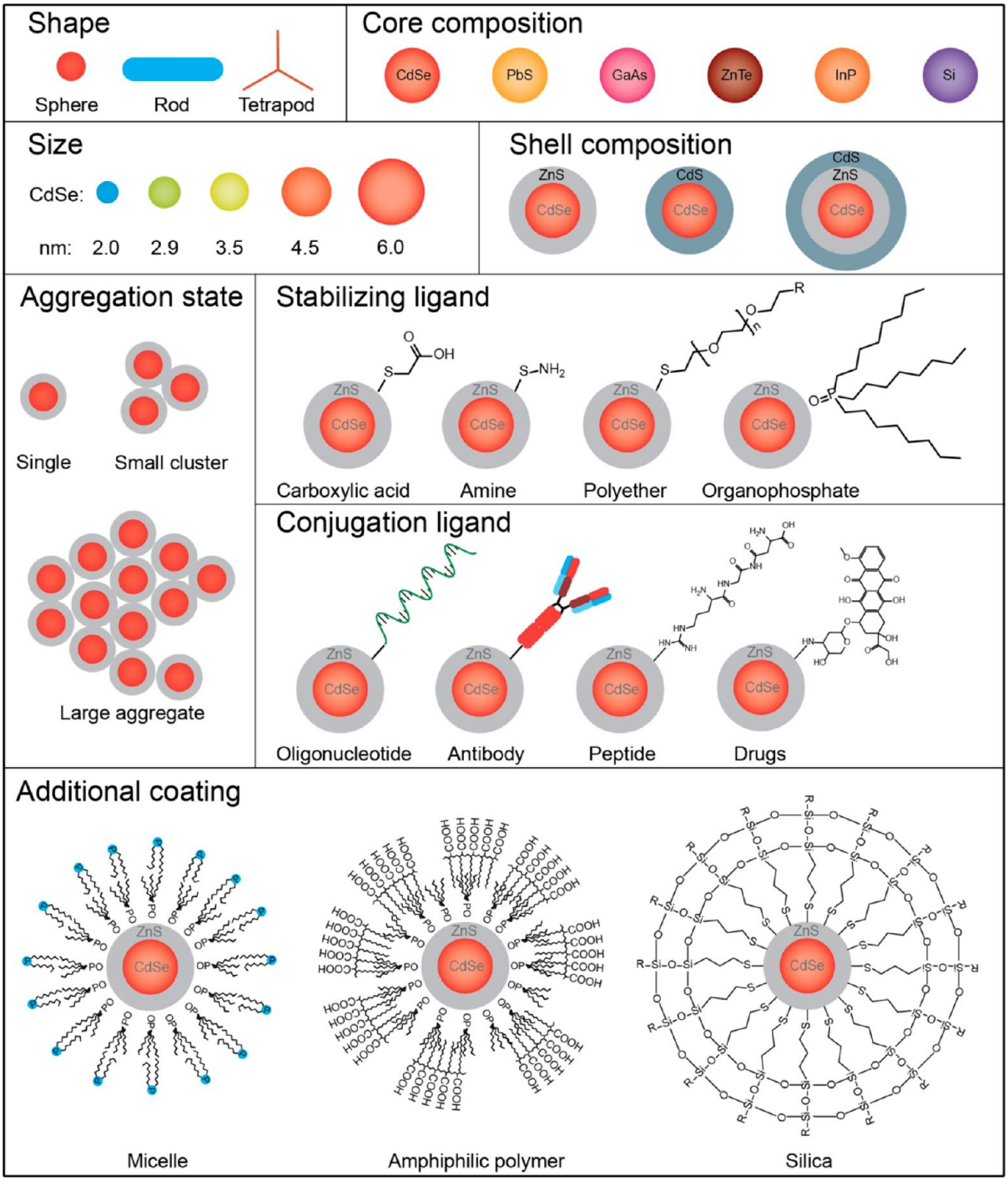


FIGURE 1. Quantum dots (QD) are a heterogeneous group of materials. Biological fate and toxicity depend on QD physicochemical properties. Shape, core composition, size, and shell composition can be manipulated during QD synthesis. Postsynthesis, surface ligands are added to solubilize and target the particles. An additional coating can further protect the QD core from oxidation. Surface chemistry influences the quantum dot's propensity to aggregate, particularly in biological solutions.

mechanism, potentially oxidative stress, may have combined with cadmium release to cause QD toxicity.[7]

A unique aspect of nanoparticle toxicity is their size-dependent intracellular routing. Nanoscale particles are able to reach organelles that are inaccessible to metal ions. This may result in unique patterns of cytotoxicity compared to their constituent metals. Following uptake by a cell, QDs are packaged into small intracellular vesicles and transported from the cell periphery to the perinuclear region.[8] Contrastingly, cadmium ions are predominantly located in the

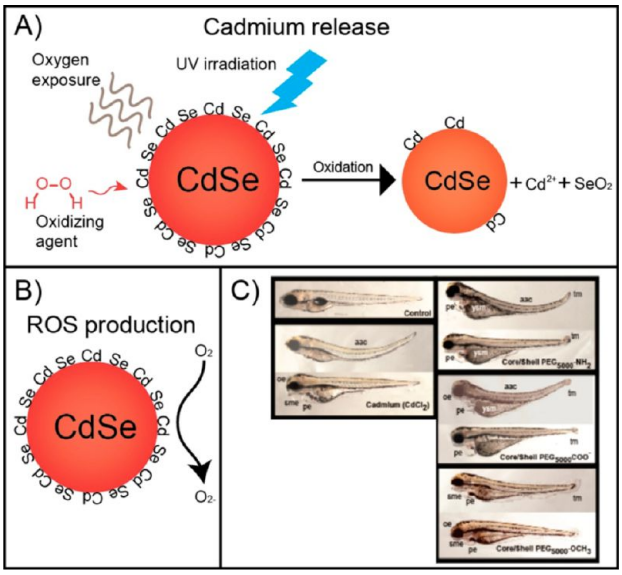


FIGURE 2. Cell culture experiments demonstrate that quantum dots (QD) induce cytotoxicity via two mechanisms: (A) QD degradation with release of free cadmium and (B) Generation of reactive oxygen species. These mechanisms likely combine to cause toxicity. (C) Zebrafish embryos exposed to either 20 μM $CdCl_2$ or 2 μM ZnS-capped CdSe QDs experienced similar mortality (3−6%) but different toxicity end-points. Ionic cadmium caused altered axial curvature (aac), pericardial edema (pe), ocular edema (oe), and submaxillary edema (sme). QDs caused these "cadmium-like" responses in addition to "not cadmium-like responses". "Not cadmium-like responses" were as follows: tail malformation (tm) and yolk sac malformation (ysm). This discrepancy suggests additional mechanism(s) combined with cadmium release to cause QD toxicity. Reprinted with permission from ref 7. Copyright 2009 American Chemical Society.

