Applications of quantum dots in biomedicine

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1. Introduction

Research and development in nanotechnology has become an increasingly popular trend in the last 5 years as the demand and production of nanometer-sized materials continue to grow. Nanotechnology is an area of research encompassing multi-disciplinary studies (including chemistry, physics, engineering, and biotechnology), and has diverse applications in agriculture, automobile, clothing, defense and more recently, biology and biomedicine [1, 2]. Among many different nanotechnological products, quantum dots (QD) have gained a lot of popularity as imaging probes in biology due to their very special physico-chemical and optical properties [3, 4]. They are stable, highly fluorescent, tunable and can be functionalized via surface modifications. Despite the numerous ongoing studies on QD synthesis to improve their physical properties, the biological effects of QDs are poorly investigated. Thus far, it is known that QD biocompatibility is largely dependent on their size, surface charge, core and surface materials [2]. Currently, extensive studies on the interactions (or interference) of QDs with cellular processes are under investigation in many scientific centers.

The understanding of cellular processes and molecular mechanisms is essential for drug discovery, particularly for disease diagnosis and treatment; however current development in biomedicine is hindered by the lack of tools to visualize cellular events and signaling of individual molecules [5]. Integration of nanotechnology in biomedicine is thus timely and inevitable, as high resolution biomedical imaging, from microscopic to nanoscopic and from two-dimensional to spatio-temporal [6, 7], is rapidly progressing.

2. Quantum dots as imaging tools in biology and medicine

2.1 Advantages and limitations of quantum dots and fluorescent dyes. Among the current array of nanotechnology products, semiconductor nanocrystal quantum dots were first reported to be a very promising tool for cellular imaging by two groups of scientists (Alivisatos and Nie) in 1998 [8, 9]. The colloidal QD core typically ranges from 2 to 10 nm in diameter, and is typically composed of

atoms from groups II–VI (e.g. CdTe, CdSe) and III–V (e.g. InP, InAs) of the periodic table. These QD cores are often capped with an additional layer or "shell" of inorganic material (e.g. ZnS) to enhance their quantum yield, resulting in enhanced signal-to-noise ratio (robust signal). Depending on the size and composition, QDs can emit at distinct and different wavelengths, all the way from UV through visible to near-infrared (NIR). Unlike traditional organic fluorophores, QDs absorb wavelengths from a broad spectrum and in turn, emit in symmetrical and narrow spectra. Taken altogether, QDs of different sizes can be excited simultaneously by a single wavelength and emit with distinctly different colors, allowing for concurrent labeling of multiple species [10, 11]. In addition to their novel and unique optical properties, QDs are also highly photostable due to their inorganic composition, rendering them less susceptible to photobleaching and providing them with significantly longer fluorescent lifetimes (10–40 ns) compared to organic fluorescent dyes, thereby permitting their use for long-term, repeated imaging [9, 12].

QDs are often synthesized in an organic environment, and in order for biological applications, QD surfaces must be modified with hydrophilic material (e.g. mercaptoproprionic acid, cysteamine) or micelle-forming polymeric materials to enhance their water solubility [13, 14]. To prevent aggregation of these nanoparticles, surface conjugations with synthetic polymers such as polyethylene glycol (PEG) are often advantageous, allowing QDs to remain as finely dispersed individual nanoparticles (Fig. 1).

The extent of cellular internalization and subcellular distribution of non-functionalized, hydrophilic QDs is largely dependent on nanoparticle size and surface charge. Studies from our group showed that different charges on CdTe QD surfaces can regulate the extent of nanoparticle uptake such that the more positively charged (cysteamine-capping) nanoparticles are taken up more readily [15]. QD internalization can also be enhanced by surface conjugation with phospholipids, synthetic polymers (i.e. PEG) [14] or other synthetic material like silica [16]. Lovrić et al. showed that non-functionalized, cationic CdTe QDs (cysteamine-capping) are internalized readily, within 1 h of incubation with cells, suggesting uptake mechanisms involving phagocytosis in microglia and macrophages in peripheral sites [17]. The larger, red-fluorescing QDs (~5 nm in diameter) are retained in the cytoplasm,

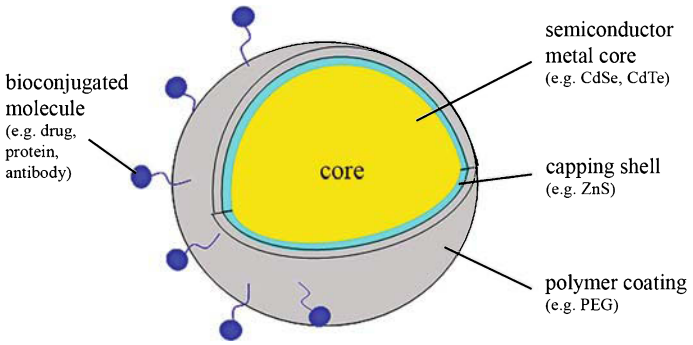

Fig. 1. Quantum dot "anatomy"

whereas the smaller, green-fluorescing QDs (~2 nm in diameter) are localized in the nucleus. These findings were corroborated by the recent work by Volkov et al., showing that non-functionalized CdTe QDs exploit the cellular active transport machinery for delivering these QDs to specific intracellular destination [18]. Both of these studies point towards a critical role of the size, charge and surface properties of QDs which together with the cell-type specific properties will determine the fate of these nanocrystals.

