Nanotoxicity assessment of quantum dots: from cellular to primate studies

Ken-Tye Yong,*a Wing-Cheung Law,b Rui Hu,a Ling Ye,c Liwei Liu,d Mark T. Swihart,e and Paras N. Prasad*abdfg

Tremendous research efforts have been devoted to fabricating high quality quantum dots (QDs) for applications in biology and medicine. Much of this research was pursued with an ultimate goal of using QDs in clinical applications. However, a great deal of concern has been voiced about the potential hazards of QDs due to their heavy-metal content. Many studies have demonstrated toxicity of various QDs in cell culture studies. However, in a smaller number of studies using small animal models (mice and rats), no abnormal behaviour or tissue damage was noticed over periods of months after the systemic administration of QDs. Nevertheless, the correlation of these results with the potential for negative effects of QDs on humans remains unclear. Many urgent questions must be answered before the QDs community moves into the clinical research phase. This review provides an overview of the toxicity assessment of QDs, ranging from cell culture studies to animal models and discusses their findings. Guidelines for using various nonhuman primate models for QD toxicity studies are highlighted. This review article is intended to promote the awareness of current developments of QD applications in biology, the potential toxicity of QDs, and approaches to minimizing toxicity.

1. Introduction

Before 1998, QDs were only considered as materials for photonic or electronics applications, based upon their unique optical and electronic properties. For example, QDs of multiple emission colors with minimal spectral overlap can be simultaneously excited with a single light source.1 They can be manipulated to emit in a range of wavelengths by changing their size, shape, and composition. These features were viewed from the perspective of potential use in displays and related optoelectronic applications. This paradigm changed upon the first demonstrations of QDs as fluorescent probes in biological applications by two independent research groups.2,3 Since then, QDs, especially cadmium-based QDs, have been extensively used in biomedical research, based upon the same unique optical properties.4 Over the past decade, tremendous research efforts have been devoted to producing high quality QDs by optimizing synthetic procedures, functionalizing the QD surface to enhance biocompatibility, and coupling the QDs to agents with complementary functions (e.g. targeting molecules or therapeutic agents).5,6 Much of this work was pursued with an ultimate vision of utilizing QDs in clinical settings.7 At the same time, a great deal of concern has been raised about the potential hazards of QDs because of their heavy-metal content.8 Several studies have argued that QDs are nontoxic when their surfaces are fully passivated with proper coatings.9,10 Multiple research groups have demonstrated toxicity of QDs in cell culture studies.8,11 Toxicity of QDs has also been studied using small animal models.12,13 Despite the fact that no abnormal behaviour or tissue damage was noticed in mice and rats over periods of months after the systemic administration of QDs, the correlation of these results with the potential for negative effects of QDs on humans remains unclear. Table 1 summarizes in vivo toxicity studies of various types of QDs using different animal models. Many questions remain to be answered before the QDs community moves into the clinical research phase. For safe application of QDs in a clinical context, understanding the response of humans to QDs is critical.
Table 1 Different types of quantum dots used for in vivo toxicity studies in various animal models

<table>
<thead>
<tr>
<th>Type of QD</th>
<th>Cells</th>
<th>Zebrafish</th>
<th>Xenopus embryo</th>
<th>Mouse</th>
<th>Rat</th>
<th>Macaque</th>
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<tbody>
<tr>
<td>CdSe</td>
<td>Toxic after oxidation, ~0.0625 mg ml⁻¹ (ref. 16)</td>
<td>—</td>
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<tr>
<td>CdSe–ZnS⁺</td>
<td>Toxic after exposed to UV irradiation, ~1 mg ml⁻¹ (ref. 16)</td>
<td>LC₅₀ values: 7–42 μM</td>
<td>Non-toxic at 4.2 × 10⁹ particles per cell (ref. 18)</td>
<td>Non-toxic at 360 pmol⁻¹–6 nmol⁻¹ (ref. 26)</td>
<td>Non-toxic at 15 nmol⁻¹</td>
<td>—</td>
</tr>
<tr>
<td>CdSe–CdS–ZnS⁺</td>
<td>LC₅₀: 0.7 μM (ref. 21)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 125 nmol⁻¹</td>
<td>Non-toxic at 3 nmol⁻¹</td>
<td>Non-toxic at 25 mg kg⁻¹ (ref. 14)</td>
</tr>
<tr>
<td>CdTe</td>
<td>LC₅₀: 50–100 nM (ref. 24)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 48 pmol⁻¹</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CdTe–ZnSe</td>
<td>Non-toxic at 1 mg ml⁻¹ (ref. 27)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 5 mg kg⁻¹ (ref. 27)</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CdTe–ZnTe</td>
<td>Relatively Non-toxic at 150 μg ml⁻¹ (ref. 26)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 5 mg kg⁻¹ (ref. 26)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CdTe–CdS–ZnS⁺</td>
<td>Non-toxic at 3.0 μM (ref. 31)</td>
<td>—</td>
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<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CdTe–HgTe–ZnS⁺</td>
<td>Adopted for cell imaging, no toxicity study was performed</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>CdSe–CdTe</td>
<td>Non-toxic at 1–10 μg ml⁻¹ loaded in PLGA nanospheres (ref. 29)</td>
<td>—</td>
<td>—</td>
<td>Sentinel lymph node mapping in mouse (dosage ~0.01 nmol) and pig (dosage ~0.4 nmol); no toxicity study was performed</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CdTe,Se₁₋ₓ–CdS</td>
<td>Non-toxic at 200 μM (ref. 33)</td>
<td>—</td>
<td>—</td>
<td>Nontoxic at 5 mg kg⁻¹ (ref. 31); Intratumor administration of 74 μg for imaging, no toxicity study was performed</td>
<td>—</td>
<td>—</td>
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<tr>
<td>InP–ZnS⁺</td>
<td>Non-toxic at 100 μg ml⁻¹ (ref. 32)</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Mn:ZnSe</td>
<td>Non-toxic at 1.2 μM³⁻¹⁴</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 0.5 mg⁻¹</td>
<td>—</td>
<td>—</td>
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<tr>
<td>ZnS:Mn–ZnS⁺</td>
<td>Non-toxic at 400 μg ml⁻¹ (ref. 35)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Mn: CdSe,Te₁₋ₓ–CdS</td>
<td>Non-toxic at 800 μg ml⁻¹ (ref. 36)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 10 mg kg⁻¹ (ref. 36)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>InAs,P₁₋ₓ–InP–ZnSe</td>
<td>Non-toxic at 150 pmol⁻¹</td>
<td>—</td>
<td>—</td>
<td>Used for imaging, no toxicity study was performed</td>
<td>Sentinel lymph node imaging, no toxicity study was performed</td>
<td>—</td>
</tr>
<tr>
<td>InAs–ZnSe</td>
<td>—</td>
<td>—</td>
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<tr>
<td>InAs–InP–ZnSe</td>
<td>LC₅₀: ~100 nmol L⁻¹ (ref. 39)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 0.5 nmol⁻¹</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CuInSe₂</td>
<td>—</td>
<td>—</td>
<td>Sentinel lymph node imaging, no toxicity study was performed</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>CuInS₂</td>
<td>LC₅₀: ~300 μg ml⁻¹ (ref. 41)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 0.1–0.2 μmol (based on Cu atoms), 0.1–0.2 μmol</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Ag₃Se</td>
<td>Non-toxic at 47.4 μg ml⁻¹ (ref. 43)</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 0.15 mg⁻¹</td>
<td>—</td>
<td>—</td>
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<tr>
<td>PbSe</td>
<td>Non-toxic at 100 μg ml⁻¹ (ref. 44), SiO₂ coated</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PbS</td>
<td>Non-toxic at 220 μg ml⁻¹ (ref. 45)</td>
<td>Caused malformations above 40 μg ml⁻¹ (ref. 46)</td>
<td>—</td>
<td>Non-toxic at 25 mg kg⁻¹ (ref. 45); or 0.3 mg⁻¹</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ZnS</td>
<td>Non-toxic at 100 μM³⁻⁸</td>
<td>—</td>
<td>—</td>
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</table>
2. Preparation, biofunctionalization and potential toxicity of QDs