cytoplasm, where they are sequestered by metallothionein.[9] The observation that QDs can localize to the cell's nuclear compartment has led researchers to investigate their potential genotoxicity. If QDs cause DNA mutations without cell death, their effect is propagated through future generations of cells and can ultimately lead to disease. Green and Howman showed that biotin-coated ZnS-capped CdSe QDs could nick supercoiled DNA.[10] Choi et al. demonstrated that QDs could induce an epigenetic response (a functionally relevant DNA modification that does not involve a change in nucleotide sequence) in cell culture. They determined that CdTe QDs caused histone hypoacetylation in human breast carcinoma cells resulting in increased cell death via upregulation of p53-related proapoptotic genes.[11]

3. Validation of QD Toxicity Using In Vivo Animal Models

Researchers have attempted to validate the QD toxicity observed in cell culture studies with animal models. While cytotoxicity can easily be measured in vitro via metabolic activity or membrane permeability assays, toxicity is not as straightforward to quantify in animals. In addition to measuring organism viability, sublethal toxicity such as organ damage must be considered. Early rodent QD bioimaging studies identified body areas at risk for toxicity. Researchers observed that the organs of the reticuloendothelial system (RES) nonspecifically took up and retained the majority of injected QDs.[12,13] The RES is part of the body's defense system to eradicate foreign materials and consists of phagocytic cells located primarily in the liver, spleen, and lymph nodes. Data then suggested that QDs are degraded within these organs. Fitzpatrick et al. followed BALB/c and nude mice for 2 years postinjection of ZnS-capped CdSe QDs and observed blue-shifted emission peaks in the liver, spleen, and lymph nodes, reflective of particle retention and breakdown within these organs.[14] As QDs become smaller during the degradation process, the fluorescence emission shifts from red to blue and the excitonic fluorescence peak becomes broader. Hauck et al. performed the first comprehensive in vivo toxicity study and demonstrated that ZnS-capped CdSe QDs were not harmful to Sprague−Dawley rats in both the short- (<7 days) and long-term (>80 days). There were no alterations in animal behavior, animal weight, or hematological markers in comparison to controls. Despite detecting QDs, or their degradation products in the liver, spleen, and kidney, no organ damage or inflammation were found through either histological or biochemical analysis.[15] To date, no studies have reproducibly documented QD toxicity in animals at physiologically appropriate doses.

These results lead to an obvious question: why are QDs apparently toxic in vitro but safe in vivo? A discrepancy between in vitro and in vivo toxicity study results is not unique to QDs.[16] This lack of agreement can partially be attributed to the nature of the nanoparticle−cell interaction in culture conditions versus in an animal. To conduct an in vitro study, a monolayer of cells is seeded in a culture dish and incubated with nanoparticles diluted in media. Consequently, cells experience a relatively constant dose of nanoparticles whose physicochemical properties are similar to what they were at time zero of the experiment. In vivo, nanoparticle dose and structure are in constant flux. Only a fraction of injected nanoparticles will interact with the cells of interest and parameters such as size and surface chemistry will be altered by nanoparticle passage through the body. Another contributing factor is the difference in complexity between these two models. In vitro, cells are isolated from the three-dimensional architecture and cell−cell

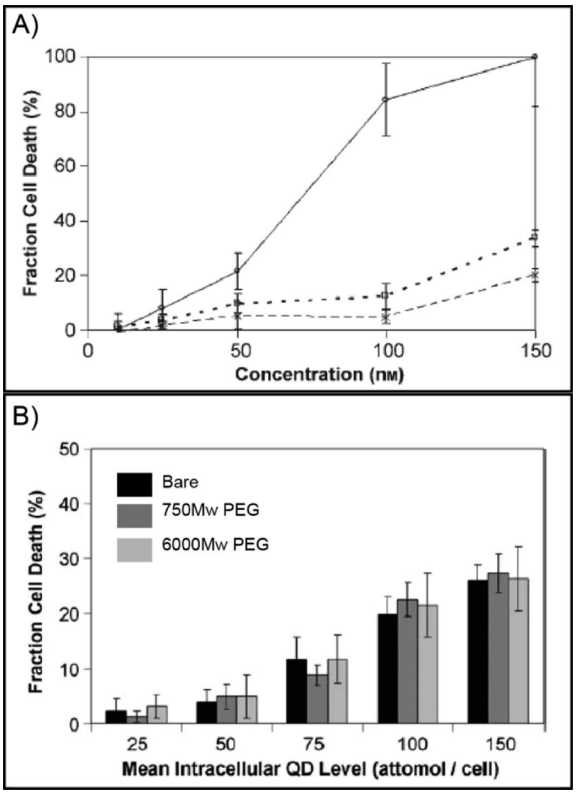


FIGURE 3. Quantum dot cytotoxicity correlates with intracellular, not extracellular particle concentration. (A) Bare (○) CdS-CdSe QDs appear more toxic than poly(ethylene glycol)-substituted (□, 750 M_w; ×, 6000 M_w) QDs to SK-BR-3 cells when extracellular QD concentration is reported. (B) Cytotoxicity is equivalent when normalized to the number of QDs per cell. Reprinted with permission from ref 18. Copyright 2006 John Wiley and Sons.

interactions present in vivo. This isolation may alter a cell's response to a toxic agent and therefore affect the result of an in vitro assay. Though it is tempting to classify animal toxicity studies as more relevant, the utility of cell-based experiments should not be underestimated. Advantages of in vitro over in vivo studies include lower cost, higher throughput and the ability to study mechanisms at the cellular level. These features render in vitro assays preferable when screening large numbers of nanoparticle formulations in order to identify candidates for detailed in vivo investigation.