2.2 Imaging of cellular and subcellular structures. Mammalian cells are typically 10 μm in diameter and contain a variety of subcellular machineries in the sub-micron range, which act to control cellular function and maintain homeostasis [19]. Pathak et al. showed that QDs bioconjugated with cell-type specific antibodies can be used to distinguish between neurons and glia in primary cultures without the use of secondary antibodies [20]. Antibodies against β-tubulin (ubiquitous cytoskeletal protein specific to neurons) and glial fibrillary acidic protein (GFAP, specific to glia) were conjugated to streptavidin-conjugated QDs to label primary cortical cultures. Compared to the blurry signals obtained from traditional fluorophore-tagged secondary antibodies, cells labeled with QDs were brighter and exhibited sharper and finer features. Although these are the pioneering studies exploiting QD-conjugates to explore neurons and glia, such approach has a number of limitations for in vivo studies in whole animals. For instance, the size of the QD-antibody structure may be too large to cross the blood brain barrier unless a targeting moiety with penetrating properties (e.g. TAT peptide, transferrin receptor) was added to facilitate the transport. Secondly, stability of the QD-antibody bond may not be adequate to preserve the integrity of the complex long enough for delivery to the destination (e.g. in deeper structures of the central nervous system).

Biological function cannot be determined by simply elucidating cellular and molecular structures without studying the spatio-temporal organization and distribution of intracellular molecules, and more importantly, tracking dynamic molecular interactions in real-time. Intracellular organelles are composed of and are regulated by nanometer-sized molecules such as proteins (1–20 nm) (Fig. 2). Current imaging techniques, including electron and confocal microscopy, have helped to elucidate the structure and the specific localization of these nanomolecules. Highly fluorescent and photostable QDs can allow live imaging of individual cellular

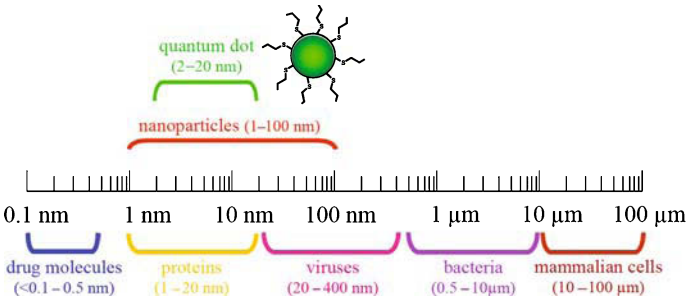

Fig. 2. QD sizes relative to drug molecules and mammalian cells

components with high resolution, selectivity, precision and bright fluorescence. Dahan et al. studied and compared the dynamic action of individual glycine receptors (GlyR) in rat spinal cord neuronal cultures, using an antibody against the GlyR α1 subunit, tagged either with QDs or a commonly used fluorescent dye (Cy3) [21]. In addition to the enhanced brightness in fluorescence of QD-GlyR (almost an order of magnitude higher than Cy3-GlyR), the authors were able to extend the live tracking of GlyR lateral dynamics in the neuronal membranes to 20 min using QD probes, compared to the much shorter 5 s fluorescence lifetime of the Cy3 probe. Diffusion coefficients of the QD-GlyRs localized within the synaptic cleft were also found to be larger compared with bead-GlyRs, suggesting that there is little or no interference of receptor dynamics by QDs compared to beads.

Imaging cell surface receptors dynamics is only one of the many aspects of signal transduction; trafficking and transport of ligands are also important for localizing the function of a specific molecule in real-time. Cui et al. conjugated QDs with nerve growth factor (NGF) and tracked the uptake and retrograde transport of NGF in rat dorsal root ganglia cultures (DRG) [22]. DRG cultures are often used as a model system in neuroscience to explore signal transduction pathways involved in nerve growth and survival [23, 24]. These primary cultures consist of mixed neurons and Schwann cells and provide a superior model over the immortalized cell line, as the mixed cultures conserve the interactions between the cell types, thereby better representing the actual environment cells are normally exposed to [25]. NGF-QDs were found to be taken up by the TrkA receptors and these receptors were transported along the axon by endosome-like vesicles ranging between 50 and 150 nm in diameter. The rate of uptake was comparable to studies using radiolabeled NGF (^{125}I-NGF), suggesting that QDs do not restrict or profoundly alter NGF structure, and more importantly, do not hinder NGF trafficking. In addition, the studies show that colocalization and activation with TrkA receptors, and phosphorylation of Erk1/2 were not abolished, indicating that the functionality of NGF was not impeded by the QDs [25].

As shown by these studies, target-conjugated QDs can be and has been used not only as a cellular marker, but as a molecular marker which can track the live, dynamic action of a molecule with bright fluorescence for a relatively long time without interfering with their endogenous function or motion. Similar approaches can be taken to explore other receptors, their distributions and functional changes under experimental conditions. Such studies are invaluable to gain insights into molecular mechanisms at the cellular level and how they are conducted under relatively controlled conditions. The limitation of such studies is that it does not provide functional connections and communications in situ, as it is in a living whole animal. In the next section, we will highlight several studies and discuss some of the advantages and limitations of whole animal studies with QDs.

