Chapter 8
Quantum Dot (QD)-Induced Toxicity and Biocompatibility

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8.1 Introduction

The foundation for quantum nanotechnology in form of quantum nanomaterials was laid much earlier with the discovery of semiconductor nanocrystalline solution, which was later on termed as Quantum dots (QDs). They are most commonly referred as "artificial atoms." QDs are nanocrystals belonging to group II–VI semiconductors with unique optical properties, like sharp emission bands and broad absorption along with size-tunable photoluminescence present in the visible spectral range [1–3]. Presently, QDs are not only composed of CdSe or CdS but of various semiconducting materials that are derived from II–VI elemental groups (e.g., zinc selenide [ZnSe], zinc sulfide [ZnS], and cadmium telluride [CdTe]), or the semiconductors from III to V elemental groups (e.g., indium arsenate [InAs] and indium phosphide [InP]), or the IV–VI elemental groups (e.g., lead sulfide [PbS] and lead selenide [PbSe]) (Table 8.1). Additionally, due to the advances in synthesis of semiconductors, novel QDs such as core/shell of CdTe/CdSe, CdSe/ZnTe core/shell, cadmium-free QDs, and Mn-doped ZnSe have been developed as well. Due to the adverse effects of II–IV binary nanocrystals, some nontoxic I–III–VI ternary QDs are also beginning to find use in bioapplications (Table 8.1).

QDs attracted prodigious attention as alternatives. The effortless designing of fluorescent semiconductor-based electrochemical or biosensor using quantum dots can be one such self-sustained integrated device/system which can be employed for

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G. Korotcenkov (ed.), *Handbook of II-VI Semiconductor-Based Sensors and Radiation Detectors*, https://doi.org/10.1007/978-3-031-19531-0_8

Table 8.1 Bioapplications of various groups of QDs

Element category	Example of interest	Application
Group II–VI elements	CdS, CdSe, CdTe, ZnSe, ZnS, ZnTe	Analysis through sensor
Group III–V elements	InP or InAs	Limited biological applications; analysis through sensor
Group I–III–VI2 elements	$CuInS_2$ or $AgInS_2$	Used for biological applications and light-emitting diodes
Group IV–VI elements	PbS, PbSe, PbTe	Important for contrast imaging
Group IV elements	C, Si, Ge	Sensor applications; biological applications

sensitive and specific analytical information processing in biologics, where recognition of desired biological element can be accomplished with the direct spatial contact of it with electrochemical transduction element. Such uniqueness can also render distinct advantages over conventional luminous dyes in terms of tunable spectral range (excitation and emission spectra), detection of signal intensity or as an amplifier, quantum yield, electrocatalytic activity, electron transport properties, and photon-based stability, etc. [4, 5]. These features have enabled researchers to proceed with QDs in diverse research applications for fluorescence imaging [6] biosensing [7] in research areas such as medicines, clinical research, etc.; further it can also aid in development of personalized detection care also known as "point-of-care" (POC) QDs nanosensors. In non-biogenic research areas such as engineering, it has also enabled researchers to fabricate improved light-emitting diodes (LEDs) with diverse emission spectra [8], designing of solar cells with enhanced photovoltaic power conversion efficiency [9]. In this chapter, we have briefly discussed QDs structures, physiochemical properties, processing and biocompatible performance of QDs, and toxicity related to it in multimodal applications in diverse research fields.

8.2 Properties of Quantum Dots (QDs)

Semiconductor nanocrystals (NCs) or QDs are tiny nanoscale particles with the size ranging from a few nanometers to tensor even hundreds of nanometers. In the case of bulk materials, the optical, chemical, and physical properties are not influenced by the size. Conversely, the NCs have characteristics that are greatly determined by their morphology, size, and shape. Once the radius or size of NCs becomes smaller or equivalent to related bulk Bohr radius, then the electrons undergo "quantum confinement" in all three spatial dimensions. During this condition, the KBT value (KB is Boltzmann constant) becomes lower than the energy disparity between two levels of QDs, which results in a restricted hole and the electron mobility in the crystal dimension. Also, according to the radius of NCs, the QDs show absorption and emission based on size coupled with distinct electronic shifts [10, 11]. This effect is known as *quantum confinement*. This effect can influence various characteristics of QDs such as conductivity, magnetic properties, and the size and shape-

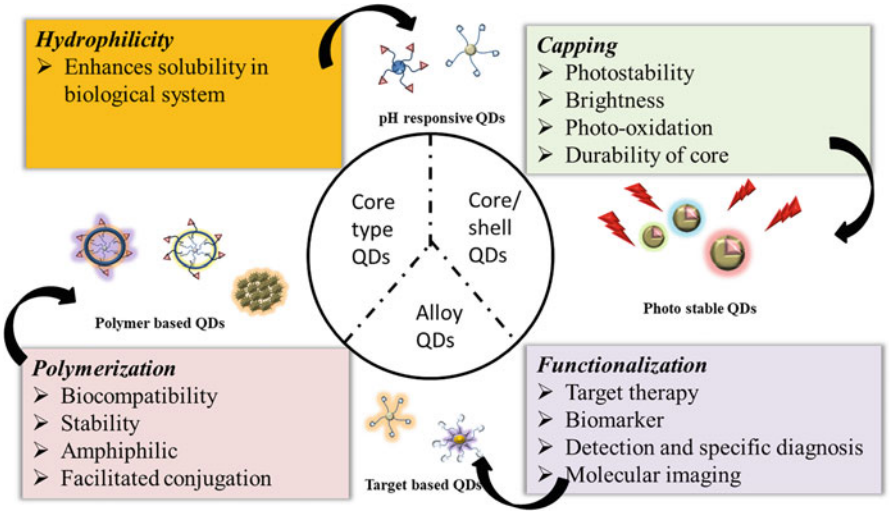

Fig. 8.1 The structure and classification based on physiochemical properties of quantum dots (QDs). They can be classified based on the core of QDs, core having a shell or alloy type

reliant optoelectronic characteristics. The reduction in size of NCs results in their free energy boost that makes them more reactive and dynamic than their bulk equivalents. It alters the various properties of NCs and makes them easily soluble in solvents that will further assist to functionalize their surface. This is the utmost attractive characteristic of NCs that can be utilized to create targeted bio-drugs and optoelectronic tools. So, the foremost physical characteristic that is desired to understand is the crystalline assembly. Analogous to their bulk equivalents, the class II–VI semiconductor QDs generally possess zinc blende or wurtzite constructs. However, occasionally, based on the conditions employed to assemble the particles, ligand-particle interaction, and impurities, many materials can possess both the crystalline assemblies [12]. The next is the matrix disparity between sowed core matter and the deposited shell matter during epitaxial development. The matrix disparity has a major role in the fabrication of core-shell structures and also can significantly influence the shell conditions and the optical characteristics [13] (Fig. 8.1). The third is morphology and shape of the semiconductor QDs. Mostly, the relatively low-temperature conditions will create nanocarriers with low dimensionality such as nano-plates or nano-disks. The intermediate temperature with weak or moderate directing ligands will generate spherical QDs, whereas the high-temperature conditions with durable directing ligands will create rod-like QDs. However, with the support of robust binding ligands such as phosphonic acid, the shell on zinc blende and wurtzite tend to fabricate dot-tetrapod and dot-rod morphologies, respectively [14, 15].