4. "The Dose Makes the Poison"

Dose is the most important factor determining a substance's toxicity. This principle also applies to QDs. Dubertret et al. first demonstrated dose-dependent QD toxicity by injecting *Xenopus* frog embryos with ZnS-capped CdSe QDs encapsulated within phospholipid block-copolymer micelles. At a concentration of 2×10^9 QD/cell, no phenotypic alterations were observed; however, at a concentration of $>5 \times 10^9$ QD/cell, abnormalities were apparent.[17] It is important to define the term "dose". Equating dose with extracellular concentration is insufficient and may be misleading. Chang et al. provided evidence that intracellular, not extracellular, QD concentration correlates with cytotoxicity. At exposure concentrations between 0 and 150 nM, "bare" CdS-capped CdSe QDs were more toxic than poly(ethylene glycol)-substituted QDs to the human breast cancer cell line SK-BR-3 (Figure 3). When the dose was altered to achieve the same number of QDs per cell for both the "bare" and poly(ethylene glycol)-substituted QDs, cytotoxicity was equivalent.[18]

This study illustrates an important relationship. QD endocytosis and, therefore, the effective intracellular dose, are dependent on particle physicochemical properties such as surface chemistry and size. Osaki et al. added saccharide moieties to CdSe QDs and demonstrated that cellular uptake by HeLa cells was maximal at a hydrodynamic diameter of 50 nm.[19] Nabiev et al. showed that QD size also dictates intracellular distribution. While green-emitting (2.1 nm in diameter) mercaptoacetic-acid-stabilized CdTe QDs were able to access the nuclear compartment, their larger red-emitting (3.4 nm) counterparts were restricted to the cytoplasm in human macrophage THP-1 cells.[20] Duan and Nie demonstrated that QD intracellular localization also depends on surface chemistry (Figure 4). The authors developed poly(ethylene glycol)-grafted polyethylenimine (PEI-*g*-PEG) copolymer ligands and by reducing the number of PEG grafts per PEI molecule from four to two were able to alter QD localization. While one QD formulation (PEI-*g*-PEG_4) was trapped within cellular vesicles and transported close to the nucleus, a slightly different formulation (PEI-*g*-PEG_2) allowed QDs to escape the endosomes and be released into the cytoplasm.[21]

It is probable that particle shape and aggregation status also influence cellular uptake, but these associations have not yet been studied for QDs. Using gold nanoparticles, Chithrani et al. found that aspect ratio was inversely correlated with uptake into HeLa cells. Cells took up 375% more 14 nm spherical than 74 × 14 nm rod-shaped gold nanoparticles.[22] Albanese and Chan showed that particle aggregation decreased receptor-mediated endocytosis in HeLa cells. NaCl was added to a solution of 16 nm transferrin-coated gold nanoparticles, yielding a range of aggregate sizes. A 25% decrease in the uptake of both 49 and 98 nm aggregates in comparison to monodisperse particles was measured.[23] QDs are equally at risk of aggregation. Biological solutions such as cell culture media contain ions, which

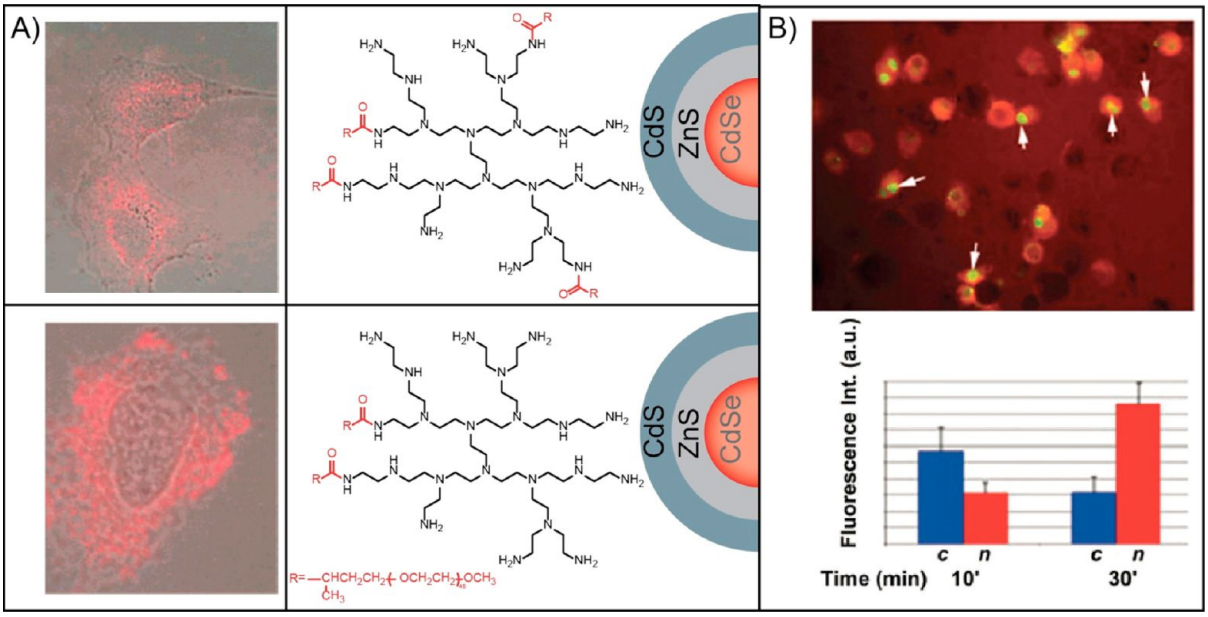


FIGURE 4. Intracellular QD localization varies with QD surface chemistry and size. (A) Effect of QD surface chemistry. Top: PEI-*g*-PEG_4-coated CdS/ZnS-capped CdSe QDs are trapped within intracellular endosomes and accumulate in the perinuclear region of HeLa cells. Bottom: PEI-*g*-PEG_2-coated QDs escape from the endosomes are are distributed throughout the cytoplasm. (B) Effect of QD size. Top: After 30 min, green (2.1 nm) CdTe QDs have completely translocated into the nucleus of THP-1 cells while larger red (3.4 nm) CdTe QDs are restricted to the cytoplasm. Bottom: Green QDs shift from the cytoplasm (c) to the nucleus (n) over time. Reprinted with permission from refs 21 and 20. Copyright 2007 American Chemical Society.

can displace or interact with surface ligands and thereby destabilize QDs in suspension.