Hot colloidal synthesis remains the most popular approach to fabricating high-quality QDs. The QDs can be prepared easily by injecting appropriate chemical precursors (e.g. cadmium-complex and selenium-complex) into a hot reaction mixture containing surfactants (e.g. TOPO, oleic acid, stearic acid, etc.) and high boiling point solvents. Upon manipulating the synthesis parameters, such as growth temperature, precursor concentrations, growth time, and the types of surfactants, one can vary the size and crystallinity of the QDs. In general, the surfactants are coated on the surfaces of the QDs, creating a hydrophobic layer that prevents the QDs from agglomerating in nonpolar organic solvents. Surfactants that bind strongly to the surface of the nanocrystals will generate greater steric hindrance that slows the growth of the nanocrystals and allows production of smaller nanocrystals with better size control. When the nanocrystals reach the desired size, addition of organic solvents such as chloroform or toluene into the hot colloidal mixture is carried out to cool the solution and prevent further growth of the nanocrystals. Cycles of collection by centrifugation, followed by redispersion, are most often used to separate the nanocrystals from unreacted precursors and byproducts. For this, a semi-polar solvent such as ethanol, methanol, or butanol is introduced into the colloidal nanocrystal dispersion, decreasing the dispersibility of the nanocrystals in the mixed solvent, and causing them to weakly aggregate into clusters large enough to be collected by centrifugation. Methods for coating a semiconductor nanocrystal core with a second and sometimes a third semiconductor shell are well documented, and different kinds of core–shell QDs have been demonstrated (e.g. CdSe–ZnS, CdTe–CdSe, CdTe–ZnSe, CdHgTe–ZnS, and InP–ZnS core–shell nanocrystals). Common guidelines for the preparation of high-quality core–shell nanocrystals have been provided in several reviews.

<table>
<thead>
<tr>
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<th>Rat</th>
<th>Macaque</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si</td>
<td>Non-toxic at 0.2 mg ml⁻¹</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 380 mg kg⁻¹ or 60 nmol¹⁰</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CdSe–CdS Quantum rods</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CdSe–CdS–ZnS Quantum rods</td>
<td>Non-toxic at 500 μM³</td>
<td>—</td>
<td>—</td>
<td>Non-toxic at 1 mg²¹</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

³ Core–shell structure. ² Core–shell–shell structure.

Recently, our group has reported the first set of non-human primate data on QD toxicity. That study serves to dampen some of the fears over the toxicity of QDs intended for applications in humans. We have systemically injected phospho-lipid-micelle-encapsulated cadmium-based QDs into rhesus macaques. Standard hematological and biochemical markers were monitored for 3 months and no abnormalities were noted. This work has elicited constructive discussions among the research community on the possibilities of clinical translation of QDs. Therefore, in this review, we present an overview of the various sources of both in vitro and in vivo nanotoxicity. The toxicity assessment of QDs, ranging from cell culture studies to animal models, and their findings will be discussed and presented (see Table 1). We will highlight the guidelines for using various nonhuman primate models for QD toxicity studies. This review article is intended to promote awareness on current developments of QD applications in biology, the potential toxicity of QDs, and the approaches to minimizing toxicity. It succinctly discusses key in vitro and in vivo studies in various animal models. This discussion will encourage the QD community to think about possible clinical applications of QDs, where their immediate benefits may outweigh their possible long-term biological effects, which mostly remain unknown.

2.1. Variety of QDs

Here, we briefly discuss the types of QDs most often used in, or proposed for, biological applications. Emission peaks of QDs can be tuned from 450 to 1800 nm by manipulating their size, shape, composition, and structure. Each type of QDs has its own advantages and disadvantages, and the discussion below aims to provide useful guidelines for researchers to engineer QD probes meeting their own specific needs.

To date, CdSe–ZnS, CdTe–ZnS and CdTe–CdSe core–shell nanocrystals have been the most frequently used QDs for bioimaging and therapy research. The preparation of high quality CdSe–ZnS QDs by hot colloidal synthesis is well documented. It generally involves the injection of cadmium and selenium precursors into a surfactant solution at high temperature (typically above 300 °C) to generate monodisperse CdSe QDs. The CdSe QD emission peak can be tuned from 500 to 650 nm. The CdSe QD core is then capped with ZnS, a higher band gap material, to passivate the CdSe QD surface trap sites, and confine photogenerated excitons to the CdSe core, away from the surface of the core–shell nanocrystal. This dramatically improves the quantum yield of the QDs. For more than a decade, CdSe–ZnS QDs have been employed for in vitro and in vivo bioimaging applications, starting with two Science papers from Nie’s and Alivisatos’ groups in 1998, both showing that CdSe QDs can be conjugated to biomolecules for targeted imaging in vitro.
High quality CdTe QDs, for which the emission wavelength can be tuned from 490 to 850 nm, are generally synthesized in aqueous solution.\(^6\) However, the aqueous CdTe QDs cannot be used directly for biological applications, because long term contact with biological fluids results in partial desorption of the hydrophilic moieties, thus exposing the “naked” nanocrystal surface. The exposed surface is likely to leach toxic cadmium ions into the surrounding fluid. To overcome this challenge, Tsay \textit{et al.} reported a two-step method to prepare CdTe–ZnS QDs.\(^7\) CdTe cores were prepared in water and then transferred into an organic solvent, where the CdTe cores were capped with a ZnS shell. This synthesis method allows one to prepare relatively small (<5 nm) near-infrared-emitting QDs.

CdSe–ZnS and CdTe–ZnS are type-I semiconductor core–shell nanocrystals, where the conduction band of the shell material is at higher energy than that of the core, and the valence band of the shell material is at lower energy than that of the core. In this case, both electrons and holes are confined to the QD core. In contrast, CdTe–CdSe nanocrystals are type-II QDs, which are engineered to have both the valence and conduction bands in the core higher than in shell material. As a result, holes (positive charge carriers) are mostly confined to the core, while electrons are mostly confined to the shell. Bawendi’s group has demonstrated the synthesis of Type II CdTe–CdSe QDs using a hot colloidal synthesis technique,\(^9\) and has demonstrated the use of biopolymer-coated CdTe–CdSe QDs for sentinel lymph node mapping in mice and pigs.\(^5\)

InP QDs are III–V semiconductor nanocrystals with better structural robustness than CdSe and CdTe QDs made up of group II–VI elements. This structural robustness, which arises from the greater degree of covalent bonding in III–V materials compared to II–VI materials, improves the InP QDs optical stability and minimizes their toxicity by reducing degradation in biological fluids. The emission peak of the InP QDs is strongly influenced by the addition of a shell layer. By capping the InP core with ZnS, ZnSe, or CdSe shells of varying thickness, the emission peak of InP QDs can be manipulated from 500 to 650 nm with a quantum yield ranging from 40 to 80%, depending on the type of the shell coated on the InP core.\(^6\) Our group has reported the use of bioconjugated InP–ZnS QDs for live cancer cell imaging and two-photon bioimaging.\(^3\,^6\)