2.3 Functional cell imaging in living animals in real-time. Whole animal imaging was limited for a long time mainly because of the poor signal resolution, resulting from the photounstable dyes, despite the quality of the microscopes used. With the advances of improved contrast agents, new opportunities arose and provided

a better handle to explore normal and diseased tissues, as well as the entire body of experimental animals and humans.

One of the objectives in imaging normal and pathologic sites in the body is not only to detect the site, but also to provide means of detecting dynamic changes as a response of progressive tissue deterioration or gradual recovery from the injury. In this regard, our laboratory has recently devised a way of merging nanotechnology with transgenic technology and investigated the responsiveness of glial cells in living mice. QDs were administered directly into brain parenchyma [26]. The objective of this work was to establish a sensitive in vivo assay for the responsiveness of astrocytes to the

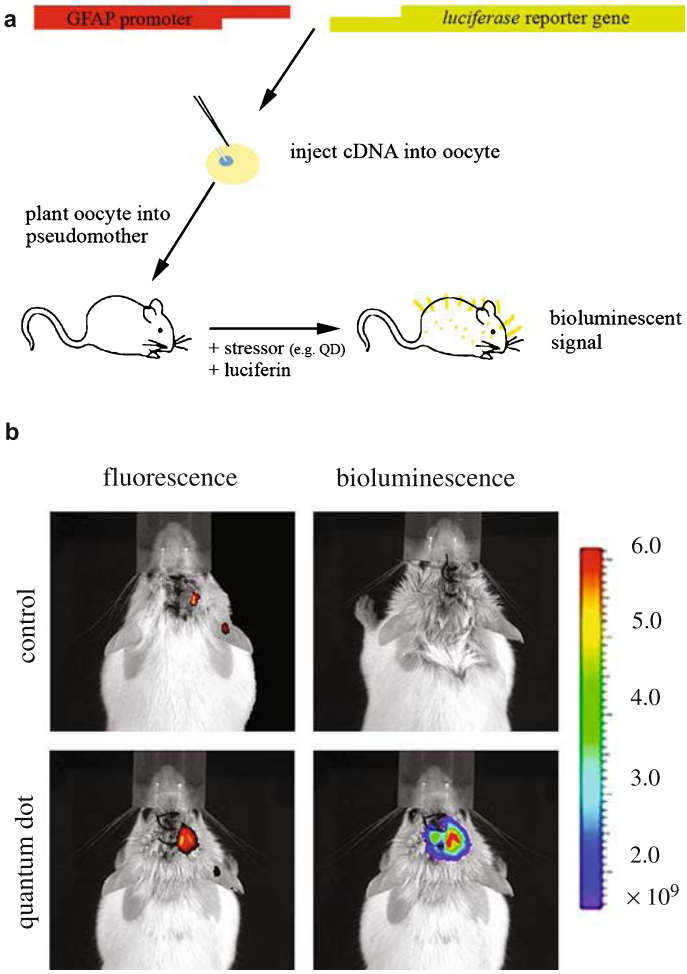

Fig. 3. In vivo neuroimaging of injected quantum dots in transgenic animals. Expression of luciferase (Luc) is driven by the glial fibrillary acidic protein (GFAP) promoter in the GFAP-Luc transgenic mice (promoter is activated in response to stress) (**a**). The substrate for luciferase (i.e. luciferin) is injected and bioluminescence is detected. **b** GFAP-Luc mice (Xenogen-Calipers LS, Alameda, CA) were imaged using the IVIS in vivo imaging system, 24 h after injection with 16 pM PEGylated CdSe/ZnS QDs (emission wavelength 705 nm) [26]

nanoparticle-induced brain injury. Astrocytes are glial cells which are activated around the site of injury or more widely in brain inflammation [27]. Currently, there are commercially available a number of transgenic mice expressing luciferase (Luc) under the control of different promoters, allowing for real-time imaging of specific tissues in the whole animal, depending on the specificity of the promoters.

The transgenic animals (GFAP-Luc; Xenogen-Caliper LS) used in our study express luciferase under the control of a promoter specifically expressed in astrocytes (i.e. GFAP). Once activated, the GFAP promoter induces luciferase expression and upon injection of the substrate (i.e. luciferin), a strong luminescent signal is generated and can be quantified. The illustration shows the principle of the luciferase expression and detection in GFAP-Luc mice (Fig. 3a) and provides an example of astrocyte activation surrounding the QD administration (Fig. 3b). It was noted that the rate of astrocyte activation is very different depending on the type of surface on the QDs [26]. This finding underlines the importance of thorough characterization of nanoparticles to be used since QDs with even comparable sizes and core materials but different surface properties can markedly change the kinetics and intensity of astrocyte activation.