QD physicochemical properties also impact in vivo biodistribution, a term that refers to QD absorption, distribution, metabolism, and excretion following administration to an animal. Fischer et al. compared the plasma half-life and relative organ uptake of mercaptoundecanoic-acid-coated ZnS-capped CdSe QDs that were cross-linked with either lysine and bovine serum albumin (QD-BSA) or lysine alone (QD-LM). Following intravenous injection into Sprague–Dawley rats, the half-life of QD-LM was 58.5 ± 17.0 min, significantly longer than that of QD-BSA ($t_{1/2}$ = 38.7 ± 3.5 min; $p < 0.05$). After 90 min, the accumulation of QD-LM (36.4 ± 8.1% dose) in the liver was significantly lower that that of QD-BSA (99.5 ± 9.2% dose) while the opposing trend was observed in the lung and kidney.[24] Though not explicitly studied by the authors, the relationship between biodistribution and QD physicochemical properties is likely mediated by protein opsonization. When exposed to blood, nanoparticles including QDs are rapidly opsonized, or coated with a complex and dynamic assembly of plasma proteins which influence how the particle is "seen" by cells in vivo. Protein opsonization has been shown to be highly dependent on nanoparticle physicochemical properties.[25]

While the dependence of biological behavior on QD design may complicate QD toxicity research, it also suggests a strategy for synthesizing safer particles in the future. The optimal QD would selectively accumulate in the in vivo target, such as a tumor, would avoid uptake by RES organs, and would ultimately clear from the body. The ability to manipulate cellular uptake, intracellular localization, and protein opsonization by modifying QD size and surface chemistry suggests that this may be possible.

5. Do QDs Clear from the Body?

Many authors have reported that QDs accumulate in the organs of the RES, but little focus has been placed on QD clearance from the body. There are two major excretion pathways available to metal-containing particles: passage through the kidneys into urine or via the liver's biliary system into feces (Figure 5). Choi et al. demonstrated that small, zwitterionic QDs could be excreted into the urine. Cysteine-coated ZnS-capped CdSe QDs were labeled with $^{99\ m}$Tc and injected into Sprague–Dawley rats. By measuring the radioactivity of both excreted and pre-excreted urine four hours postadministration, they observed that a hydrodynamic diameter of 5.5 nm or less allowed for clearance of a minimum 50% of total body QD content.[26] We want to point out that the renal filtration threshold for nanoparticles is likely greater than 5.5 nm as the authors did not take protein opsonization into account. Regardless, most QDs under investigation for biomedical applications have an ex

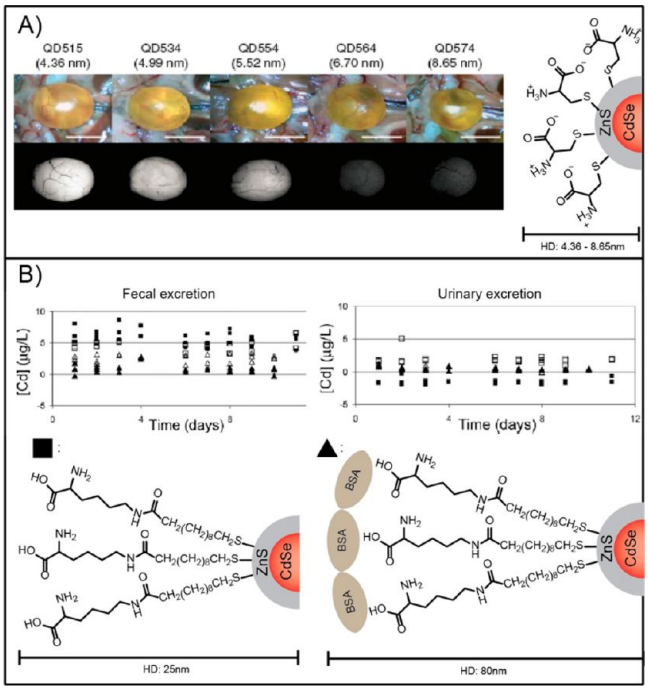


FIGURE 5. In vivo QD excretion is size-dependent. (A) Cysteine-coated ZnS-capped CdSe QDs with a hydrodynamic diameter ≤ 5.5 nm were able to pass through the kidney into the bladder for excretion in the urine. Shown are color video (top) and fluorescence (bottom) images of surgically exposed CD-1 mouse bladders following injection of different size QDs. (B) Larger QDs are not excreted in either the feces (left) or urine (right). Mercaptoundecanoic acid-stabilized ZnS-capped CdSe QDs cross-linked either with lysine and bovine serum albumin (▲, hydrodynamic diameter, HD 80 nm) or lysine alone (■, HD 25 nm) were injected into Sprague–Dawley rats. Cadmium content in urine and feces was not significantly different between QD-exposed and control (□ and △) animals over a 10 day period. Reprinted with permission from refs 26 and 24. Copyright 2007 Nature Publishing Group, 2006 John Wiley and Sons.

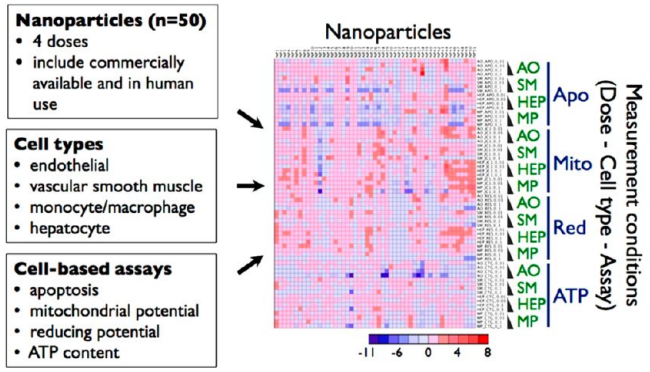


FIGURE 6. High-throughput platform for nanoparticle toxicity screening. Fifty different nanoparticles were assessed at four doses in four cell types using four assays (i.e., 64 different conditions per nanoparticle). The data set is presented as a heat map where the color of each square reflects the *Z* score for an experimental condition (*Z* score: $Z_{\text{nanoparticle}} = (\mu_{\text{nanoparticle}} - \mu_{\text{control}})/\sigma_{\text{control}}$, where μ = mean and σ = standard deviation). Abbreviations: AO, aortic endothelial cell; SM, vascular smooth muscle; HEP, hepatocyte; MP, monocyte/macrophage; Apo, apoptosis assay; Mito, mitochondrial potential assay; Red, reducing equivalents assay; ATP, ATP content assay. Reprinted with permission from ref 31. Copyright 2008 National Academy of Sciences, USA.