Hinds \textit{et al.} have demonstrated the synthesis of water-dispersible PbS QDs for biological applications.\(^6\) The PbS QDs, with tunable emission from 800 to 1400 nm, were synthesized using hot colloidal chemistry at relatively low reaction temperatures (~180 °C). Hyun \textit{et al.} showed that water-dispersible PbS QDs can be functionalized with mercapto ligands for near-infrared imaging of cancer cells.\(^6\)

Silicon QDs are group IV semiconductor nanocrystals that are expected to be far less toxic than heavy-metal-based QDs. However, the quantum yield of silicon QDs is generally lower than that of cadmium-based QDs and their absorbance cross-section is also much smaller. Li \textit{et al.} demonstrated the fabrication of silicon QDs grafted with poly(acrylic acid) for cancer cell imaging.\(^6\) Erogbobgo \textit{et al.} fabricated water-dispersible silicon QDs using PEGylated phospholipid micelles where emission intensity was maintained for weeks.\(^6\) In this study, the silicon QDs were made by laser-pyrolysis of silane, followed by acid etching. The silicon QD surfaces can be linked with styrene, octadecene, or ethyl undecylenate, allowing them to be dispersible in organic solvents. These hydrophobic silicon QDs can then be encapsulated with PEGylated phospholipid micelles, creating a hydrophilic shell with PEG groups on its surface. These micelle-encapsulated silicon QDs were used for imaging of pancreatic cancer cells. More recently, the same group has demonstrated that properly encapsulated Si QDs can be used in multiple cancer-related \textit{in vivo} applications, including tumor vasculature targeting, sentinel lymph node mapping, and multi-color NIR imaging in living mice. The micelle-encapsulated silicon QDs were reported to have stable luminescence and long (>40 h) tumor accumulation time \textit{in vivo}.\(^5\)

In addition to the QDs mentioned above, other types of QDs have been synthesized recently for biological applications. For example, Santra \textit{et al.} have prepared manganese-doped Mn:CsS–ZnS core–shell QDs with fluorescence, radio-opacity, and paramagnetic properties for labeling brain blood vessels in small animals.\(^6\) Qian \textit{et al.} reported the fabrication of CdHgTe–CdS QDs by an aqueous phase synthesis method and used them as probes for small animal imaging.\(^6\) Yong \textit{et al.} reported the synthesis of ternary CuInS\(_2\) QDs that were conjugated with folic acid for targeted imaging of tumors \textit{in vivo}.\(^4\) In addition, the preparation of manganese-doped CdTe\(_{0.25}\)Zn\(_{0.75}\) core–shell QDs for near-infrared imaging of pancreatic cancer cells was also demonstrated.\(^3\) These works have laid an important foundation for others to create innovative QDs for biomedical and medicinal applications.

2.2. Surface functionalization of QDs and their biodistribution

To date, the best quality QDs have been generated in organic solvents and are not dispersible in biological buffers without further modification. Thus, innovative approaches to make water-dispersible QDs are important if these nanocrystals are to be utilized for \textit{in vitro} and \textit{in vivo} applications. Two general methods have been used to make the hydrophobic QDs dispersible in biological fluids: (1) ligand exchange processes; and (2) coating of QDs with biocompatible materials. For ligand exchange, an excess of one or more heterobifunctional surfactants is added to the suspension of hydrophobic QDs. Functional groups (e.g. thiol or amine group) from the heterobifunctional surfactants bind to the QD surface, displacing the surfactants used in synthesis. The hydrophilic functional group at the free end of the surfactant (e.g. carboxyl or hydroxyl group) will favourably interact with water, rendering the QDs dispersible in aqueous media.\(^6\) This requires the availability of a bifunctional molecule with a hydrophilic group that does not bind strongly to the QD surface, and another group that binds more strongly than the surfactants used in synthesis.

In the second approach, the hydrophobic QDs are coated with amphiphilic biocompatible polymers. The polymer encapsulation allows QDs to have excellent colloidal stability in...
biological fluids due to the strong hydrophobic interaction between the hydrophobic moieties bound to the QD surface and the hydrophobic segment of the polymer.\textsuperscript{18}

Regardless of which method is used for suspending the QDs in biological media, the suspension should be purified of excess ligands and amphiphiles before it can be used for any biological experiments. When selecting a method to prepare water-dispersible QDs, one must remember that the biological and physical properties of the QDs will be strongly influenced by the surface coating, and the hydrodynamic diameter of the QDs is directly related to the coating thickness. In many applications the volume of organic molecules on the QD surface can greatly exceed the volume of the inorganic QD itself.

Many hydrophilic QDs can be conjugated with biomolecules using well-known conjugation methods that have long been used for tagging biomolecules with dyes and radioactive labels. QDs terminated with carboxyl groups can be reacted with amines using carbodiimide chemistry. Alternatively, amine-terminated QDs can be reacted with active N-hydroxysuccinimide (NHS) esters or converted to maleimide groups for conjugation with thiolated peptides, cysteine-tagged proteins, or thiolated antibodies. Third, the hydrophobic coating of QDs can be directly displaced with thiolated peptides or proteins to provide a direct linking of targeting ligands to the nanocrystal surface. Finally, nanocrystal conjugation can also be achieved using a non-covalent self-assembly method with engineered proteins.\textsuperscript{57}

To date, PEGylated QDs have been most commonly used for both \textit{in vitro} and \textit{in vivo} applications. PEG molecules on the QD surface can significantly reduce the uptake of QDs in the liver, spleen, and bone marrow, increasing circulation time of the QDs and increasing the possibility of targeted uptake elsewhere. Nonetheless, a substantial fraction of the QDs are ultimately accumulated in the liver, spleen, and bone marrow, even when the QDs have been PEGylated. Several PEGylated QD formulations have been employed for live animal luminescence imaging. In those studies PEGylated QDs were observed in the spleen, bone marrow, and lymph nodes of mice, but did not produce any ill effects. Many surface coatings (e.g. polyethylene glycol, lysine, dihydrodiolapoic acid, PEGylated phospholipids, and cysteine) have been employed in making colloidal stable QD formulations.\textsuperscript{26,57,69} PEGylated QDs terminated with amine groups have circulation half-lives that depend on the molecular weight of PEG. Similarly, when a “pure” PEG coating was applied to QDs (without functional groups), the biodistribution of QDs was reported to depend on the length of the PEG molecule.\textsuperscript{70}