8.3 Core/Shell Nanostructures

Depending on the core material, they can be roughly divided into cadmium, silver, indium, carbon, and silicon QDs. They are different types of core/shell QDs as depicted in Fig. 8.2 based on edge alignment of conduction and balance bands [16].

From biological applications, the most frequently synthesized QDs are the core/shell nanocrystals, wherein the core nanocrystal is over-shelled by another semiconductor material to fortify and enhance its optical properties. Undeniably, CdSe/ZnS cores/shells are the "flagship" QD material, but the common representatives of Group II–VI QDs are CdSe, CdTe and other core/shell analogues such as CdSe/ZnS and CdTe/ZnS [17]. Basically, these core/shell QDs are like the earth structure that consists of a core with multiple layers surrounding it, called a shell. The core of QDs regulates the inclusive characteristics of the QDs and also refines the lights that illuminate through the QDs. The most frequently used cores are indium phosphide (InP) or cadmium selenide (CdSe) cores, which have multiple properties that make them more suitable than other materials. Although CdSe QDs are more competent and bear high quantum yield, the reported acute toxicity makes them unusable for biological purposes [18]. In the succeeding section, we discuss the cytotoxicity associated with QDs II–IV groups.

8.4 Surface Modifications

Given that the QDs are composed of toxic heavy-metal ions, the surface coating becomes a crucial parameter to ensure their biocompatibility. Owing to their non-dissolubility, photoluminescence instability, and metallic toxicity in water, QDs are usually required to be modified by passivation process, wherein additional hydrophilic coating materials coordinate or bind to the surface of QDs, to render

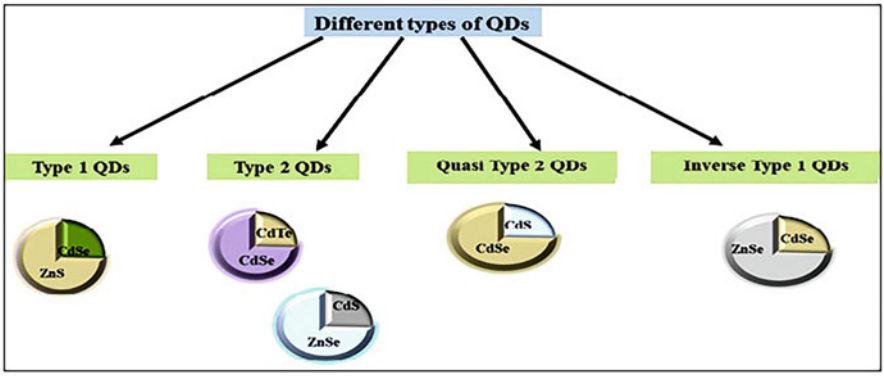

Fig. 8.2 A schematic representation of different types of QDs

them biocompatible and biostable. The chemically prepared QDs generally have the outermost surface containing an organic ligand, as the nonpolar capping agent that facilitates its solubility is only in organic solvent, which restricts their direct application in biological fluids. Therefore, it is imperative to select an appropriate strategy for stabilization and solubilization of QDs in aqueous solutions to be used in physiological conditions. Two key approaches are used to achieve surface modifications of QDs (i) hydrophobic interaction of amphiphilic molecules with QDs and (ii) interaction of the polar groups of the coating moieties with the surface of QDs (the ligand exchange/modifiers) [19]. Among the bifunctional molecules, thiols (–SH) are used as anchoring groups to bind to the QDs surface, while carboxyl (–COOH) groups are used as the hydrophilic ends. The suitability of a specific strategy is determined by the chemical properties of QDs that depend on their process of synthesis.

8.4.1 Polymer Coating

Polymers with exceptional biocompatibility and reduced toxicity are widely and effectively used to engineer biocompatible QDs composites for varied medical and biological applications. Existing synthetic strategies of biocompatible QDs for in vivo applications typically involve high-temperature organometallic approaches [16] and subsequent solubilization in aqueous solution using amphiphilic polymers [20, 21] or phospholipid micelles [22]. Different polymer coatings are reported to enhance the water solubility and to provide multifunctionality for targeted delivery or for use as biosensors to detect low concentration of molecules (Fig. 8.3).

Generally, QDs are coated with the amphiphilic graft and block copolymers [23, 24]. When a solution of QDs that are coated with hydrophobic surfactants like tri-octyl phosphine (TOP) and tri-n-octyl phosphine oxide (TOPO) is mixed with polymers, the polymeric hydrophobic chains tend to intercalate in between the surfactant moieties, making the hydrophilic groups visible on the surface, helping the QDs to stabilize in aqueous milieu (Fig. 8.4). Further, the polymers present on the QDs surface can be additionally cross-linked to enhance their stability. Generally, while solubilizing, the nonpolar solvent such as chloroform is dissolved with the amphiphilic polymer along with the QDs. Then the organic solvent is evaporated

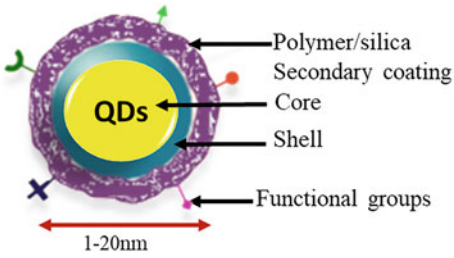

Fig. 8.3 Illustration of a generic QD that is coated with a polymer to reduce toxicity and functionalized for targeted delivery