vivo hydrodynamic diameter larger than 5.5 nm and may not be rapidly cleared by either pathway. Fischer et al. collected urine and feces from Sprague–Dawley rats injected with 25 and 80 nm hydrodynamic diameter protein-coated ZnS-capped CdSe QDs. Neither QDs nor their byproduct were detected via inductively coupled plasma atomic emission spectroscopy in either excretion product up to 10 days postinjection.[24] The tendency toward accumulation rather than clearance seems to also hold true at the cellular level. QD exocytosis has been poorly investigated but multiple studies infer retention of intracellular QDs over time.[27,28] If they are released, they are released at a very slow rate.

Lack of QD clearance poses a problem for long-term toxicity, particularly if repeat dosing is required. One potential consequence is on reproductive health. Chu et al. observed that mercaptopropionic-stabilized CdS-capped CdTe QDs, or their constituent cadmium ions, could be transferred across the placenta of Kun Ming mice resulting in dose-dependent pup death. For instance, following a perinatal injection of 125 μg of QD solution, 71.8 ± 40.4 ng of cadmium per gram body weight was detected in the pups causing a mortality rate of 66.3%.[29]

6. Perspective and Future Outlook

We should no longer ask "Are quantum dots toxic?" Rather, the question should be "At what dose are 5 nm CdSe QDs that are stabilized with mercaptoacetic acid and conjugated to the antibody herceptin toxic to HeLa cells?" Both in vitro and in vivo studies have demonstrated that QD physicochemical properties influence the particle–cell interaction and the potential for toxicity. Each QD design is a unique combination of size, shape, surface chemistry, and propensity to aggregation. Consequently, each QD design exhibits different uptake kinetics, biodistribution patterns, degradation rates, and potential for generating reactive oxygen species than another formulation. A more rational and cost-effective strategy may be to focus on clinically promising QD formulations on a case-by-case basis. This would require a high-throughput platform to screen large numbers of nanoparticle designs using multiple toxicity assays and cell types in order to separate formulations that are safe from ones that are toxic (Figures 6 and 7). QDs that "pass" the initial screen could then undergo more rigorous cell-based mechanistic and animal toxicity studies. The ability of an in vitro high throughput screen to accurately predict in vivo toxi-

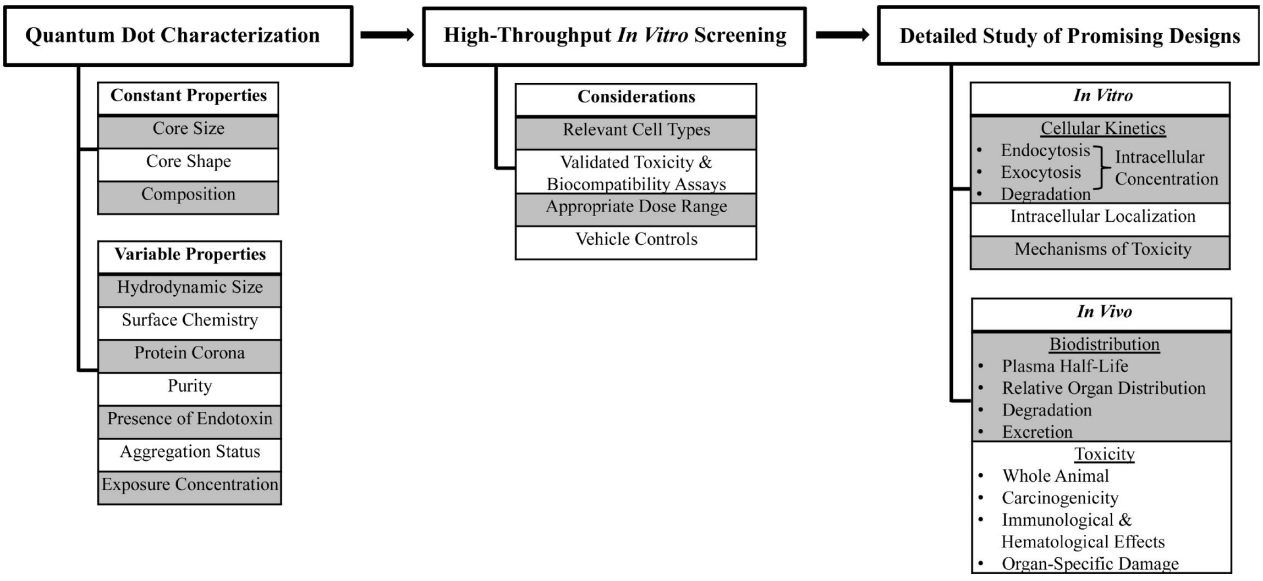


FIGURE 7. Multistage strategy to identify "safe" QD formulations.

city has been demonstrated for pharmaceutical agents.[30] Nanoparticle-suitable methods are currently in development.[31]

QD toxicity research is defined by its diversity. The material of interest is heterogeneous, but so are the investigators conducting the studies. In many ways, this diversity has been positive as the perspective of many stakeholders, namely, material scientists, pharmaceutical researchers, clinicians, bioengineers, and toxicologists, has been included. However, it has also resulted in detrimental variability in experimental methodology. We advocate that four areas in particular need to be addressed in order to properly assess QD toxicity in the future.