2.3. Potential toxicity of QDs

For the past several years, concern about QD toxicity has been a major roadblock to the translation of QDs toward clinical research and applications. Reports of QD toxicity in the literature have been quite confusing due to the wide variation in the types of QDs being tested and in the biological models in which they have been tested. Each type of QD has its own unique physicochemical properties that determine its potential toxicity. Along with composition, the physical and surface characteristics of the QDs play a major role in determining toxicity. These characteristics include size, shape, surface charge, and surface coverage (coatings, chemically conjugated molecules). Because the surface-to-volume ratio in QDs is very large compared to the corresponding bulk or even microscopic form of the same material, surface initiated processes are significantly enhanced. Thus, toxicity studies performed with larger-size semiconductor particles cannot be used to assess the toxicity of QDs. Similarly, the surface charge on the QDs has a pronounced impact on the toxicity produced. For example, some QDs have been found to be cytotoxic only after oxidative and/or photolytic degradation of their core.\textsuperscript{71} QD dosages and concentrations reported in the literature vary in their units (e.g., mg ml\textsuperscript{-1}, mol, mg kg\textsuperscript{-1}, number of QDs per cell), and correlating dosage across current studies is usually difficult and sometimes impossible. As mentioned above, the majority of the mass of a QD formulation is often made up of the organic molecules encapsulating the inorganic nanocrystal, but dosages and concentrations are often reported in terms of the total mass, including the organic component. Thus, there is a need to formulate a set of characterization protocols to standardize the relevant tests and assays for reproducible and intercomparable characterization of nanotoxicity. One must fully characterize the QDs prior to evaluating their toxicity. Only then can one draw meaningful conclusions from comparisons of one type of QDs to another. The key components required for characterization of QD formulations are typically: (i) inductively coupled plasma mass spectroscopy (ICP-MS) analysis to determine the precise elemental composition of the formulation; (ii) transmission electron microscopy (TEM) to determine the actual core size, shape, and monodispersity of the QDs; (iii) dynamic light scattering to evaluate the overall hydrodynamic size of the QDs formulation; and (iv) zeta potential measurement to characterize the surface charge of QD formulations. To accurately evaluate the toxicity of a specific kind of nanomaterial, the general procedures are suggested to be: (i) optimizing the synthetic procedures to obtain highest monodispersity of nanoparticles; (ii) mapping out a calibration curve between concentration and a readily measurable physical parameter (e.g. molarity vs. absorption) using ICP-MS and TEM; (iii) modifying the surfaces as desired for the biological application; (iv) measuring the hydrodynamic diameter and zeta potential; (v) conducting \textit{in vitro} assays (e.g. cell viability assessment); (vi) carrying out \textit{in vivo} model evaluations. In the following section, examples of commonly used techniques or models are provided as guidelines to evaluate the toxicity of nanomaterials for biological applications in general, as well as the current toxicity status of cadmium-based QDs.

2.4. Mechanisms of QD induced cytotoxicity

Cytotoxicity refers to toxicity at the cellular level introduced by the interaction of QDs with the cell membrane or intracellular constituents and organelles.\textsuperscript{4} Scheme 1 illustrates some of the main toxicity mechanisms which the QDs could potentially induce. For example, the intracellular uptake of QDs could disturb the oxidative balance of the cell and cause oxidative stress. The production of reactive oxygen species (ROS) such as superoxide (\(O_2^-\)), hydroxyl radicals (\(\text{HO}^*\)), peroxide radicals...
In vitro nanotoxicity

In vitro nanotoxicity assesses toxicity based on the effect of the nanomaterial on cultured cells. It is extremely crucial because it can pre-determine the potential toxicity and the underlying mechanism before administering the nanoparticle to any animal subjects. The nanoparticles must demonstrate a degree of biocompatibility and safety prior to in vivo studies. The early in vitro studies used formulations that did not show good biocompatibility and therefore were not used in vivo. Later in vivo studies used better-passivated QD formulations. The common in vitro assessments include the evaluation of cell viability, expression of specific genes, and induced immune-responses.4

3.1. Cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is the most widely used assay for cell viability measurement of the toxicity of nanoparticles. The MTT molecule is reduced to a purple formazan product by mitochondrial activity. The viability of cells is obtained by quantifying this purple product using optical absorbance measurements. Absorbance is usually interpreted as being proportional to the number of viable cells, although factors other than the number of cells can also influence mitochondrial activity. A targeted cell line is treated with different concentrations of nanoparticles and incubated for a defined period of time (often 48 and 72 hours). Many reports have shown that the size, shape, surface coating, charge and structure (core–shell) of nanoparticles can affect the cytotoxicity. Using the MTT assay, Derfus et al. first revealed that CdSe-core QDs were degraded under UV irradiation and led to cell death due to the release of cadmium ions (Fig. 1).16 However, with proper surface coating such as a ZnS shell or bovine serum albumin corona, the toxicity of CdSe QD can be drastically decreased. Surface chemistry has played a very important role in reducing the toxicity of CdSe QDs. In addition to the high band gap semiconductor shell, polymeric coatings (e.g., PEG) and other inorganic coatings, such as silica, can serve as a barrier to prevent the cadmium-containing core from being exposed to the biological media. The most commonly used PEG derivative is phospholipid-PEG (DSPE-PEG) because it provides excellent stability to QD and can be quickly adapted to in vivo studies. Our group found that the safe dosage of DSPE-PEG encapsulated QD for in vitro experiments is ~150–200 μg ml⁻¹.27

3.2. Oxidative stress induction

Sometimes, a nanomaterial may not directly affect the viability of treated subjects but triggers a sequence of inflammatory mediator formation. Thus, studying the level of reactive oxygen species (ROS) is very important to evaluate the toxicity of QDs.

Scheme 1 Various mechanisms of QD induced cytotoxicity.

(ROO*), hydrogen peroxide (H₂O₂) and singlet oxygen can adversely affect cellular functions. At a high level of oxidative stress, the antioxidant defense system will be overwhelmed by the excessive ROS and eventually lead to mitochondrial malfunctions, as well as apoptosis. High ROS level could also disrupt DNA function. DNA fragmentation, breakage of the DNA double strand, and suppression of DNA functions, such as replication and transcription, can be induced by oxidative stress and cause genotoxicity. Neurotoxicity could also be a potential hazard of using Cd-based QDs, as exposure to heavy metals can kill neuronal cells which are involved in the signal transmission processes from the brain to the rest of the nervous system. Thus, toxicity assessments related to the cell viability, oxidative stress, genotoxicity and neurotoxicity are extremely important. In the next section, several in vitro assays for evaluating these important parameters will be presented.

Fig. 1 Toxicity assessment of CdSe quantum dots in liver cell culture model. (A) Hepatocyte viability of QD-treated cultures relative to untreated controls. Thirty minutes of exposure to air renders TOPO-capped QDs highly toxic at all concentrations tested. Ultraviolet light exposure also induces toxicity that increases with exposure time and is QD-concentration dependent. (B) Phase contrast microscopy image of control hepatocyte cultures exhibited distinct intercellular boundaries, well-defined nuclei, and polygonal morphology. (C) Nonviable cultures exposed to cytotoxic QDs exhibited granular cytoplasm, indistinct intercellular boundaries, undefined nuclei, and evidence of blebbing. Scale bars represent 100 μm. Copyright © 2004, American Chemical Society. Reproduced with permission from Derfus et al.16
ROS are well known to play significant roles in the regulation of cellular events. High levels of ROS can cause cell damage and unusual cell death or proliferation. Clift et al. studied the formation of oxidative stress caused by QDs with different surface coatings.\textsuperscript{72} By measuring the reduced glutathione and oxidized glutathione levels in J774.A1 macrophage cell line, their study demonstrated that both carboxyl- and amine-terminated QDs can cause oxidative stress that leads to the modulation of intracellular Ca\textsuperscript{2+} signalling. Electron paramagnetic resonance (EPR) spectroscopy has also been used to evaluate the generation of free radical species when QDs were irradiated under UV light.\textsuperscript{73,74} Expression levels of some genes (e.g. heme oxygenase-1 and GADD45) or transcription factors (e.g. AP-1 and NF-κB) are also responsive to the increase in oxidative stress. Expression of these genes also increased when the cells were treated with high Cd dosage.\textsuperscript{75}

3.3. Genotoxicity
QDs have been extensively explored in cancer research for \textit{in vivo} imaging and delivery of therapeutics. In this context, evaluating the genotoxicity risk of this engineered material, which may potentially cause DNA damage and promote carcinogenesis factors, is essential. Expression of p53 is a good indicator of DNA damage. p53 is a tumor suppressor that regulates the cell cycle and is described as a “guardian of the genome”. It prevents the conversion of damaged DNA to genome mutation.\textsuperscript{76} Choi et al. revealed that uncoated CdTe QD activated the p53 genotoxic stress pathways and resulted in the upregulated transcription of \textit{Puma} (p53-upregulated modifier of apoptosis) and \textit{Noxa} (NADPH oxidase activator 1), which are involved in apoptosis.\textsuperscript{77} Anas et al. and Green et al. used the plasmid nicking assay to demonstrate that ZnS-coated QDs can also cause DNA breakdown and nucleobase damage due to the generation of free radicals.\textsuperscript{73,78} While there are very few studies that have studied the genotoxic potential of QDs as a function of their physico-chemical properties, there is still a growing interest in investigating any negative impact of QDs on these human building blocks.\textsuperscript{79}