Among the many QDs and other fluorescent or non-fluorescent nanoparticles, infrared-emitting QDs were used in our in vivo studies. Emission in the near-infrared is necessary to overcome autofluorescence, to penetrate the skull and to provide for long-term, repeated live imaging of the brain [26]. We recently reported that NIR QDs can be used for deep tissue imaging and can penetrate up to 6 mm of tissue [28]. As promising as these initial in vivo results with QDs may be, further research is necessary to characterize and optimize QD properties and to study how these properties are altered in an in vivo system, where these QDs end up, how long they stay at one site or whether they are eliminated and do not present any hazard to the normal functions of the surrounding tissues and of the whole organism.

3. Quantum dots as diagnostic tools

Investigation of nanoparticles for diagnostic purposes is currently the most advanced and well-studied in the field of oncology [29–34]. Cancer is presently the leading cause of death in North America and the number of new cases in the United States is expected to be over 1.5 million in 2008 (American Cancer Society Inc.). Current cancer therapies are lacking due to inadequate understanding of the multimodality disease, particularly failing to detect tumor formation with early diagnosis and accurate prognosis, and in turn impeding the effectiveness of anticancer drugs.

Existing diagnostic approaches are mostly limited to the detection of relatively large, solid tumors, which often involve invasive techniques such as tissue biopsies [29]. In most cases, this detectable tumor is at a late stage, at which the cancer has metastasized to other tissues, resulting in a greater challenge for both tumor detection and proper therapeutics. In addition, tissue biopsies are difficult to obtain from deep tissues, bioanalytical assays from urine and blood samples are often not providing reliable results, and imaging with contrast agents are limiting as current dyes cannot distinguish between the highly invasive and benign types of tumor. More recently, high throughput genomic and proteomic analyses have revealed that many of these subtypes can be distinguished based on expression profile rather than presence of a

single protein [30]. It would therefore be useful to have diagnostic tools which could allow for simultaneous detection of multiple proteins with sufficient sensitivity. QDs, among other nanotechnological products, could become versatile tools for screening cancer markers in biological fluids (urine, blood) and tissue biopsies, as well as high resolution contrast agents for medical imaging of metastatic tumors [31].

The rationale for using QDs as diagnostics in cancer are the following: (i) they are highly fluorescent and can be used for deep tissue imaging in vivo, (ii) they can serve as sensitive probes for multiple cell types because of their multiplexing abilities and wide range of tunable emissions [3], and (iii) utilization of functionalized QDs to target tumors in experimental animals shows promising results for future developments and eventual applications in humans.

Voura et al. reported the use of non-functionalized CdSe/ZnS QDs for multiphoton tracking of "metastasis" of different tumor cell populations in animals [31]. Different populations of murine lung melanoma (B16F10) were matured in vitro and transfected with QDs emitting in different wavelengths (510, 550, 570, 590, and 610 nm), after which these were injected into syngeneic mice. Tumor cell invasion into the lung was assessed using fluorescence emission-scanning microscopy 5 h after QD injection, and the individual tumor populations could be clearly identified. Another study by Stroh et al. also reported the use of non-functionalized CdSe/ZnS QDs in imaging murine mammary adenocarcinoma vasculature in vivo [32]. QDs with different colors were encapsulated into micelles and injected into GFP-transgenic animals xenografted with tumors and multiphoton microscopy was used to track the uptake of these nanoparticles into the tumor vasculature. The group reported that the QD-labeled vasculature could be clearly distinguished from perivascular cells in vivo, and labeling of the bone marrow with another type of QDs also showed recruitment of precursor cells to the vasculature. These studies not only again emphasize the promising implications of QD imaging in vivo, but also show the ready uptake of QDs by tumors via passive targeting mechanisms. It is well documented that macromolecules and nanoparticles can progressively accumulate in tumors due to the hypervasculature and enhanced permeability, a process known as enhanced permeability and retention (EPR) [33].

In addition to passive uptake of nanoparticles by the EPR effect, active targeting of tumors in vivo had also been reported using highly fluorescent QDs. Gao et al. reported in their study the use of QDs, conjugated with a tumor-targeting ligand, to actively localize at tumor sites in live animals [34]. CdTe/ZnS QDs were encapsulated in a polymer micelle conjugated with an antibody targeting the prostate-specific membrane antigen (PSMA), and injected systemically in mice xenografted with a prostate tumor. In vivo fluorescence of the brightly fluorescent QD-PSMA Ab probes was measured with a high signal-to-background ratio, and QD probes were found to localize specifically at the sites of tumor growth (i.e. prostates). Additionally, microbeads (0.5 μm in diameter) linked to different color QD probes were injected into three adjacent locations in the animal and imaged with multiphoton microscopy, suggesting the possibility of in vivo tracking of therapeutic action of drugs linked with QDs.

Despite the success of in vivo imaging studies with QDs, currently the application of QDs in diagnostic assays yielded more practical results. Immunoassays using bio-

conjugated QDs have been developed to assess variety of cellular states and functions, including protein–protein interaction [35], protein function [36], and more relevant to cancer, cell motility [37]. Pellegrino and colleagues reported in three studies the use of CdSe/ZnS QDs to track the motility of tumor cells in vitro, which in turn could be useful in determining the invasiveness of the cancer cells [37–39]. Based on the concept of EPR, cancer cells engulf the highly photostable QDs readily, and combined with real-time tracing of the fluorescent trail (or the disappearance of this trail), the metastatic potential of the tumors can be staged.