First, dose metrics must be standardized. A wide range of techniques has been used to measure QD dose, contributing to the inconsistency in the field. For example, QD concentration is often determined via Beer's law, $A = \varepsilon CL$, where A is the absorbance measured at the first exciton peak, ε is molar absorptivity ($cm^{-1}\ M^{-1}$), L is path length (cm), and C is molar concentration (M) (Table 1). As there is no accepted value for molar absorptivity, the reported QD dose depends on which study a researcher selects.[32−34] We advocate that a more objective method is required. Researchers have started to assess QD elemental content via analytical techniques such as inductively coupled plasma atomic emission spectroscopy (ICP-AES). It would not be difficult to normalize QD exposure based on a pertinent metal, such as cadmium, and this would improve our ability to compare the results of one study to the next. Furthermore, intracellular along with extracellular QD concentration should be reported. Intracellular QD concentration may more accurately reflect cellular exposure, as it takes both endocytosis and exocytosis into account. Its dynamic nature renders it more difficult to measure. Some researchers have characterized time-dependent intracellular QD content based on fluorescence. This approach is unreliable as photobleaching, alterations to the QD surface, and QD degradation also affect fluorescence intensity. Instead, we suggest that elemental analysis techniques such as ICP-AES also be used for this purpose.

TABLE 1. QD Concentration As Determined by Beer's Law Depends on Which Molar Absorptivity Study Is Selected[a]

source	molar Absorptivity, ε ($cm^{-1}\ M^{-1}$)	QD concentration, C ($\times 10^9$ mol/L)
Yu et al.[32]	262 595	381
Striolo et al.[33]	740 000	135
Leatherdale et al.[34]	1 331 732	75

[a]For a hypothetical solution of 4.2 nm diameter CdSe QDs, there is a five-fold difference in calculated concentration between studies ($A = 0.1$; $L = 1$ cm).

Second, QD characterization must reflect the QD at the time of delivery, not the QD that is synthesized. Most studies now report composition, shape, zeta potential, as well as core and/or hydrodynamic diameter values. These measurements are often conducted postsynthesis in water or buffer. Contrastingly, cells are exposed to QDs that have been diluted in media, potentially after sitting on a shelf for a few months, experiencing bacterial contamination or being oxidized in air. Cell culture media is a complex solution of ions and serum proteins that can affect QD hydrodynamic diameter and aggregation. Environmental conditions can induce QD degradation or endotoxin formation. Consequently, the QD assessed in an in vitro study may have

different properties than those reported by the authors. Another commonly overlooked variable is QD solution purity. QD synthesis and surface modification steps require certain agents, such as the solvent tri-*n*-octylphosphine oxide or the surface ligand mercaptoacetic acid, that may themselves be toxic to cells. Studies often fail to account for contaminants and/or use appropriate controls. It is impossible to attribute toxicity results to the QDs themselves if impurities are not measured and if a buffer rather than the QD vehicle solution is used as the control. Finally, commercially available QDs should be characterized before use in toxicity experiments because they may have different properties than those reported by the company and are susceptible to lot-to-lot variability.[35]

Third, in vitro model selection must be more reflective of in vivo biodistribution. To date, cell types appear to have been chosen almost at random. This is problematic because cells can vary substantially in their sensitivity to nanoparticle exposure.[36] Purposeful selection of cell types shown to be relevant in animal biodistribution studies would help resolve the discrepancy between the in vitro and in vivo studies. For example, it is accepted that QDs accumulate in the liver following intravascular injection. A logical next step would be to conduct studies based on liver cells, such as hepatocytes and Kupffer cells. This approach would improve the odds of identifying mechanisms of toxicity and cellular kinetics relevant to animal and potentially human exposure. While intravascular injection is the mode of exposure most relevant to biomedical applications, QD toxicity research will only be complete when transdermal, inhalational and oral exposure are also understood. As QDs become more popular, the chance of unintentionally touching, breathing, or ingesting these materials rises. Biodistribution will vary with route of entry and a different subset of organs and cell types will need to be studied.

Finally, an appropriate set of sublethal and lethal toxicity end points must be included. Assays to measure cytotoxicity, ROS generation/oxidative stress, and genotoxicity are commercially available and have been reviewed elsewhere.[37] Protocols to evaluate immunological properties and blood biocompatibility have been published by the Nanotechnology Characterization Laboratory.[38] It is important to note that due to their high adsorption capacity, optical properties, and catalytic activity, QDs have the potential to interfere with assay components or detection systems. This can cause false negative or false positive results.[39] The high adsorption capacity of another nanoparticle type, single walled carbon nanotubes, was shown to precipitate MTT-formazan crystals, producing a false positive result in the commonly used MTT cell viability assay.[40] It is recommended to validate any cytotoxicity experiment with more than one test.

We are still a long way from transitioning a QD-based technology from the bench to a patient's bedside. While significant research effort over the past decade has advanced our understanding of QD cellular transport kinetics, toxicity mechanisms, and biodistribution, its main contribution has been to provide guidance for the future. The traditional toxicological paradigm, useful for evaluation of chemicals, can no longer be applied to QDs. Existing techniques must be adapted and novel approaches introduced that are more appropriate for this diverse and unique group of materials. Additionally, new strategies to engineer safer QDs through either changes in chemical composition (e.g., indium phosphide)[41] or surface coatings (e.g., polystyrene)[42] should be explored. Innovative thinking combined with improved methodological quality are important steps forward in bridging the gap between in vitro and in vivo testing and, ultimately, finding the answer to QD toxicity.

W.C.W.C. would like to acknowledge CIHR, NSERC, CFI, and MRI for funding support. K.T. would like to acknowledge the Surgeon-Scientist Program at the University of Toronto and NSERC for graduate funding.

BIOGRAPHICAL INFORMATION

Kim M. Tsoi received a Bachelor of Applied Science and a medical degree from the University of Toronto. She is an orthopaedic surgery resident pursuing doctoral studies in Biomedical Engineering through the Surgeon-Scientist Program, also at the University of Toronto. Her research interest is in quantum dot hepatotoxicity.

Qin (Bill) Dai received a Bachelor of Science degree from McMaster University in Hamilton, Canada. He is now pursuing doctoral studies in Biomedical Engineering at the University of Toronto. His research interests are nanoparticle engineering, tumor targeting, and therapeutic delivery.