3.4. Neurotoxicity
Due to the unique optical properties and robustness of QDs, their usage as an optical contrast agent has been extended to brain imaging. Our group has demonstrated that transferrin conjugated quantum rods can facilitate transport across a model of the blood brain barrier (BBB, Fig. 2).\textsuperscript{80} Gao and co-workers investigated the use of lectin-functionalized QDs for \textit{in vivo} brain targeting.\textsuperscript{81} However, there have been few studies concerning the potential neurotoxicity of QDs. Neurotoxicity can originate from excess ROS that trigger apoptosis or inflammatory responses in the neural cells or central nervous system (CNS). ROS induced transcription factors, such as NF-κB, have been implicated in neurodegenerative diseases.\textsuperscript{82} Cytokines such as TNF-R and IL-1β and gene expression of IL-6 and IL-8 are also related to the dopaminergic neuron death. Through analysing these parameters \textit{in vitro}, one can potentially map out the signalling pathway and underlying mechanism of the neurotoxicity of QDs.

4. In vivo nanotoxicity evaluations: animal models
\textit{In vivo} studies using animal models take into account an entire spectrum of biological interactions in living organisms and are the natural next step, after \textit{in vitro} testing, in evaluating and understanding the toxicity of QDs. These experiments can measure an overall effect, including cytotoxicity and organo-toxicity, on an entire organism. This \textit{in vivo} toxicity can be organ-specific and its manifestation can depend upon the mode of introduction of QDs, or the specific organ being targeted. However, in a systemic delivery, where QDs formulations are directly introduced in the bloodstream, the high blood flow organs such as the liver and spleen are the main sites of QD uptake. In addition, translocation of QDs to secondary organs and tissues which are not targeted may also produce toxicity. The various animal models used to study \textit{in vivo} toxicity of QDs are described in the following sections.

4.1. Zebrafish
Commonly, \textit{in vivo} toxicity is tested in mammalian models, such as mice or rats. However, even these small animal studies can be time-consuming and expensive. In recent years, some groups have used zebrafish as an alternative model for toxicity testing of QDs. Zebrafish \textit{(Danio rerio)} have been used to study almost every aspect of vertebrate biology, including the development and function of the cardiovascular system, the central nervous system and the digestive system, because they are small, easy to care for, inexpensive to maintain, and produce large numbers of transparent embryos for testing. The ability to culture large numbers of zebrafish embryos and larvae in small volumes of media facilitates rapid testing of compounds for toxicity while using a minimal dosage of nanoparticles. Embryonic, larval and adult zebrafish have all been used for
distinguish different types of toxicity produced by CdSe QDs.\textsuperscript{17} Effects and (B) presents the statistical results. CdCl\textsubscript{2} caused four toxic responses: altered axial curvature (aac), pericardial edema (pe), ocular edema (oe), and submaxillary edema (smoe). Submaxillary edema was strongly associated with the surface ligands. End points of toxicity that were not cadmium-like consisted of: tail malformation (tm), yolk sac malformation (ysm), and opaque tissue indicative of tissue necrosis in the head, body, and yolk sac. This combination of cadmium-like and not cadmium-like responses to functionalized QD exposure suggested that both Cd and the QD coating. At sub-lethal concentrations, many QD preparations produced characteristic signs of Cd toxicity that weakly correlated with metallothionein expression, suggesting that QDs were slightly degraded \textit{in vivo}. QDs also produced distinctly different toxicity that could not be explained by Cd release. Using Cd\textsuperscript{2+} ions, they found that zebrafish larvae showed clear signs of Cd toxicity. However, nanoparticles were even more potent and produced end points of toxicity distinct from that of Cd\textsuperscript{2+}. Also, by using MT gene induction as an indicator of Cd\textsuperscript{2+} release, they were able to detect breakdown of QDs after absorption by the larvae.

Zhang \textit{et al.} reported the impact of cadmium selenide (CdSe) QDs and copper ion (Cu\textsuperscript{2+}) joint exposure on zebrafish embryo and larvae.\textsuperscript{83} The study was performed to determine the developmental toxicities to zebrafish exposed to combined pollution with CdSe QDs and Cu\textsuperscript{2+} compared to the impact of a single exposure. Their findings revealed that: (i) QDs facilitated the accumulation of copper ions in zebrafish; (ii) QDs caused higher mortality, lower hatch rate, and more malformations of the exposed zebrafish; (iii) junction, bifurcation, crossing, particles, and aggregation of the exposed FLI-1 transgenic zebrafish larvae could be observed; (iv) embryo cell apoptosis occurred in the head and tail region; and (v) synergistic effects played an important role during joint exposure. These observations provide a basic understanding of CdSe QDs and copper ions joint toxicity to aquatic organisms.

More recently, Truong \textit{et al.} employed the embryonic zebrafish to evaluate the importance of QDs surface functionalization in both nanoparticle stability and \textit{in vivo} biological responses.\textsuperscript{46} The authors tested two PbS QD formulations with the same core sizes but surface functionalized with either sodium 3-mercaptopropanesulfonate (MT) or sodium 2,3-dimercaptopropanesulfonate (DT) ligand. Exposure to these QDs formulations was begun at 6 hours post fertilization (hpf). Exposure to the MT-PbS formulation led to 100% mortality by 120 hpf while exposure to DT-PbS formulation produced less than a 5% mortality rate at the same concentration. Exposure to the MT and DT ligands alone did not produce any adverse developmental effects. Inductively coupled plasma-mass spectrometry (ICP-MS) was used to confirm that the embryos took up both MT-PbS and DT-PbS upon exposure. The results suggested that the toxicity of the QDs was strongly associated with the surface ligands.

\subsection*{4.2. Aquatic frogs}

The frog embryo teratogenesis assay (\textit{Xenopus protocol}) is an established and commonly used method for assessing harmful pollutants. \textit{Xenopus laevis} tadpoles are normally less sensitive to many chemicals than other amphibians. Dubertret \textit{et al.} reported the preparation of encapsulated individual QDs in phospholipid block-copolymer micelles and demonstrated \textit{in vivo} imaging of xenopus embryos.\textsuperscript{14} They found that upon injection into xenopus embryos, the micelle-coated QDs were stable, nontoxic ($< 5 \times 10^9$ nanocrystals per cell), cell autonomous, and slow to photobleach (Fig. 4).

The QD fluorescence could be followed to the tadpole stage, allowing lineage-tracing experiments in embryogenesis. The authors reported that the micelle-coated QDs have very little toxicity.
The toxicity was sufficiently low that cell lineage could be traced by fluorescence visualization. For typical QD injections (2 × 10^9 QDs per cell), injected embryos displayed an unaltered phenotype and their health was statistically similar to that of uninjected embryos. At higher concentrations (>5 × 10^9 QDs per cell), abnormalities became apparent. The cause of these defects is not yet known, but they may result from changes in the osmotic equilibrium of the cell. The QD-micelles were stable in vivo.