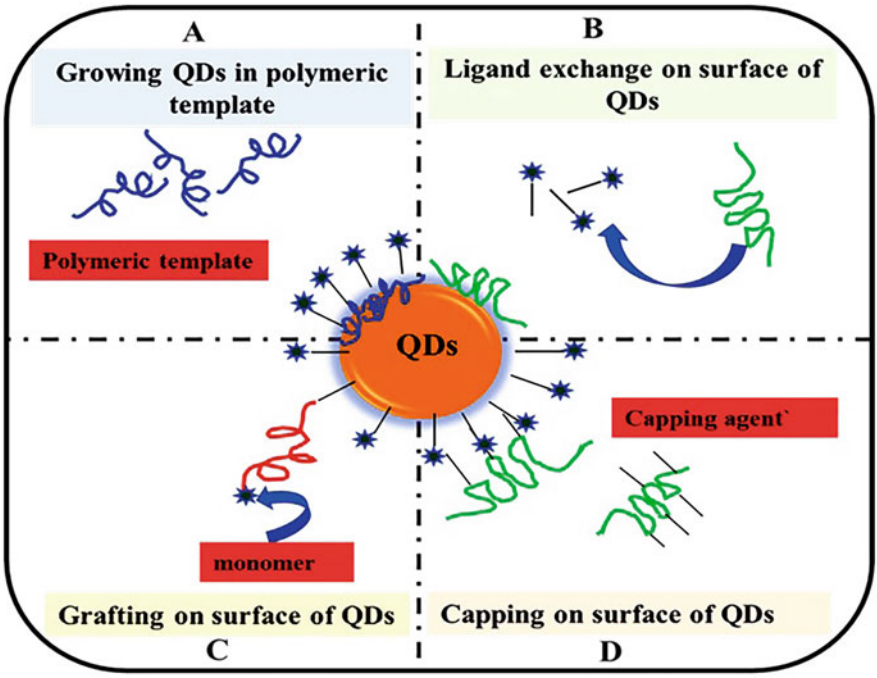

Fig. 8.4 Strategies for surface modifications of QDs. (**a**) Grafting QDs to polymer template; (**b**) ligand exchange on surface of QDs; (**c**) grafting polymer on surface of QDsl (**d**) capping on surface of QDs

from the mixture, and an aqueous buffer solution is added [25]. To maintain the colloidal stability of polymer-coated QDs, few parameters are critical, and they influence (i) the molecular weight of the polymer, (ii) the QD to polymer ratio, (iii) the ratio between the hydrophilic and hydrophobic molecules of the polymer and the number of hydrophobic chains per polymer moiety and the length of the polymer, and (iv) the number of charged groups that are exposed in the QD coating of polymer. Innumerable methodical investigations on QD-polymer interactions are necessary for each specific experiment, to optimize and regulate the optimal conditions for solubilization of QDs [26, 27].

Introducing polyethylene glycol (PEG) to QDs enhances the circulation time, which is important in biodistribution studies using QDs as contrast agents [28] or for drug delivery. QDs may be coated with amphiphilic molecules where the coating happens via the hydrophobic exchanges of the nonpolar parts of the amphiphilic molecules with TOPO present on the QDs surface. The ligand exchange approach differs as the QDs are directly bound with the coating molecules, typically a thiol- or amine-containing compound. Both the strategies have advantages and disadvantages. The direct addition of the thiol-containing moieties to QDs, rather than the bulky TOPO/polymer coating, produces ultrasmall nanoparticles. This is vital for various in vivo applications; the size of QDs may drastically alter the

pharmacokinetics, and biodistribution [29, 30] may affect the efficacy of FRET-based assays that are extremely sensitive to the donor-acceptor distance [31]. Innumerable methodical investigations on QD-polymer interactions are necessary for each specific experiment so as to optimize and regulate the optimal conditions for solubilization of QDs [26, 27].

8.4.2 Silanization

Silanization is an effective, low-cost covalent coating process that helps modify the surface that may be rich in hydroxyl groups, like titanium, metal oxide surfaces, and hydroxyapatites. The silica shell on QDs is chemically inert, optically transparent, safe, and innocuous. The silica surface can easily be modified to form silica-coated QDs that are ideal for conjugating various biomolecules such as antibody, antigen, aptamer, etc. Additionally, the monodispersed silica QDs confirmed high fluorescence intensity and are less likely to induce aggregation [32]. The surface of QDs is covered by a protective silica coating that prevents its exposure to the biological milieu, thereby enhancing the biocompatibility of QDs for in vivo applications. Therefore, silanization becomes a critical parameter to reduce the toxicity of QDs.

The initial step of silanization is to interchange the surface ligand with a thiol-derived silane, i.e., mercaptopropyltris (methyloxy) silane (MPS) (Fig. 8.5). This silica shell can be further modified with a variety of silicon to endow multifunctionality, and the most commonly used ones are aminopropylsilanes (APS), phosphosilanes, and polyethylene glycol (PEG) silane. Initially, Chen et al. synthesized silica-coated CdTe QDs and functionalized covalently with α-fetoprotein antibody, anti-AFP (secondary antibodies denoted Ab_2) to establish a novel technique for the ultrasensitive detection of biomarkers based on CdTe quantum dots [33]. The cytotoxicity of PbSe QDs that were functionalized with silica was verified on two different cell lines and showed that the silica-coated PbSe QDs were much less cytotoxic than the polymer-coated PbSe QDs [34]. Because the silica shells are exceedingly cross-linked, the silanized QDs are very stable. Therefore, silanization is favored as it is less toxic when compared to other ligands. Even though numerous groups have successfully reported silica-coated core-shell QDs, different types of surface-silanized QDs can be categorized into three classes, like core-shell QDs, single QDs, and multiple-layered QDs being coated by silica shell.

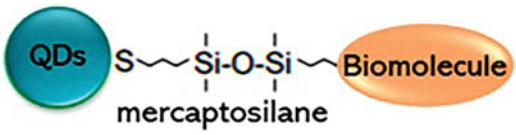

Fig. 8.5 Silanization of QDs and bioconjugation using a mercaptosilane compound

8.5 Biocompatibility

Biocompatibility is an important aspect for the success of biological and biomedical applications of QDs and is based on its solubility, stability, and toxicity. It is extremely crucial to understand and address the increasing biological complexity keeping in mind the progress toward clinical translation of QDs. Further to evaluate whether the surface-modified QDs are biocompatible, their intracellular fate, mechanism of internalization, and toxicity caused by them that may depend on the core material, surface modification, size, shape, and surface charge need to be understood (Fig. 8.6).

8.5.1 Intracellular Fate of QDs in Cells

The extracellular environment is separated from the intracellular milieu by the cell membrane that acts as a natural barrier. The process of QDs adhering to the cell surface may be internalized by transmembrane, cell membrane, transporters, distribution patterns, mean residence time, and elimination, all of which are closely linked to their application and subsequently associated toxicities. Alternately, for therapeutics, the adverse effects induced by QDs can be correlated with the therapeutic regimen, dosage, or the internalized concentrations [35].