In summary, QDs together with transgenic animals as presented in this section could be used as versatile screening platforms for the assessments of effectiveness of chemotherapeutic, surgical, and radiation therapies to facilitate diagnosis and possibly treatment of solid and metastatic tumors. Combining genomic, proteomic and nanomedical tools for in vitro and in vivo imaging, will eventually contribute to future developments in achieving more personalized medicine in cancer and other diseases.

4. Quantum dots as nanotherapeutics

4.1 Quantum dots as drug delivery systems. Unfortunately, poor diagnosis and prognosis are only small parts of the overall "cancer problem," as this inevitably leads to inadequate development of treatments. Current chemotherapeutic agents are highly cytotoxic; however, most are lacking in specificity to cancerous cells and resulting in systemic toxicity and adverse side effects [40]. Nanoparticles, such as QDs and the often reported liposomes and polymeric micelles, may not only improve tumor targeting, but may also act as a new drug delivery tool, and even as direct therapeutics against tumor cells [41].

There is a growing trend for the development of multifunctional nanoparticles to image, diagnose and deliver treatment to cancer cells. The major struggle underlying the design of drug delivery systems is the same problem encountered in developing tools for imaging and diagnosis, and that is, target specificity. Encapsulating drugs in nanosized micelles was a big step forward in the research and development of drug delivery systems, as these nanoparticles can be easily surface-conjugated with ligands for targeted delivery, but more importantly, the release of drugs can be localized at the targeted region, reducing side effects due to non-specific drug action [42]. However, the current trend in the design of nano-delivery systems is focused on yet another level, which is the monitoring of drug action. QDs, among the array of nanomaterials, may be the optimal tool for all these purposes: imaging, diagnosis, drug delivery and tracking drug action [43].

Bagalkot et al. recently proposed in their study the design of a cancer imaging, therapy, and sensor system based on functionalized CdSe/ZnS QDs [44]. QDs were first surface conjugated with A10 RNA aptamers, which target the PSMA specific on prostate tumors, and subsequently, a fluorescent anticancer agent, doxorubicin (Dox), was intercalated with the aptamer to yield the QD-Apt(Dox) probe. Based on the concept of a bi-fluorescence resonance energy transfer (Bi-FRET), both QD and Dox fluorescence are quenched by their close proximity with each other in the intact

QD-Apt(Dox) probe. Upon the PSMA-mediated internalization of the nanosystem into the tumor cell, there is a release of the drug from the QD, thereby unquenching the fluorescence of both. The group reported that this nanosystem is indeed functional and specific to PSMA(+) cells in vitro, and showed that the fluorescence can be unmistakably distinguished. Another study by Derfus et al. described the use of QDs to deliver and monitor the delivery of siRNA, with the potential to knockout overexpressed oncogenes [45]. QDs, multiconjugated with a tumor-homing peptide (F3) and siRNA against an artificially transfected gene (enhanced green fluorescent protein, EGFP), were added to HeLa cells in vitro and the expression of EGFP was measured as the outcome of the knockdown. Fluorescence micrographs showed that cells containing the functionalized QDs also had no EGFP fluorescence, indicating the effectiveness of the system.

4.2 Photodynamic therapy using quantum dots. The photophysical properties, specifically the high photoluminescence and the energy-transfer potential, of QDs can be harnessed for therapeutic purposes, especially in the case of cancer, and this have been shown by a number of studies [46–48]. QDs are photosensitive energy donors, which can offer useful photodynamic therapy tools (PDT), at least for now in experimental animals. The principles of such a therapeutic approach has been proposed by several teams [47, 49, 50] and it is briefly summarized: in response to light and in the presence of oxygen, energy is released from QDs and transferred to cellular molecules, leading to the formation of reactive oxygen species (ROS) [51, 52]. Excessive production of ROS can induce cell apoptosis via oxidative stress-linked mechanisms, which when targeted to tumors, can lead to destruction of the specific tissues in a non-invasive manner [53]. In fact, a number of studies, including those from our laboratory, have shown evidence of the photosensitive and oxygen-sensitive nature of QDs, leading to the degradation of the QD core and subsequent release of free metal ions, and ultimately inducing cell death via apoptotic ROS signaling [15, 51, 54–56] (Fig. 4). Despite the lack of concrete evidence of the actual effectiveness of QDs as a PDT agent in anticancer therapy, one can envision the potential application of QDs conjugated with a targeting molecule (i.e. against an oncogene such as epithelial growth factor) in targeting and imaging the tumor sites.

One potential problem in this regard is that QDs may induce cytotoxicity and damage the surrounding and distant tissue at the initial photoactivation site. To avoid such undesirable effects, thorough biodistribution analyses and pharmacokinetic studies are required for every new biotechnological product to be used in nano-oncology, including QDs.