Dr. Benjamin Alman is the A.J. Latner Professor and Chair of Orthopaedics and Vice Chair of Research for the Department of Surgery at the University of Toronto. He is a senior scientist in the Research Institute of The Hospital for Sick Children, with an appointment in the Program in Developmental and Stem Cell Biology. His group studies the role of developmental signaling pathways in musculoskeletal tumors and repair processes.

Dr. Warren Chan received his Bachelor of Science degree from the University of Illinois, his PhD from Indiana University, and completed postdoctoral training at the University of California-San Diego. He is currently an Associate Professor at the Institute of Biomaterials and Biomedical Engineering at the University of Toronto. His group is interested in understanding the interaction

of nanoparticles with cells and in animals, engineering novel nanostructures that can effectively target tumors, and the design of handheld molecular diagnostic devices for cancer and infectious diseases.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: warren.chan@utoronto.ca.
The authors declare no competing financial interest.

REFERENCES

1 Derfus, A. M.; Chan, W. C. W.; Bhatia, S. N. Probing the Cytotoxicity of Semiconductor Quantum Dots. *Nano Lett.* **2004**, *4* (1), 11–18.

2 Godt, J.; Scheidig, F.; Grosse-Siestrup, C.; Esche, V.; Brandenburg, P.; Reich, A.; Groneberg, D. A. The Toxicity of Cadmium and Resulting Hazards for Human Health. *J. Occup. Med. Toxicol.* **2006**, *1*, 22.

3 Clift, M. J. D.; Brandenberger, C.; Rothen-Rutishauser, B.; Brown, D. M.; Stone, V. The Uptake and Intracellular Fate of a Series of Different Surface Coated Quantum Dots In Vitro. *Toxicology* **2011**, *286* (1−3), 58–68.

4 Mancini, M. C.; Kairdolf, B. A.; Smith, A. M.; Nie, S. Oxidative Quenching and Degradation of Polymer-Encapsulated Quantum Dots: New Insights into the Long-Term Fate and Toxicity of Nanocrystals in Vivo. *J. Am. Chem. Soc.* **2008**, *130* (33), 10836–10837.

5 Ipe, B. I.; Lehnig, M.; Niemeyer, C. M. On the Generation of Free Radical Species from Quantum Dots. *Small* **2005**, *1* (7), 706–709.

6 Lovrić, J.; Cho, S. J.; Winnik, F. M.; Maysinger, D. Unmodified Cadmium Telluride Quantum Dots Induce Reactive Oxygen Species Formation Leading to Multiple Organelle Damage and Cell Death. *Chem. Biol.* **2005**, *12* (11), 1227–1234.

7 King-Heiden, T. C.; Wiecinski, P. N.; Mangham, A. N.; Metz, K. M.; Nesbit, D.; Pedersen, J. A.; Hamers, R. J.; Heideman, W.; Peterson, R. E. Quantum Dot Nanotoxicity Assessment Using the Zebrafish Embryo. *Environ. Sci. Technol.* **2009**, *43* (5), 1605–1611.

8 Parak, W. J.; Boudreau, R.; Le Gros, M.; Gerion, D.; Zanchet, D.; Micheel, C. M.; Williams, S. C.; Alivisatos, A. P.; Larabell, C. Cell Motility and Metastatic Potential Studies Based on Quantum Dot Imaging of Phagokinetic Tracks. *Adv. Mater.* **2002**, *14* (12), 882.

9 Beyersmann, D.; Hechtenberg, S. Cadmium, Gene Regulation, and Cellular Signalling in Mammalian Cells. *Toxicol. Appl. Pharmacol.* **1997**, *144* (2), 247–261.

10 Green, M.; Howman, E. Semiconductor Quantum Dots and Free Radical Induced DNA Nicking. *Chem. Commun.* **2005** (1), 121–123.

11 Choi, A. O.; Brown, S. E.; Szyf, M.; Maysinger, D. Quantum Dot-Induced Epigenetic and Genotoxic Changes in Human Breast Cancer Cells. *J. Mol. Med.* **2008**, *86* (3), 291–302.

12 Akerman, M. E.; Chan, W. C. W.; Laakkonen, P.; Bhatia, S. N.; Ruoslahti, E. Nanocrystal Targeting In Vivo. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (20), 12617–12621.

13 Ballou, B.; Lagerholm, B. C.; Ernst, L. A.; Bruchez, M. P.; Waggoner, A. S. Noninvasive Imaging of Quantum Dots in Mice. *Bioconjugate Chem.* **2004**, *15* (1), 79–86.

14 Fitzpatrick, J. A. J.; Andreko, S. K.; Ernst, L. A.; Waggoner, A. S.; Ballou, B.; Bruchez, M. P. Long-term Persistence and Spectral Blue Shifting of Quantum Dots in Vivo. *Nano Lett.* **2009**, *9* (7), 2736–2741.

15 Hauck, T. S.; Anderson, R. E.; Fischer, H. C.; Newbigging, S.; Chan, W. C. W. In vivo Quantum-Dot Toxicity Assessment. *Small* **2010**, *6* (1), 138–144.

16 Sayes, C. M.; Reed, K. L.; Warheit, D. B. Assessing Toxicity of Fine and Nanoparticles: Comparing in Vitro Measurements to In Vivo Pulmonary Toxicity Profiles. *Toxicol. Sci.* **2007**, *97* (1), 163–180.

17 Dubertret, B. In Vivo Imaging of Quantum Dots Encapsulated in Phospholipid Micelles. *Science* **2002**, *298* (5599), 1759–1762.

18 Chang, E.; Thekkek, N.; Yu, W. W.; Colvin, V. L.; Drezek, R. Evaluation of Quantum Dot Cytotoxicity Based on Intracellular Uptake. *Small* **2006**, *2* (12), 1412–1417.

19 Osaki, F.; Kanamori, T.; Sando, S.; Sera, T.; Aoyama, Y. A Quantum Dot Conjugated Sugar Ball and Its Cellular Uptake. On the Size Effects of Endocytosis in the Subviral Region. *J. Am. Chem. Soc.* **2004**, *126* (21), 6520–6521.