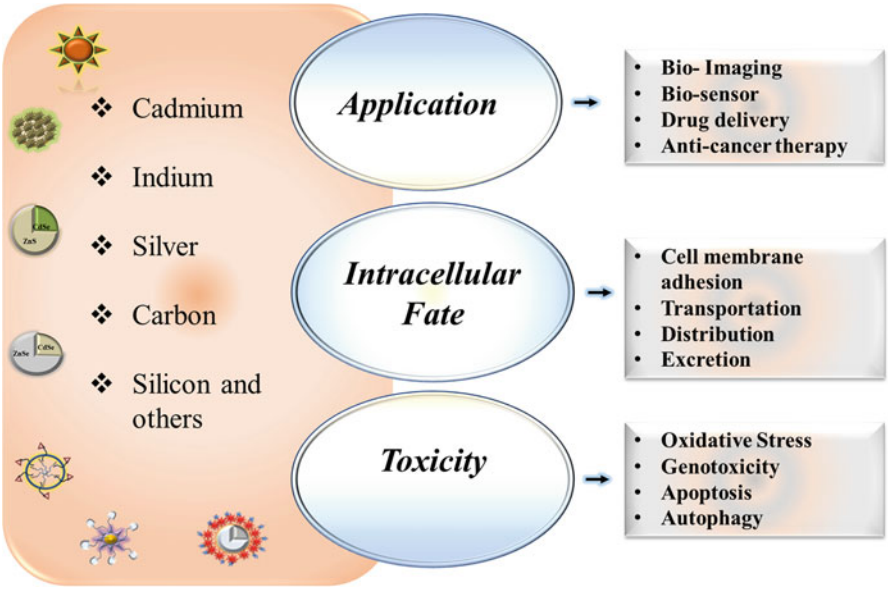

Fig. 8.6 Overview of the biological interaction of QDs

8.5.1.1 Cellular Uptake of QDs

The factors that determine the cellular uptake of QDs depend on the cell type, morphology, size, and surface coating (independent of the core) of QDs. Therefore, for the given QDs, there may be one size and concentration parameter of the highest cell internalization efficiency.

Direct penetration of QDs via the cell membrane is possible Fig. 8.7 [36, 37]; but mostly QDs are internalized by endocytosis. Endocytosis includes phagocytosis by specialized cells, such as neutrophils, monocytes, and macrophages; Pinocytosis involves the uptake of fluids containing solutes and particles by vesicles of smaller size than those generated during phagocytosis. This endocytic mechanism can be classified in macropinocytosis and receptor-mediated endocytosis. Figure 8.8 elucidates the various pathways for internalization of QDs.

Following steps for endocytosis are observed: initially, QDs bind to the plasma membrane receptors, which causes formation of coated pits, but mostly QDs are internalized by endocytosis. Macropinocytosis is observed in almost all cell types. It generates large macropinosomes that contain the extracellular fluid and soluble protein, which eventually fuse with lysosomes or recycle its content to the surface; clathrin/caveolin further makes intracellular buds to create endosomes or endocytic vesicles. Clathrin-dependent endocytosis comprises the assembly of clathrin and adaptor proteins on a region of the plasma membrane where specific receptors are clustered to form a budding vesicle ordained for internalization. Caveolin-dependent endocytosis includes the assembly of caveolin coats on portions of plasma membrane that are rich in lipid rafts to form the budding vesicle to be internalized. Later, the QDs are transported to the subcellular targets like the late endosomes/lysosomes or eliminated from the cytosol. Both macropinocytosis and phagocytosis are dependent on remodeling of the actin cytoskeleton and clathrin-independent processes [38].

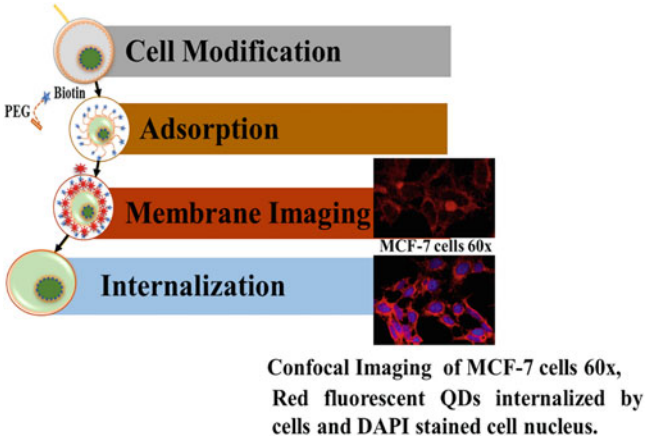

Fig. 8.7 Cellular uptake of QDs

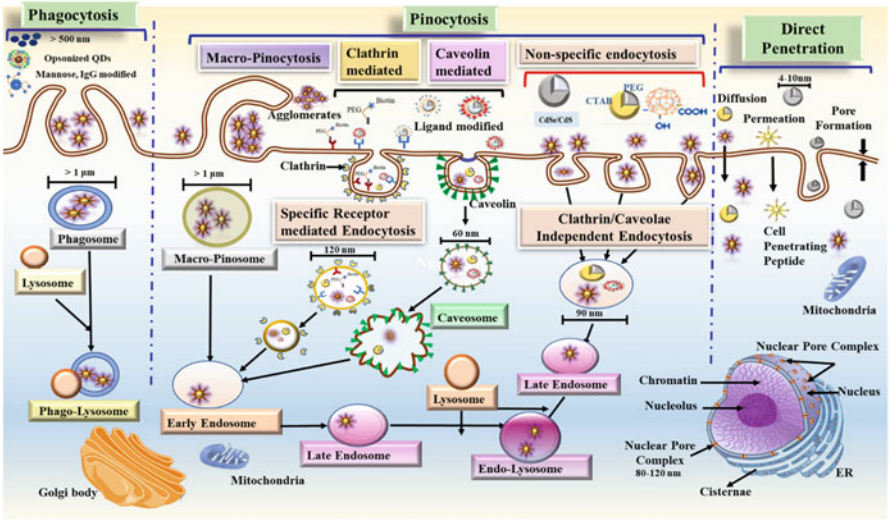

Fig. 8.8 Intracellular trafficking of QDs by distinct pathways by cells

Hence, for any given QDs, there may be a uniform parameter of both size and concentration that indicates the maximum internalization efficiency in a particular cell type. A widely used method for studying the uptake mechanism is to observe the changes of cell uptake efficiency by pretreating the cells with specific inhibitors. Since QDs emit fluorescence, they can be directly tracked in biological systems when compared to other nanomaterials. Therefore, it is feasible to observe the mechanism of cellular internalization of QDs using various endocytic inhibitors (Table 8.2) and cell transfection techniques, and quantitative and qualitative analysis of sub-localization of QDs can also be assessed.