4.3. Mice

In vivo studies of toxicity of QDs have also employed various mouse models (e.g. Balb/C mice, Kunming mice, and nude mice). Relative to other mammals, mice are easy to care for, inexpensive to maintain, and produce large numbers of offspring. Most importantly, standardized mice can be obtained from well-established companies in most countries. Thus, they are the most suitable model for evaluating various kinds of potentially toxic substances. Rzigalinski et al. have reviewed early mouse studies of toxicity of Cd-based QDs, which all showed the absence of any significant toxicity at low dosage. Fitzpatrick et al. reported the observation of fluorescence of QDs injected into Balb/c and nude mice for up to two years post injection using both whole-body and microscopic fluorescence techniques. Two-photon spectral microscopy was used to verify the existence of quantum dots within two-year tissues, but also revealed a range of significantly blue-shifted emission peaks with increased bandwidths (Fig. 5). A total of 23 mice were imaged at intervals from 15 min to two years after tail-vein injection of quantum dots and only the five mice which survived for two years were reported. After imaging, mice were necropsied; tissues removed for examination included those from the liver, spleen, bone marrow, and lymph nodes. Immediately after the injection, QD fluorescence was seen in the circulation, liver, spleen, lymph nodes, and bone marrow. With increasing time post-injection, the liver fluorescence rapidly faded (2–5 days) and the fluorescence in the bone marrow faded more slowly (3–6 months), while the lymph nodes retained QD fluorescence for a longer period (up to 2 years). The authors suggested that the blue shift seen in QD fluorescence after two years in vivo is due to the slow breakdown of the QD. It may be a degradation of the ZnS surface and/or slow loss of core, a cationic exchange mechanism or a change in the size and shape of the QDs. They have noted that the range of observed

Fig. 4 Using phospholipid micelle encapsulated green CdSe–ZnS core–shell QDs for the labeling of Xenopus embryos at different stages and specific QD intracellular localizations. (A) Schematic showing the experimental strategy. QDs were injected into an individual blastomere during very early cleavage stages. (B) to (E) show merged transmission and fluorescence images of embryos injected with QDs (in green) at different time post injection. (F) Intracellular labeling of an axon (arrow) and somites at tadpole stage 40. (G) QDs localized in the nucleus during mid-blastula stages. (H) Labeled neural crest cells migrating into the branchial arches. (I) QD fluorescence observed in the gut of an injected embryo. Scale bars: (B) to (E), (H), and (I), 0.5 mm; (F) and (G), 30 μm. The injection of 2.1 × 10^9 to 4.2 × 10^9 particles per cell does not induce any observable toxicity. Copyright 2002, American Association for the Advancement of Science. Reproduced with permission from Dubertret et al.

Fig. 5 Fluorescent and spectral analysis of frozen lymph node sections from Balb/C mouse treated with CdSe–ZnS core–shell quantum dots after 2 years. (a) Background and autofluorescence collected in the range of 500–550 nm, pseudo-colored in green. (b) Transmitted light DIC image taken of the same tissue region in (a) to illustrate the morphology of the lymph node. (c) Fluorescent signals collected in the range of 620–670 nm from the same frozen section pseudocolored red, which encompasses the majority of the quantum dot emission. (d) Overlay of the green and red spectral regions. (e) Normalized spectral data from frozen tissue sections. The spectra are represented as: black dotted line, directly from 655 nm emitting quantum dots in solution; black open squares, mouse lymph node 24 h after injection; diamonds (blue, orange and red), mouse lymph node 2 years after injection; green circles, 2 year injected mouse autofluorescence; and open green circles with dashed line, 1.5 year uninjected control mouse autofluorescence. The autofluorescence intensity was a factor of 10 less than that of the quantum dots and both the autofluorescence from the 2 year injected mouse and the 1.5 year uninjected mouse have similar spectral signatures. Copyright 2009, American Chemical Society. Reproduced with permission from Fitzpatrick et al.
blue shifts is tissue-type invariant, eliminating organ-specific breakdown mechanisms. This suggests that the degradation mechanism may be specific to subcellular location (i.e., vesicle physiology, presence of peroxide, pH level) or simply a result of long-term infinite dilution. These QDs caused no acute toxicity when injected in quantities that would normally be expected to show lethal toxicity as soluble cadmium ions. However, the appearance of a wavelength shift strongly argues that the particles, in spite of both a ZnS and polymer coating, are degraded over time in vivo. Yang et al. made similar observations.86 They have used ICP-MS analysis to show that after intravenous injection of cadmium-based QDs in mice, the cadmium level in the kidneys and liver eventually (over 28 days) reached nearly 10% and 40% of the injected dose, respectively. The redistribution of cadmium over time may signify the degradation of QDs in vivo, because the natural accumulation sites of Cd ions are the liver and kidneys.

More recently, Hu et al. demonstrated the use of PEGylated phospholipid micelles to encapsulate near infrared emitting ultra-small lead sulfide (PbS) QDs for in vivo imaging.15 Over 4 weeks of evaluation, the authors did not observe any signs of toxicity in the mice. Even though there are some advantages of using PbS QDs for biological research, toxicity concerns based on their lead content are likely to prevent their development for clinical applications. Nonetheless, PbS QDs can be carefully tailored for some specific applications such as cell labelling, small animal imaging, and flow cytometry, where toxicity concern will not be a major issue.

4.4. Rat

To date, very few groups have used rat models to evaluate the QDs toxicity in vivo, mainly because they are not as easy to care for as mice in long term toxicity studies. However, the rat model does have advantages over mice, including the ability to obtain larger volume blood samples for evaluation, and to more accurately monitor body mass and behavioural changes due to their larger size. Hauck et al. studied toxicity of CdSe–ZnS core–shell QDs in Sprague-Dawley rats.12 Biodistribution, animal survival, animal mass, hematology, clinical biochemistry, and organ histology were characterized at different concentrations (2.5–15.0 nmol) over short-term (<7 days) and long-term (>80 days) periods. Throughout the study, rats exhibited no unusual responses or behaviors (lethargy, weight-loss, etc.) compared to controls. The Cd levels detected in tissues from rats not treated with QDs were negligible, below 0.1 μg Cd g⁻¹ wet tissue for both buffer and vehicle controls. This is expected, as Cd has no known biological function, and laboratory rats have a highly regulated food supply and are not exposed to typical environmental sources of Cd.

The biodistribution profile (Fig. 6) indicated that QDs initially accumulated in the liver and spleen and after several days to several weeks, QDs, or at least their constituent metals, were found in the kidneys. At one day after injection, the liver contained 0.92 μg Cd g⁻¹ and the kidneys contained 0.09 μg Cd g⁻¹, and by 30 days after injection the liver contained 1.69 μg Cd g⁻¹ and the kidneys contained 3.00 μg Cd g⁻¹. The accumulation of QDs in the liver and spleen is expected to occur via the clearance of QDs from the blood by cells of the mononuclear phagocytic system. The authors suggested that the QDs may be breaking down and behaving at least partially as their constituent ions.

In another study, Tiwari et al. investigated in vivo toxicity of anti-HER2ab-conjugated CdSe–ZnS QDs in Wistar rats.87 For toxicity evaluation of injected QDs, body weight, organ coefficients, complete blood count, biochemistry assay, comet assay, reactive oxygen species, histology, and apoptosis were determined. Complete blood count and biochemistry assays showed insignificant changes in the anti-HER2ab-QD treated rats, but significant changes were observed in the group treated with non-bioconjugated QDs. No tissue damage, inflammation, lesions, or QD deposition were found in histology and TEM images of the anti-HER2ab-QD treated group. Apoptosis in the liver and kidneys was also absent in the anti-HER2ab-QD treated group, in contrast to animals treated with nonconjugated QDs, which showed comet formation and apoptosis. Cadmium deposition was also confirmed in the nonconjugated-QD-treated group, but was not observed in the anti-HER2ab-QD treated group.