20 Nabiev, I.; Mitchell, S.; Davies, A.; Williams, Y.; Kelleher, D.; Moore, R.; Byrne, S.; Rakovich, Y. P.; Donegan, J. F.; Sukhanova, A.; Conroy, J.; Cottell, D.; Gaponik, N.; Rogach, A.; Volkov, Y. Nonfunctionalized Nanocrystals Can Exploit a Cell's Active Transport Machinery Delivering Them to Specific Nuclear and Cytoplasmic Compartments. *Nano Lett.* **2007**, *7* (11), 3452–3461.

21 Duan, H.; Nie, S. Cell-Penetrating Quantum Dots Based on Multivalent and Endosome-Disrupting Surface Coatings. *J. Am. Chem. Soc.* **2007**, *129* (11), 3333–3338.

22 Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. W. Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells. *Nano Lett.* **2006**, *6* (4), 662–668.

23 Albanese, A.; Chan, W. C. W. Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity. *ACS Nano* **2011**, *5* (7), 5478–5489.

24 Fischer, H. C.; Liu, L.; Pang, K. S.; Chan, W. C. W. Pharmacokinetics of Nanoscale Quantum Dots: In Vivo Distribution, Sequestration, and Clearance in the Rat. *Adv. Funct. Mater.* **2006**, *16* (10), 1299–1305.

25 Walkey, C. D.; Chan, W. C. W. Understanding and Controlling the Interaction of Nanomaterials with Proteins in a Physiological Environment. *Chem. Soc. Rev.* **2012**, *41* (7), 2780–2799.

26 Choi, H. S.; Liu, W.; Misra, P.; Tanaka, E.; Zimmer, J. P.; Ipe, B. I.; Bawendi, M. G.; Frangioni, J. V. Renal Clearance of Quantum Dots. *Nat. Biotechnol.* **2007**, *25* (10), 1165–1170.

27 Jaiswal, J. K.; Mattoussi, H.; Mauro, J. M.; Simon, S. M. Long-Term Multiple Color Imaging of Live Cells Using Quantum Dot Bioconjugates. *Nat. Biotechnol.* **2002**, *21* (1), 47–51.

28 Fischer, H. C.; Hauck, T. S.; Gómez-Aristizábal, A.; Chan, W. C. W. Exploring Primary Liver Macrophages for Studying Quantum Dot Interactions with Biological Systems. *Adv. Mater.* **2010**, *22* (23), 2520–2524.

29 Chu, M.; Wu, Q.; Yang, H.; Yuan, R.; Hou, S.; Yang, Y.; Zou, Y.; Xu, S.; Xu, K.; Ji, A.; Sheng, L. Transfer of Quantum Dots from Pregnant Mice to Pups Across the Placental Barrier. *Small* **2010**, *6* (5), 670–678.

30 O'Brien, P. J.; Irwin, W.; Diaz, D.; Howard-Cofield, E.; Krejsa, C. M.; Slaughter, M. R.; Gao, B.; Kaludercic, N.; Angeline, A.; Bernardi, P.; Brain, P.; Hougham, C. High Concordance of Drug-Induced Human Hepatotoxicity with In Vitro Cytotoxicity Measured in a Novel Cell-Based Model Using High Content Screening. *Arch. Toxicol.* **2006**, *80*, 580–604.

31 Shaw, S. Y.; Westly, E. C.; Pittet, M. J.; Subramanian, A.; Schreiber, S. L.; Weissleder, R. Perturbational Profiling of Nanomaterial Biologic Activity. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (21), 7387–7392.

32 Yu, W.; Qu, L.; Guo, W.; Peng, X. Experimental Determination of the Extinction Coefficient of CdTe, CdSe, and CdS Nanocrystals. *Chem. Mater.* **2003**, *15* (14), 2854–2860.

33 Striolo, A.; Ward, J.; Prausnitz, J. M.; Parak, W. J.; Zanchet, D.; Gerion, D.; Milliron, D.; Alivisatos, A. P. Molecular Weight, Osmotic Second Virial Coefficient, and Extinction Coefficient of Colloidal CdSe Nanocrystals. *J. Phys. Chem. B* **2002**, *106* (21), 5500–5505.

34 Leatherdale, C. A.; Woo, W. K.; Mikulec, F. V.; Bawendi, M. G. On the Absorption Cross Section of CdSe Nanocrystal Quantum Dots. *J. Phys. Chem. B* **2002**, *106* (31), 7619–7622.

35 Ioannou, D.; Griffin, D. K. Nanotechnology and Molecular Cytogenetics: The Future Has Not Yet Arrived. *Nano Rev.* **2010**, *1*, 5117–5131.

36 Bregoli, L.; Chiarini, F.; Gambarelli, A.; Sighinolfi, G.; Gatti, A. M.; Santi, P.; Martelli, A. M.; Cocco, L. Toxicity of Antimony Trioxide Nanoparticles on Human Hematopoietic Progenitor Cells and Comparison to Cell Lines. *Toxicology* **2009**, *262* (2), 121–129.

37 Stone, V.; Johnston, H.; Schins, R. P. F. Development of In Vitro Systems for Nanotoxicology: Methodological Considerations. *Crit. Rev. Toxicol.* **2009**, *39* (7), 613–626.

38 National Cancer Institute Nanotechnology Characterization Laboratory Assay Cascade Protocols. http://ncl.cancer.gov/working_assay-cascade.asp (accessed Apr 29, 2012).

39 Dobrovolskaia, M. A.; Germolec, D. R.; Weaver, J. L. Evaluation of Nanoparticle Immunotoxicity. *Nat. Nanotechnol.* **2009**, *4*, 411–414.

40 Wörle-Knirsch, J. M.; Pulskamp, K.; Krug, H. F. Oops They Did It Again! Carbon Nanotubes Hoax Scientists in Viability Assays. *Nano Lett.* **2006**, *6* (6), 1261–1268.

41 Chibli, H.; Carlini, L.; Park, S.; Dimitrijevic, N. M.; Nadeau, J. L. Cytotoxicity of InP/ZnS Quantum Dots Related to Reactive Oxygen Species Generation. *Nanoscale* **2011**, *3*, 2552–2559.

42 Chou, L. Y. T.; Chan, W. C. W. A Strategy to Assemble Nanoparticles with Polymers for Mitigating Cytotoxicity and Enabling Size Tuning. *Nanomedicine* **2011**, *6* (5), 767–775.