8.5.1.2 Dynamic Process of Uptake and Elimination of QDs

Biological systems are dynamic, and the mechanism of QDs uptake and removal is continuous. Therefore, different QDs will behave differently. Based on the material of the core, QDs can be roughly categorized as cadmium, indium, silver, silicon, and carbon QDs and have been briefly discussed in terms of internalization and toxicity observed.

8.5.1.2.1 Cadmium-Based QDs

The amount of QDs internalized by cells largely depends on the surface modifications and surface charge [43]. Reports suggested that D-penicillamine-coated CdSe/ZnS QDs adhere to the plasma membrane of cells [44], and only when the

Table 8.2 A list of inhibitors that are generally used by researchers

Endocytosis	Description	QDs	Localization	Inhibitor	Ref.
Phagocytosis	Plasma membrane forms a pseudopod to entrap the particles into the cells.	Graphene QDs (GQDs)	Cytoplasm, mitochondria, lysosome and ER, nucleus	Colchicine Nocodazole Cytochalasin D Cytochalasin B	[39]
Macropinocytosis	Macropinosomes that are irregular endocytic vesicles are formed by the folds of the cell membrane. Large vesicle with a diameter of 0.5 μm–2 μm sometimes 5 μm with caveolin coating.	$Eu^{3+/}Mn^{2+}$-co-doped ZnSe@ZnS core/shell QDs	Cytoplasm Endosome	Ly294002 (LY); Wortmannin (WMN); 5-(N,N-dimethyl)-amiloride (DMA)	[40]
Clathrin-mediated endocytosis	Involvement of the membrane-associated protein clathrin in forming membrane vesicles that become internalized into the cell.	Streptavidin-conjugated QDs	Early to late endosomes (lysosomes)	Chlorpromazine Nocodazole Cytochalasin D Bafilomycin A1 Nystatin	[41]
Caveolin-mediated endocytosis	Bulb-shaped, 50–60 nm plasma membrane invaginations called caveolae (or "little caves"). Caveolae formation is driven by integral membrane proteins called caveolins as well as peripheral membrane proteins called cavins.	QDs-labeled GCRV particles	Cytoplasm Caveolin-1	Methyl-β-cyclodextrin Nystatin Genistein	[42]

concentration of QDs exceed the threshold, the process of internalization is triggered in human cervical cancer cells-HeLa cells. For example, the negatively charged (–COOH) CdSe [36, 45] and CdTe QDs [46, 47] were greater than the internalization of PEG-modified QDs that were neutral in charge as well as the positively charged (–NH_2) ones. Some surface modifications weaken the internalization process such as N-acetylL-cysteine (NAC) [47] and Gum arabic (GA)/TOPO [48]. But, reports also reveal that the amount of internalized QDs was equal [49] in all QDs irrespective of surface charge such as positively charged (polydially-dimethyl ammonium chloride (PDDA)), negatively charged carboxylic acid (CA), and the neutrally charged PEG QDs.

Different cells have varied internalization mechanisms of QDs. Macrophages can internalize by phagocytosis and significantly higher amount of CdSe/ZnS QDs than epithelial cells [50]. But higher internalization of CdSe/ZnS QDs was observed by epithelial cells when compared to lymphoblastoid (TK6) and fibroblasts (HFF-1) cells [51]. Additionally, more uptake of CdTe/CdS lipids QDs was observed by HepG2 cells when compared to normal hepatocytes HL-7702 [42]. Generally, CdTe and CdSe QDs have been reported to be internalized by macropinocytosis [52], caveolae/lipid raft-dependent pathway [53], and clathrin-mediated endocytosis [47, 54, 55]. Likewise, human embryonic kidney (HEK) cells were observed to uptake of CdSe/ZnS-COOH without caveolin, clathrin, macropinocytosis, melanosome-transfer, and F-actin pathway but by lipid rafts that could be associated with G-protein-coupled receptor (GPCR) and low-density lipoprotein receptor/scavenger receptor (LDLR/SR). Also, the distribution and uptake of QDs by cells are time-dependent and dynamic processes [56]. Based on reports, cysteine (Cys)-CdTe QDs primarily remain adhered to the cell membrane in the first 5 min, and most of the QDs were found in cytoplasm within the next 40 min [57].

Similarly, after 6 h, the crystalline core (Cd/Se/Te QDs) with shell of ZnS (QD705) was sited at the lysosome, endosome, and endoplasmic reticulum (ER) of mouse renal adenocarcinoma and transported to mitochondria at 24 h [58]. Usually, CdSe and CdTe QDs were localized in cytoplasm and organelles, especially in the perinuclear region [52], rather than the nucleus [56–60]. More specifically, when compared to ER, mitochondrion, [50] and Golgi apparatus, QDs were localized in the lysosomes [54, 61]. Further, the subcellular localization of CdTe QDs was based on their size, the red QDs (~5 nm) were dispersed throughout the cytosol of microglia (N9 cells), and the 2 nm, green QDs were predominantly localized in the nuclear compartment [62].

Remarkably, irrespective of the different surface coatings (–COOH, –PEG, and –NH_2) affected the efficiency of uptake of CdSe/CdTe/ZnS QDs by J774.A1 macrophage cell line and did not govern the localization [63]. The QDs only partially entered the cells and were eliminated via exocytosis, and maximum were retained within the cells [54]. It is important to mention that exocytosis of QDs from cell was energy dependent and was again dependent on the size, shape, surface charge, dose [64], coating group, [65], and cell type [66].

8.5.1.2.2 Indium-Based QDs

The cellular uptake of InP/ZnS was observed to be dependent on type of cell, its surface modification, and its concentration (optimal range ~20–100 nM) [67]. Enhanced uptake of $–NH_2$- and –COOH-coated InP/ZnS QDs was observed in human lung cancer cells (HCC-15) after being incubated for 4 h with alveolar type II epithelial cells (RLE-6TN), when compared to InP/ZnS-OH at a concentration of 2 μg/mL. Further, HCC-15 cells had significantly improved uptake of QDs than RLE-6TN cells [68]. Also, InP/ZnS QDs were predominantly localized in the cytosol and the perinuclear region in human neuroblastoma cells SH-SY5Y, lung adenocarcinoma A549, RLE-6TN, and HCC-15 cells [69]. There is insufficient data on the mechanism of cellular uptake of In-based QDs.