5. Biodistribution of quantum dots

A major concern regarding the safe use of QDs is their accumulation in the body and the poor understanding of the pharmacokinetics of nanoparticles after different routes of administration. The first in vivo imaging study of QDs was reported by Ballou et al., and they showed the distribution of intravenously (tail vein) injected non-functionalized QDs in mice and found that QD fluorescence can be measure

in vivo for at least 4 months [57]. Live animal imaging with fluorescence microscopy shows that QDs distribute to different sites in the body immediately after injection, and the circulation lifetime of QDs was monitored and found to vary greatly (12–70 min) depending on the length of the polymer (i.e. PEG) conjugated on QD surfaces. Circulation lifetimes, in turn, determine the rates of QD deposition in the liver, spleen, lymph nodes and bone marrow. Accumulation of QDs in the liver and spleen was detected by necropsy and electron microscopy as early as 24 h after injection. After 1 month, QD fluorescence was mostly found in the lymph nodes, bone marrow and intestinal contents, with residual fluorescence in the liver and spleen, suggesting eventual excretion of these nanoparticles with time [57].

In contrast to these earlier findings, Fischer and Chan reported sequestration of non-functionalized QDs in rats after intravenous injection (jugular vein cannula) [58]. The group used a quantitative method (atomic emission spectroscopy) to assess cadmium content (correlated to QD concentration) in different organs and found that the liver alone takes up the majority of the injected QDs within 90 min (ranging from 40 to 90% depending on QD-surface conjugates) despite comparable fluorescence measured in the liver and the spleen. Daily analysis of the fecal and urinal materials for up to 10 days after injection did not yield detectable QD content, and additional experiments using transmission electron microscopy and digestion-ultracentrifugation show that intact QDs were taken up and retained by Kupffer cells after long-term circulation in the body, suggesting that these nanocrystals are poorly metabolized, retained in the reticuloendothelial system, and likely re-distributed in the body. Recent studies by Soo Choi et al. provided data on the renal clearance of intravenously injected QDs in rats [59]. The major finding from these studies shows that several requirements must be fulfilled before renal filtration and urinary elimination of these inorganic, metal-containing nanoparticles can be achieved. For instance, a final hydrodynamic diameter greater than 5.5 nm hinders renal excretion, whereas nanoparticles smaller than 5.5 nm are effectively excreted in urine. In addition, QD surfaces with zwitterionic charge are superior over positively and negatively charged surfaces, as QD interaction with plasma proteins is improved.

In summary, these studies highlight the notion that total body clearance of nanoparticles is not trivial and point towards the need to analyze biological fluids, including urine and bile, as a part of human risk assessment after environmental exposure or intended nanoparticle use for diagnostic (imaging) purposes. Analyses of these biological materials together with other routine clinical biochemical tests will help to estimate the total amount of retained nanoparticles (if the exposure dose is unknown), thereby indicating which ones are hazardous and which are harmless.

6. Nanoparticle-induced cytotoxicity

6.1 Experimental approaches to assess cytotoxicity: advantages and limitations. Conflicting results on the biodistribution and clearance of QDs are owed to the variety of methods and assays available for assessing cytotoxicity. Despite the variety in methods, one should keep in mind that most, if not all, of these assays simply evaluate the functions and structural integrity of different subcellular organelles

Table 1. Some biochemical methods for the assessment of nanoparticle-induced cell toxicity

Tools	Subcellular target	Outcome measures	References
Annexin V[a]	Plasma membrane lipid (phosphatidylserine, PS)	Extracellular PS due to "flipping" of membrane	[73]
Lactate dehydrogenase release	Plasma membrane	Membrane integrity	[74]
Propidium iodide[a] exclusion	Plasma membrane	Membrane permeability and integrity	[51, 75]
Trypan blue exclusion	Plasma membrane	Membrane permeability and integrity	[15, 76]
Alamar blue[a]	Cytosol (dehydrogenase)	Metabolic activity	[77]
JC-1[a] aggregation	Mitochondrion (membrane potential)	Membrane depolarization	[15]
MitoTracker®[a]	Mitochondrion	Morphological structure	[17, 51]
MTT (tetrazolium) reduction	Mitochondrion (dehydrogenase)	Metabolic activity	[15, 78]
LysoTracker®[a]	Lysosome	Morphological structure	[2]
DRAQ5[a]	Nucleus (DNA)	Morphological structure	[63, 79]
Dihydroethidium[a] oxidation	Nucleus (DNA)	Oxidative stress: detection of superoxide	[51, 80]
Hoechst[a]	Nucleus (DNA)	Morphological structure	[54]

[a] Commercially available fluorescent dyes

(examples of some of these methods used by our laboratory to evaluate nanotoxicity are selected and compiled in Table 1). If used individually, these techniques are restricting and results may be misleading. For example, the MTT assay is often used to assess cell viability, but an increase in formazan conversion (usually associated with improved viability) simply represents the increased activity of mitochondrial enzymes, which could very well be an initial cell defence response, as the cell struggles to boost its survival chances against the stressor [51]. It is important, therefore, to use a number of other approaches in concert with in vivo pharmacokinetic studies to evaluate the safety or the extent of toxicity of nanoparticles. A brief overview of the most commonly used techniques in assessing nanoparticle toxicity and examples of studies employing them is provided in a recent review [60].