A major limitation to usage of QDs and other inorganic nanomaterials in biomedical research is their long-term accumulation, mainly in the reticuloendothelial system (RES) organs such as the liver, spleen and bone marrow. The long-term health risks of this accumulation are not known. Recently, in an effort to overcome this challenge, researchers have developed ultrasmall renal-clearable nanoparticles and tested them on rats.88–90 QDs with hydrodynamic diameter around 5.5 nm were reported to undergo efficient urinary excretion and elimination from the body.23 Thus, minimizing the hydrodynamic diameter of QDs is an important factor to lower the overall potential for toxicity of the QD formulations, since they can be facilely excreted in the urine after performing desired tasks such as providing imaging contrast or facilitating drug or gene delivery.91

4.5. Nonhuman primates

Nonhuman primates are irreplaceable animal models for biomedical and medicinal research. Their close evolutionary
relationship to humans makes them high-fidelity models with excellent predictive abilities that may not be available in the in vitro and in vivo models discussed above. However, research that depends on nonhuman primates is becoming more difficult to perform in many countries. The supply of animals is barely sufficient to meet current demands, and ethical concerns about the use of primates in research have grown in recent years. By virtue of their taxonomic status and sentience, combined with their relatively slow rate of reproduction in captivity, primates present ethical and practical challenges as animal models. Unlike most other species used in research, primates typically have a long lifespan, spend years in captivity, and are frequently re-used in several independent studies during the course of their lives. This possibility of reuse of animals minimizes the total number of primates used in research. Thus, researchers must carefully evaluate the potential of translating their specifically engineered QD formulation to clinical studies before testing them with primate models.

Of the most frequently used primates, Chlorocebus aethiops (Grivet) was used almost exclusively as a source of commercially obtained kidney cells for biochemistry and virology investigations. Macaca mulatta (rhesus macaque) was primarily used in neuroscience, toxicity, and AIDS research, and Macaca fascicularis (crab-eating macaque or cynomolgus monkey) was used mainly in neuroscience and Ebola research. Papio spp. (Baboon) subjects were most commonly used in surgery and genetics research, and Pan paniscus (chimpanzee) and Gorilla spp. were mostly used in studies of genetics and conservation.

Despite the challenges that it presents, the use of nonhuman primate models for the toxicological evaluation of QDs is the next natural step to take given the many uncertainties and the broad range of opinion on the applicability of cadmium-based QDs for in vivo applications. The toxicity and pharmacokinetics of QD formulations can be investigated in greater detail in primate models and with minimal risk, time, and cost compared to human clinical trials. Chimpanzees are the closest species to humans and, from that perspective, may be the most suitable for preclinical research studies, especially for studying short and long term toxicity of QDs. However, we do not recommend the use of chimpanzees for any nanomaterial toxicity study because they are an endangered species and they are not easy to care for and support. Rhesus macaques are Old World monkeys that diverged approximately 25 million years ago from the lineage that led to both chimpanzees and humans. Despite these limitations, rhesus macaques remain the best practical model for QD toxicological and pharmacological evaluation prior to human clinical studies.

In addition to rhesus macaques, crab-eating macaques (Macaca fascicularis) might be another alternative primate model for QDs toxicity assessment. The crab-eating macaque is one of the most widely used surrogate animal models for studies of infectious diseases, organ transplantation, reproductive biology, and development of new vaccines.

Here, we describe a few general methods that are utilized for the assessment of in vivo toxicity in primates. The first method consists of monitoring of behaviour changes in an animal after injection of nanoparticles. Examples are loss of appetite, loss of weight, decreased activity, and sleep disorders. These behavioural changes, monitored over a period of time, in comparison with a control animal, are powerful indicators of acute toxicity. The second method involves the hematological and biochemical markers. Nanoparticle toxicity could also produce changes in populations of red blood cells, white blood cells, and platelets. Primates have sufficient blood volume to allow for complete blood analysis to be carried out at regular intervals. Histology provides a direct assessment of changes in the tissue structures of different organs, after introduction of nanoparticles. This requires taking tissue specimens from the organs, fixing and sectioning, and having them examined by qualified medical pathologists in comparison to tissues from standard collections or control animals. While in some cases these tissue samples may be obtained by biopsy, complete histology of all major organs typically requires sacrificing the animal under study. Obtaining a complete biodistribution of the introduced nanoparticles via chemical analysis of the organs (e.g. ICP-MS analysis) also requires sacrificing the subject animal. Finally, determining the excretion profile of the nanoparticle formulation can be very valuable. An examination of renal excretion and feces provides information on the removal of nanoparticles from the primate body. It is not an indication of toxicity assessment of nanoparticles. However, verification of complete nanoparticle excretion, preferably both by detection of material in the urine or feces and by observing

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**Fig. 7** Histological images from the major organs of the rhesus macaques three months after intravenous injection of the phospholipid micelle encapsulated CdSe–CdS–ZnS core–shell–shell QDs. No anomalies were observed by pathologists. (A) heart, (B) liver, (C) spleen, (D) lungs, (E) kidneys and (F) lymph node.
disappearance of the material in the organs, eliminates the possibility of any long-term health risk. Thus in the overall strategy of evaluating the benefit/risk ratio, study of excretion of nanoparticles or their component elements, whether intact or biodegraded, is extremely valuable.

Recently, we have presented the first study of QDs in primates, reporting toxicological and pharmacological responses in male rhesus macaques after intravenous injection of 25 mg kg\(^{-1}\) of phospholipid-micelle-encapsulated CdSe–CdS–ZnS QDs.\(^{14}\) Standard hematological and biochemical markers used to investigate the potential toxicity were within the normal ranges over 90 days of monitoring. In addition, the histological analysis of major organs such as the heart, liver, spleen, kidneys, lungs, and lymph nodes, displayed no signs of inflammation or injury (Fig. 7). This indicates that the acute toxicity of QDs \textit{in vivo} can be minimal, for appropriate formulations and doses. On the other hand, chemical analysis of major organs proved the presence of cadmium and selenium in the liver, spleen, kidneys after 90 days. For selenium, uptake was also highest in the kidneys, spleen, and liver. We have also determined the blood clearance profile for the QDs formulation. The QDs formulation was found to have blood circulation times around one hour. The concentrations of cadmium and selenium in the blood dropped to 0.1 and 0.2 µg ml\(^{-1}\) 24 hours after injection. No cadmium and selenium were detected in blood 72 hours after injection. After 90 days, the cadmium levels in the kidneys eventually reached nearly 35% of the injected dose, compared to the 58% in the liver and 6% in the spleen. Due to the continued presence of high cadmium and selenium levels in organs after 90 days, longer-term studies will be needed to investigate the \textit{in vivo} impact of these heavy metals and any side effects arising due to QDs presence in the body. All the results reported in our study suggest that the tested formulation has low acute toxicity and could potentially be used in specific clinical applications.

In our study, virtually the entire initial dose of cadmium was found in the liver, spleen, and kidneys after 90 days (Fig. 8). Much lower levels were observed in the other organs. For selenium, uptake was also highest in the kidneys, spleen, and liver. We have also determined the blood clearance profile for the QDs formulation. The QDs formulation was found to have blood circulation times around one hour. The concentrations of cadmium and selenium in the blood dropped to 0.1 and 0.2 µg ml\(^{-1}\) 24 hours after injection. No cadmium and selenium were detected in blood 72 hours after injection. After 90 days, the cadmium levels in the kidneys eventually reached nearly 35% of the injected dose, compared to the 58% in the liver and 6% in the spleen. Due to the continued presence of high cadmium and selenium levels in organs after 90 days, longer-term studies will be needed to investigate the \textit{in vivo} impact of these heavy metals and any side effects arising due to QDs presence in the body. All the results reported in our study suggest that the tested formulation has low acute toxicity and could potentially be used in specific clinical applications.