8.5.1.2.3 Silver-Based QDs

Cellular uptake of Ag-based QDs has been sparsely reported. The cellular uptake of Ag_2S QDs was dependent on various cell types. The highest internalization of QDs was observed in A549 and BEAS-2B cells, while the least uptake of QDs was observed in HeLa cells. Moreover, with increase in the concentration of dose and incubation time, enhanced internalization of Ag2Se QDs was observed in BV2 cells [70].

8.5.1.2.4 Silicon-Based QDs

Exposure to SiQDs at suboptimal concentration indicated substantial time-dependent intracellular accumulation in human embryonic hepatocyte HepG2, mouse embryo fibroblast 3T3-L1 [71], HUVECs [72], and HeLa cells [66]. But then again, the amount of internalized SiQDs is progressively stabilized leading to a gradual decrease in the concentration of the intracellular QDs, although maximum SiQDs were localized within the cells [68]. Prior report specified that HeLa cells internalized the alkyl-SiQDs via a cholesterol-dependent endocytosis or a caveolin- and lipid rafts-mediated endocytosis. The intracellular accumulation of alkyl-capped SiQDs was significantly higher in HuH7 and HepG2 cells and considerably reduced in HeLa cells and human colorectal adenocarcinoma CACO-2. Also, SiQDs get internalized in HeLa cells through endocytosis and eliminated via exocytosis, localizing the QDs in the lysosomes and getting trapped in the exocytic vesicles [73].

8.5.1.2.5 Carbon-Based QDs

C-based QDs preferentially locate in the nucleus, unlike all the Cd- and In-based QDs discussed so far that are normally localized in the cytosol. Aminated CQDs were mainly internalized by rat alveolar macrophages (NR8383), initially by

phagocytosis, and then by caveolin-mediated endocytosis [43]. Besides, size-dependent variation in the mechanism of internalization was observed in dendritic cells (DCs), wherein small graphene GQDs (24 nm) exhibit increased accumulation and larger sized GQDs (66 nm) show reduced internalization; the larger GQDs were internalized by the dynamin-dependent process, whereas the smaller GQDs via dynamin-independent but cholesterol-dependent pathways [74]. The cellular internalization of GQDs is a dynamic process and changes over time. When NR8383 cells were incubated with GQDs for ~12 h, the GQDs were dispersed in the cytoplasm, ER, mitochondria, and endo-lysosomes; but when the incubation was for 24 h, the GQDs were localized in the nucleus much more than the cytosol of the NR8383 cells. Post 48 h of exposure, the intracellular GQDs was drastically reduced by ~35%. Incidentally, ample amount of GQDs persisted in the nucleus and cytosol. Also, expression of the two main genes of nuclear pore complex (NPC): Nucleoporin 98 (Nup98) and karyopherin β2 (Kapβ2) have an important role in the nuclear uptake of GQDs. Surprisingly, the intracellular amount of GQDs did not vary with time, especially between 24 and 48 h, signifying that primarily GQDs internalization occurred earlier, i.e., before 24 h.

8.6 Elucidation of Potential Toxicity

QD toxicity is a complicated issue as it relies on its intrinsic properties including size, charge, surface chemistry, and chemical composition of QDs. Group III and IV quantum dots show reduced toxic effects; hence, they are widely used as optical probes and biosensors. Toxicity severely limits the potential for clinical translation of II–VI semiconductor QDs, such as CdSe and CdTe QDs as they easily disintegrate in the biological systems if their surfaces are not coated carefully with protective shells, biocompatible polymers, and biomolecules that are inert.

An ideal solubilization strategy should reduce the undesirable nonspecific uptake of QDs by living tissues and scavenging by the reticuloendothelial system. Literature suggests that few QDs have shown direct cytotoxicity, particularly after oxidative and/or photolytic deterioration of their core coatings. QDs are efficient energy donors and can easily transfer energy to nearby oxygen molecules that induce generation of reactive oxygen species (ROS), which in turn causes cell damage and eventually leads to cell death. Figure 8.9 gives a schematic view of the recent in vitro and in vivo experimental design that can be undertaken to assess the potential toxicity of QDs based on their characteristics in the biological environment.

8.6.1 Genotoxicity

Genotoxicity is the damage to DNA – the genetic material, often caused by interaction of innumerable agents that alter the cellular genetic information leading to

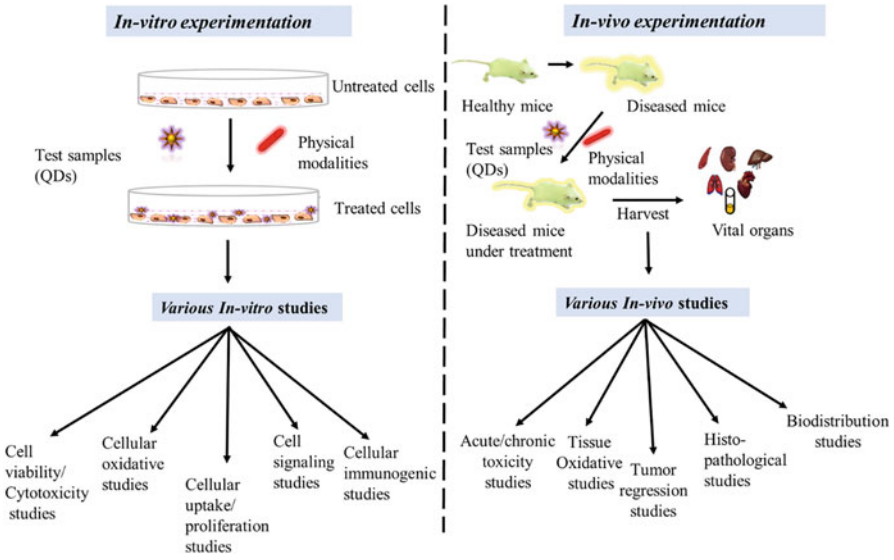

Fig. 8.9 Schematic illustration of experimental design for in vitro and in vivo studies