6.2 Molecular mechanisms in quantum dot-induced cytotoxicity. In response to the demand in establishing screening procedures for nanotoxicity, the focus is gradually moving towards the detection of early molecular changes induced by QDs, and development of pharmacological interventions to reverse or prevent the changes leading to cell death. Elucidating the mechanisms underlying QD-induced cytotoxicity is therefore an important first step as nanotoxicity is becoming a prominent concern in the scientific community, especially with the growing number of studies highlighting the toxicity of nanoproducts [60–62]. However, one must emphasize that tremendous efforts are being made to minimize and eventually eliminate current concerns regarding biohazard of some nanomaterials, especially in limiting the production of QDs with undesirable surface properties, core composition and poor stability in complex biological environments [2].

Table 2. Selected examples of biologically important reactive species

	Free radicals	Non-radicals
Reactive oxygen species (ROS)	Superoxide, $^{\bullet}O_2^-$ Hydroxyl, $^{\bullet}OH$ Peroxyl, $^{\bullet}RO_2$ Hydroperoxyl, $^{\bullet}HO_2^-$	Hydrogen peroxide, H_2O_2 Hydrochlorous acid, HOCl
Reactive nitrogen species (RNS)	Nitric oxide, $^{\bullet}NO$ Nitrogen dioxide, $^{\bullet}NO_2^-$	Peroxynitrite, $OONO^-$ Nitrous oxide, HNO_2

The cytotoxic potential of semiconductor QDs is of no surprise as their cores are composed of known toxic metals such as cadmium, tellurium and mercury [55]. However, only in the past few years had we begun to understand the mechanisms underlying the toxicity of these QDs. Studies in our laboratory first showed that QDs with different core composition, core size, surface coating, and surface charge induce different levels of toxicity [15, 17, 51, 55]. CdTe QDs enter cells readily, localize in different subcellular organelles [17], and in turn, cause lipid peroxidation [15, 54], mitochondrial damage [15], nuclear damage, epigenetic and genetic changes, even if they do not enter the organelles in detectable quantities [63]. This triggering of cytotoxic events stems, in part, from the initial degradation of QDs upon exposure to light and oxygen (photosensitization), leading to the release of free metal ions (i.e. Cd^{++}) and the formation of excessive reactive species, including reactive nitrogen species (RNS) and ROS, both intracellularly and extracellularly [55, 64] (Table 2 provides a brief list of some examples of RNS and ROS that are important for stress-activated cellular signaling). Extracellular ROS can damage the cell membrane and induce plasma membrane lipid peroxidation, leading to the production of more cell-damaging molecules such as aldehydes like ONE (4-oxo-2-nonenal), which would trigger the p53-dependent apoptotic signaling cascade [15]. Extracellular ROS can also trigger other pro-apoptotic events like the activation of cell surface Fas death receptors, which leads to subsequent activation of caspases, eventually leading to mitochondria-dependent apoptosis [65]. ROS can passively cross the plasma membrane and can lead to organelle damage. Due to the lack of choice and specificity of markers available currently, one of the urgent needs in biological sciences and nanomedicine is to develop suitable probes to detect specific types of reactive oxygen and nitrogen species.

Small, green-emitting QDs with a diameter $\leq$5 nm can enter the nucleus via the nuclear pore and induce damage including DNA nicking [66]. More recently, we suggested that cells exposed to small amounts of QDs for a prolonged period, undergo epigenetic changes which will modify gene expression [63]. The epigenome regulates the expression of genes via DNA methylation and posttranslational modifications of histones, which can have lasting effects on the organism and its offspring [67]. The epigenetic changes observed by our group further indicated that non-functionalized CdTe QDs induced upregulation of pro-apoptotic genes and a downregulation of antiapoptotic genes, thereby shifting the cellular homeostasis to be more cell death-favourable [63]. Simultaneous damage at other subcellular

organelles is also occurring, most notably at the mitochondrial and lysosomal levels [15, 55]. Mitochondrial and lysosomal enlargement was observed early following CdTe QD treatment suggesting likely functional impairment in these organelles. We observed that mitochondrial function was indeed compromised in the presence of CdTe QDs as shown by the decrease in mitochondrial membrane potential [15]. This depolarization of the membrane leads to increased permeability across the mitochondrial membrane, and the subsequent release of apoptotic factors such as cytochrome *c*, triggering caspase-dependent apoptosis [68] (Fig. 4). However, caspase-independent cell death (e.g. necrosis) and several other modes of cell death can also be detected in cells exposed for a prolonged time to poorly protected QDs, particularly in those cells which have been predisposed to trophic factor deprivation.

6.3 Ways to overcome quantum dot-induced cytotoxicity. With the above-mentioned mechanisms underlying QD-toxicity, the outlook on developing