In this study, no abnormalities in eating, drinking, grooming, exploratory behavior, activity, physical features, urination, and neurological status were observed. These measurements were taken subjectively. For example, the food and water provided were consumed at a reasonable rate. Neurological status means that the monkeys are behaving normally. They are responsive without showing unusual aggression when they are given food. Their movements of the arms and legs are normal.
Ideally, we would like to perform the experiments with a range of doses ranging from 10 to 1000× the maximum envisaged dosage. In our experience, this is not practical with our QD formulation. An IV dose of 100× the maximum envisaged dose would require approximately 20 g of QDs in our case. With current QD synthesis protocols, preparing 20 g of QDs would be a major task, but would be possible. However, the concentration of QDs in the dispersions injected in the experiments described above was already near the solubility limit. Increasing the total dose would therefore require increasing the total volume injected by the same amount. Thus, the maximum envisaged dose in a single treatment would require injecting an unreasonably large volume. We advise that one should carefully test the maximum concentration at which a specific engineered QD formulation can be prepared before testing it on animals.

In future nonhuman primate studies of QD toxicity, we suggest that cardiovascular (e.g. blood pressure, heart rate, and electrocardiography) and respiratory measurements would also be valuable; these data will help one to determine the impact on the cardiovascular and respiratory systems. In our case, we have taken heart rate and electrocardiography measurements while the monkeys were anesthetized and injected with the QD formulation. The measured heart rate and electrocardiography were normal for all the monkeys. Also, we suggest that specific immunohistochemistry assays can be used to assess hepatocytes in the liver samples where we have not observed any signs of inflammatory response. Genetic analysis such as real-time polymerase chain reaction (q-RT-PCR) can be performed on the tissue samples to investigate the inflammatory gene regulation.

In some mouse studies, removal of QDs from the systemic circulation by the liver and spleen was observed, and may have exerted a mild inflammatory response. One of the major advantages of using primate models is their close resemblance to humans both morphologically and physiologically. In the near future, liver biopsies could potentially be performed at earlier time points to evaluate potential acute hepatic toxicity. By assessing histology at multiple time points, temporal analysis of potential toxic effects of QDs may give us new insights into the interaction between QDs and the liver. However, conducting biopsies without confounding the results will be difficult. While taking the biopsies, the animal may suffer from physical and mental shock that can lead to behavioral changes.

One must also remember that QDs may agglomerate or aggregate when they are injected into the body. In general, agglomeration indicates more loosely bound QDs and aggregation suggests tightly bound QDs (typically occurring during encapsulation of QDs within a polymer micelle). QDs might agglomerate due to the high ionic strength of blood, which shields the repulsion between the charges of QDs. The QD community has not devoted much attention to agglomeration of QDs in vivo, even though agglomeration would be expected to affect nanotoxicity by changing the size, surface area, and sedimentation properties of the QDs. Many QD formulations may agglomerate in the body before they reach their desired targets, but this has not been clearly demonstrated. Thus, careful investigation of the impact of agglomeration on in vivo QD toxicity would be valuable. In our primate study, we have specifically designed a formulation of “aggregated” QDs using phospholipid micelle encapsulation. Analysis of TEM images indicates that the micelle size ranges from 40 to 55 nm. Throughout our analysis, we have found that about 120 to 180 QDs are encapsulated in a micelle. The micelle-encapsulated QDs can be dispersed in biological buffers such as PBS and HEPES. Future studies should compare the toxicity of agglomerated and aggregated QD particles in vivo. This information will be useful in creating guidelines in producing agglomerated or aggregated particles with multimodal imaging and therapy capabilities for real-time traceable drug delivery applications.

5. Summary and outlook

Toxicity of QDs is a very important issue to address in order to fully utilize the potential of QDs for healthcare and medical research applications. A great deal of concern has been raised about the possible healthcare risk of QDs, both in the press and by practicing physicians. Thus a critical assessment of risk versus benefit of use of engineered QDs for diagnostics and therapy is extremely vital for the advancement of QD use in medicine. This review has focused on nanotoxicity of QDs, emphasizing the complexity of this subject and various in vitro and in vivo models used for assessments of QD nanotoxicity. QD toxicity studies in cell culture, zebrafish, aquatic frogs, mice, and rats are relatively simple and inexpensive but these results are difficult to extrapolate to humans. Table 1 summarizes the in vivo toxicity studies of various types of QDs using different in vivo models. Also, some of these models are not able to capture the complexity of the in vivo environment in humans. Primate studies provide the most relevant approach to assess the toxicity of engineered QDs in humans, but should be carefully used on a case-by-case basis where the engineered QDs have immediate benefits that outweigh their potential long-term biological effects. Primate testing is comparatively difficult and expensive, and could raise ethical concerns. In the near future, we envision that the most likely early applications of QDs would be in areas such as image-guided surgery for tumor removal, for which functionalization may or may not be needed (e.g., lung and liver cancer that can be targeted through EPR effects), where the advantages provided by QDs can outweigh the potential risk associated with accumulation of their constituent elements in the body.

Recently, Nawrot et al. have reviewed current understanding of the impact of cadmium on human health. Significantly progress has been made in the investigation of the concentration-dependent effects of cadmium on osteoporosis and mortality. Current understanding states that environmental exposure to cadmium increases total mortality in a continuous fashion, without any evidence of critical dosage, independently of kidney function and other factors associated with mortality including age, gender and smoking. The authors pointed out
that two recent environmental population-based cohort studies discovered that for each doubling of urinary Cd concentration, the relative risk for mortality increases by 17%. They have noted that tubular kidney damage starts at urinary Cd concentrations between 0.5 and 2 μg urinary Cd per g creatinine. A recent investigation focusing on bone effects showed increased risk of osteoporosis even at urinary Cd as low as 1 μg Cd per g creatinine. The nonsmoking adult population has urinary Cd concentrations close to or higher than 0.5 μg Cd per g creatinine. These studies clearly suggest that if we are planning to use cadmium-based QDs for specific designed clinical applications such as tumor removal, we must employ minimal dosages, because the levels of Cd present in many populations through environmental exposure are already approaching levels where ill effects are expected. QDs remaining in the liver and spleen may degrade over periods of months to years as discussed above. This slow release of cadmium from the QD crystals over time may increase systemic cadmium levels in a manner similar to environmental exposures.

In the near future, the QD research community should investigate the impact of QD formulations on translocation-induced and pulmonary toxicity. The translocation induced toxicity can occur through different mechanisms. An example is the translocation of QDs into the brain. When the QDs with appropriate ligands on their surface are introduced into blood circulation by systemic injection, they may cross the BBB and enter the brain. The BBB crossing can be due to size effects and pegylation, or due to the damage of BBB by interaction with QDs. Neuronal cells in the CNS are sensitive to stresses, because of their extensive, very thin, and fragile extensions, and they are also very sensitive to the aerobic metabolic activities. Any inflammatory response developed by the QDs can cause neurological disorders. On the other hand, pulmonary toxicity refers to the side effect on the lungs developed by interaction with nanoparticles. These effects will be more pronounced when the nanoparticles are introduced by instillation or inhalation within the respiratory tract. This may be more important for workplace exposures resulting from preparation of large quantities of nanomaterials as powders that can be dispersed to the air. Fortunately, most colloidal QDs like those discussed in this review do not form dry powders. Even upon solvent evaporation (or lyophilisation) they typically remain waxy due to the large amount of organic molecules or polymers encapsulating them.

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Notes and references