mutations. The extent, type, and persistence of damage compromise the DNA integrity by various mechanisms. Absence of an inflammatory response induces primary genotoxicity, while activation of the proinflammatory cells such as macrophages and neutrophils mediates the secondary genotoxicity, which further generates significant quantities of reactive species [75]. Moreover, physical perturbations caused by interaction of particulate material with DNA can cause direct genotoxicity, and indirect genotoxicity may be a consequence of increased generation of ROS after interaction with cellular organelles such as cell membrane, mitochondria, etc., resulting in elimination of intracellular antioxidants [76]. In this way, ROS, generated by the cellular pathways, may accrue and induce DNA damage. This alteration could have a direct or an indirect effect on the DNA: the event activation that is mistimed, induction of mutations, and direct DNA damage that may also cause mutations. Known DNA mutations include mutations of the key loci in the genetic code that are characteristic molecular hallmarks of cancer. Proto-oncogenes can stimulate the cellular proliferation and growth (e.g., K-ras), and inhibition of proliferation involves the tumor suppressor genes that may also do DNA repair (e.g., p53). Apart from causing carcinogenesis, DNA mutations are responsible for causing numerous pathological conditions that can modify susceptibility to the disease. Mutations can involve relatively small sequences, involving single genes, or can occur on a larger scale. Point mutations are small-scale mutations such as transversion or transition, wherein a nucleotide is substituted by another; deletions that remove the nucleotides from DNA; and insertions, where nucleotides are added to the genetic code. The chromosomal structure may get affected by mutations that may cause genotoxicity. Clastogenic chemicals induce

chromosomal aberrations that can either be numerical (aneugenic) or structural (clastogenic). Micronucleus (MN) assay is a reliable and sensitive tool to estimate the chromosomal damage caused by DNA breakage (clastogenic) or by abnormal segregation (aneugenic) methods. The alkaline comet assay helps in identification of single and double DNA strand breaks (DSBs) [77, 78]. DSBs damage the integrity of chromosomes and subsequently affect cell viability. Mis-repaired or unrepaired DSBs induce chromosome rearrangements inducing mutations leading to cell death and cancer [78–82]. Therefore, evaluation of genotoxicity is marked as an important tool to study the potential carcinogenic risk, i.e., damages to genetic material caused by exposure to QDs. Banerjee et al. observed remarkable dose-dependent genotoxic effects by CdSe QDs along with oxidative stress in *Allium cepa* plant using the DNA comet assay [83]. Another study showed that genotoxicity induced by QDs was based on the intrinsic surface chemistry and charge. Manshian et al. assessed various surface coatings of QDs such as neutral (hexadecyl amine; negative (carboxyl), HDA) or positive (amine) polymer and evaluated their effects on human lymphoblastoid TK6 cells. Genotoxicity was analyzed using the micronucleus assay (gross chromosomal damage) and the hypoxanthine phosphoribosyltransferase (HPRT) forward mutation assay (point mutagenicity). This study concluded that HAD-QDs induced the greatest genotoxicity and cytotoxicity in comparison to carboxyl and amine-coated QDs. The possibility of this genotoxicity was caused by release of free cadmium ion [84]. Moreover, PCR (polymerase chain reaction) evaluation is used to show the upregulation of DNA damage, the responsive gene, as well as proinflammatory cytokine genes. In addition, transcriptome sequencing demonstrated the upregulation of metallothionein family of genes in cells treated with mercaptopropionic acid (MPA)-coated CdSe (MPA-CdSe) QDs in comparison to cysteamine (Cyst) CdSe (Cyst-CdSe) QDs [85]. Similarly, preparation of MPA-CdSe QDs (water soluble) was evaluated in *Escherichia coli* DH5α (gram-negative) and *Staphylococcus aureus* (gram-positive bacteria) based on agar disc, and cytotoxicity was determined by measuring ROS level in QD-treated cells. *S. aureus* was highly sensitive when compared to *E. coli*; size- and dose-dependent elevation of generation of reactive oxygen species (ROS) as well as enhanced zone of inhibition was observed in agar-disc diffusion assay. The oxidative stress analysis (glutathione (GSH), lactate dehydrogenase (LDH)) and ROS-induced DNA damage caused genotoxicity in *S. aureus* [86]. *Drosophila melanogaster* is also frequently used as a genotoxic model organism, wherein the comet assay was used to demonstrate the genotoxicity of Cd QDs and $CdCl_2$ QDs. Cd QDs exhibited both dose- and time-dependent genotoxicities in larvae by penetrating the intestinal barrier, passing to the hemolymph and interacting with the hemocytes [87].

8.6.2 Cytotoxicity

Despite numerous preclinical studies on QDs, the key question that remains unresolved is their potential cytotoxicity. It has been reported that core of QDs (Se/Cd) exhibited inherent toxicity to cell cultures and to live animals. Recently, Derfus et al. evaluated the cytotoxicity of QDs based on certain conditions such as synthesis, UV exposure, and surface functionalization as observed in primary rat hepatocytes cells and in in vivo model. This study concluded that coated Cd/Se QDs exhibited low cytotoxicity as compared to uncoated QDs, which release free cadmium ions in cells [88]. In addition, cytotoxic analysis of InP/ZnS QDs modified with three different groups (–NH_2, –COOH, –OH) was studied in two lung-derived cell lines. The results suggested that InP/ZnS-COOH QDs and InP/ZnS-NH_2 QDs exerted more cytotoxicity than InP/ZnS-OH QDs based on the concentration and surface functionality. All these QDs promoted cell apoptosis and intracellular ROS generation [67]. Similarly, it has been reported that MPA- and cysteamine-coated (Cyst) CdTe QDs were cytotoxic at 10 μg/mL, when exposed to rat pheochromocytoma (PC12) cells, while uncoated showed cytotoxicity at 1 μg/mL. This cytotoxicity was determined by chromatin condensation and membrane blebbing, the characteristic traits of apoptosis. Although positively small-sized QDs impart higher toxicity than neutral-charged large-sized QDs, because of their subcellular biodistribution, smaller QDs localized to the nuclear compartment while larger ones in the cytosol compartment. Therefore, cell death may be considered due to the release of free Cd, which leads to ROS production and ultimately causes loss of function [62]. Dussert et al. reported the cytotoxicity of the single-shelled InZnP/Zn(Se,S) core/gradient shell as well as the double-shelled InZnP/Zn(Se,S)/ZnS core/shell/shell QDs, using both the pristine form and the aging form that give a real-time conservational weathering of QDs. This concluded that aged QDs evinced significant cytotoxic and genotoxic activity, thereby regulating gene expression that is responsible for zinc homeostasis, cell redox response, and inflammation in human primary keratinocytes, whereas pristine QDs showed insignificant toxicity. Furthermore, this study focuses on the end product of InP-based QDs, which is pernicious to skin cells as observed by accidental exposure that caused oxidative stress, inflammation, and imbalance of cell metal homeostasis, specifically Zn homeostasis [89, 90]. In addition, Bhanoth et al. synthesized different combinations of core-shell QDs using ZnSe, CdS, and CdSe (CdS/CdSe, ZnSe/CdS, and ZnSe/CdSe) were analyzed in subjected to cytotoxicity and therapeutic efficacy. From these different combinations, the ZnSe/CdS QDs possessed excellent optical properties; therefore, this formulation was further used for in vitro and in vivo study for the assessment of cytotoxicity and therapeutic efficacy. Hence, ZnSe/CdS QDs displayed 65% cytotoxicity against MCF-7 cells (breast cancer) and also imparted negligible hemolysis. ZnSe/CdS QD-treated mice model represented that 34% tumor regression in comparison to mitomycin C (positive control) observed 93% with respect to PBS [91].