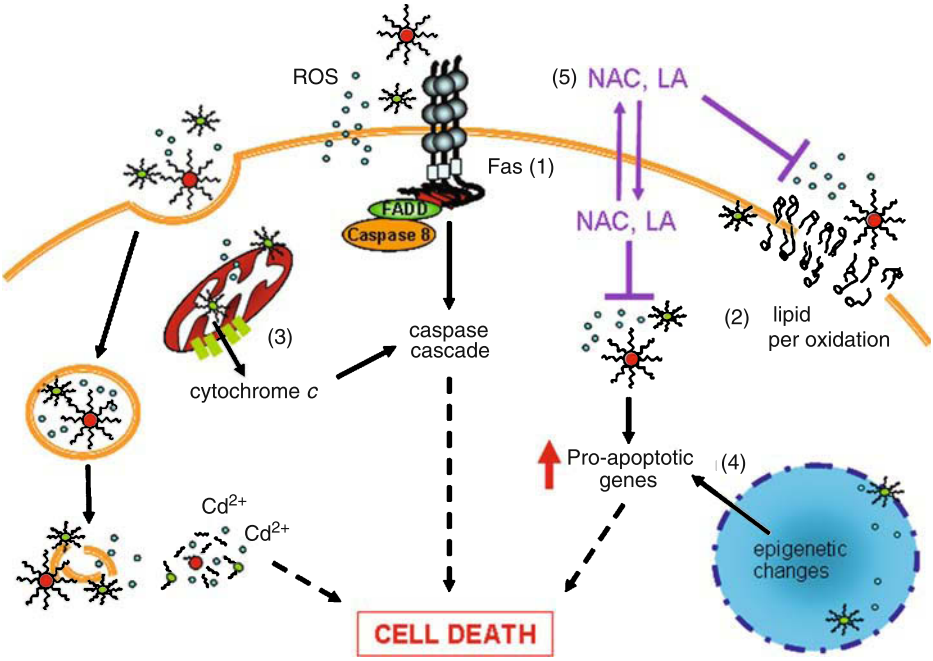

Fig. 4. Non-functionalized CdTe QDs interact and can interfere with cellular functions. (**1**) CdTe QD can upregulate the Fas death receptor, leading to the recruitment of the Fas-associated death domain (FADD) and initiating the caspase cascade [15]. (**2**) QD-induced production of reactive oxygen species (ROS) can induce lipid peroxidation of the plasma membrane and those of subcellular organelles. Internalization of CdTe QDs can be via endocytosis or active transport as well, and may result in the retention of the QDs in individual organelles. (**3**) CdTe QDs can impair mitochondrial function and enhance mitochondrial membrane permeability, thereby facilitating the release of pro-apoptotic factors such as cytochrome *c* [51]. (**4**) Nuclear damage by QDs is seen as chromatin condensation and epigenetic changes which favor the expression of pro-apoptotic genes (e.g. p53 and Bax) [63]. (**5**) Cell pre-conditioning with antioxidants such as *N*-acetylcysteine (NAC) and α-lipoic acid (LA) can protect cells from CdTe QD-induced cell death

preventive measures against QD-induced cytotoxicity is quite positive. QD-toxicity is dependent on QD stability, size, surface materials and charge among many factors. Not all QDs are toxic. In fact, most current studies now show that QDs with zwitterionic surfaces [59] or coated with synthetic polymers (i.e. PEG) are mostly inert and do not induce toxic response in most cell types under investigated conditions [16, 69, 70].

QD surface modifications can affect their cellular internalization, which in turn determines the extent of toxicity such that positively charged QDs can cross the plasma membrane very readily and induced more toxicity than negatively charged QDs. Choi et al. demonstrated that by conjugating or capping the surface of CdTe QDs with a small drug molecule, *N*-acetylcysteine, the overall charge of the QDs can be changed, in turn affecting their internalization and improving QD biocompatibility [15]. In addition, NAC can act as an antioxidant against the ROS produced by QDs. It is well documented that NAC acts with different modes of action as an antioxidant: (i) directly scavenge ROS with its cysteine moiety, (ii) regenerate endogenous antioxidants as a precursor to glutathione, (iii) regulate gene transcription to maintain cellular homeostasis, and (iv) promote cell survival by inhibiting JNK and p38 pathways [71]. Cell preconditioning with clinically relevant antioxidants such as NAC and LA can also prevent and reduce the cell damage induced by QDs [15, 51].

7. Current status and prospective

Most of the studies conducted with nanoparticles in cell cultures and animals so far, were carried out for relatively "short" time (up to several days) except some distribution studies which covered much longer time periods (several months [58, 72]). As with many pharmacological agents, it could also be the case with certain types of QDs that very small concentrations of QDs, undetectable by common chemical or imaging techniques, could lead to small changes critical to cellular function (e.g. epigenetic modifications). Advances in chemical and bioengineering approaches providing fine-tuning of the QD surfaces and other properties should allow for more positive rather than deleterious long-term effects at cellular level. Epigenetics is an evolving area of research and "nano-epigenetics" is in its infancy. Among the first studies addressing the epigenetic changes by QDs is one by Choi et al. [63]. These authors pointed out possible long-term consequences of cellular exposure to small QD concentrations and showed that histone acetylation is altered. This finding complements the more recent study pointing towards the active transport of non-functionalized nanoparticles and subsequent interaction with histone proteins [18]. Since histones play an important regulatory role in the normal cell cycle and tumor growth, consequences of epigenetic changes induced by QDs (and possibly other nanomaterials) and interactions between nanoparticles and histones remain to be explored in more detail. Our laboratory has initiated a number of studies, in both cell cultures and live animals, with the aim to provide an additional screening platform, including "nano-epigenetics," to complement common toxicological assay systems in defining hazardous versus well-tolerated nanomaterials.

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