8.6.3 Photo-Induced Toxicity

QDs stability/toxicity is the most important aspect to be considered during synthesis, storage and in biological studies. It has been reported that maximum cytotoxicity is induced by photolysis and oxidation. Under the influence of photolytic and oxidative environments, QD core-shell coatings are known to be labile and prone to degradation, thereby exposing the inherently toxic "capping" matter or the entire core metalloid compound or releasing the core metals ions (e.g., Cd, Se, Zn). Pathakoti et al. studied photo-induced toxicity of CdSe/ZnS QDs with multiple surface coating at three different wavelengths (530, 580, and 620 nm) to *E. coli* under solar irradiation. This study concluded PEI (polyethylene imine)-coated QDs exhibited maximum phototoxicity to *E. coli*, QSA (polyethylene glycol)-coated QDs and QSH QDs with amphiphilic polymer coating had negligible phototoxicity. The phototoxic activity triggered due to the oxidative stress via production of hydroxyl radicals caused lipid peroxidation and consequently a drop in the reduced glutathione level. Therefore, PEI-coated CdSe/ZnS QD was highly toxic, while non-cadmium-based QDs have remarkable biocompatibility [92].

Similarly, the effect of photo-induced ROS evaluated in differently functionalized ZnO QDs demonstrated that the highest ROS generator QDs (ZnO-GLYMO) were most deleterious toward DNA. This study showed photo-induced damages caused by various factors, not only based on ROS generation but also biomolecule interactions [93]. In another study, primary rat hepatocytes treated with 62.5 μg/mL MAA–CdSe QDs promoted cell toxicity, accounted to photodegradation and oxidation of the QD coating. Likewise, under UV light exposure, MAA–TOPO-capped CdSe QDs undergo dose-dependent cytotoxicity, and prolonged exposure enhanced toxicity by almost 91%. It was deduced that long-lasting exposure of QDs to oxidative and photolytic conditions endows degradation of MAA–TOPO-capped CdSe QDs nanocrystals [94].

8.7 Molecular Mechanisms Induced by QDs

Currently, the underlying mechanisms of toxicity caused by QDs remain unpredictable. Hyperactivation and increased oxidative stress, sudden elevation of intracellular Ca^{2+} levels, QDs, and Cd^{2+} ions released from Cd-carrying QDs were reported to be main source of toxicity (Fig. 8.10 and Table 8.3).

8.7.1 Reactive Oxygen Species and Oxidative Stress

Enhanced ROS production is considered a key mechanism for nanomaterial toxicity. The ROS molecules interact with macromolecules such as proteins, DNA, lipids,

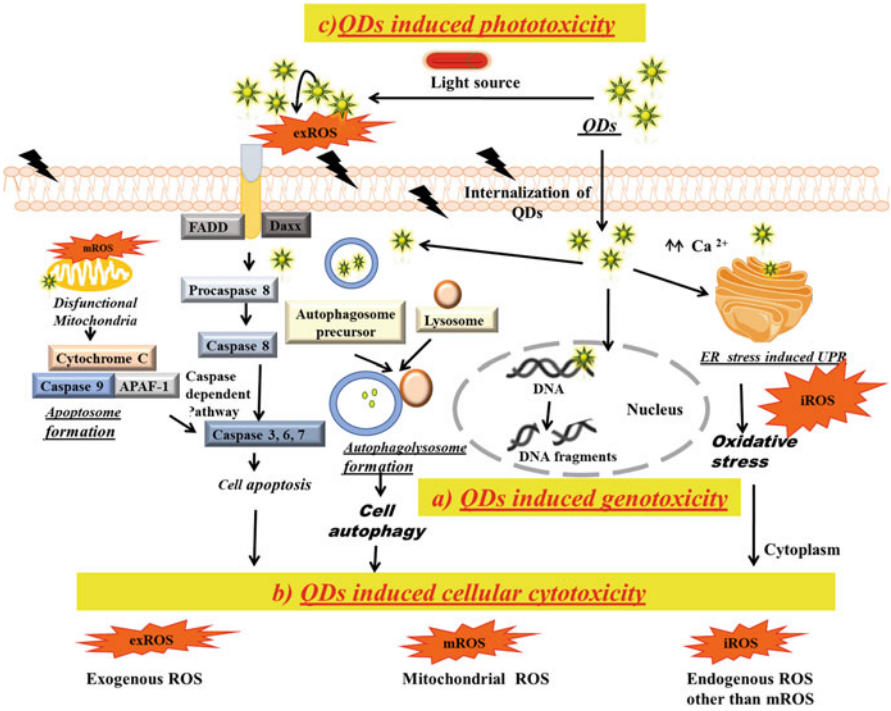

Fig. 8.10 QD-induced toxicity mechanisms. (**a**) QD-induced genotoxicity. (**b**) QD-induced cellular cytotoxicity. (**c**) QD-induced phototoxicity

etc., affecting their structural and functional integrity causing severe oxidative damage to the cells. Tang et al. demonstrated that CdTe QDs and $CdCl_2$ administered in zebrafish liver cells significantly increased ROS levels that cleave the DNA strand, upregulating the expression of antioxidant genes and inhibited DNA repair [95]. Similarly, CdTe QDs were observed to activate the apoptotic pathway too. QDs damage the mitochondria causing mitochondrial swelling, loss of cristae, thus facilitating the loss of mitochondrial membrane potential (MMP) and mitochondrial permeability transition (MPT). This ultimately results in the release of cytochrome c into the cytosol thereby, stimulating the downstream cascade resulting in apoptosis. Further, its pernicious effect shown on mitochondrial electron transfer chain (ETC) results in decreased activity of ETC complex (I, II, and IV). Lai et al. demonstrated that reduced GSH-Cd-QDs bind with the pore-forming proteins of mitochondrial membrane in human embryonic kidney cells (HEK-293), which hyperactivates the mobility of transmembrane protein. This enhances the MPT formation, which eventually elevates the intracellular ROS levels [96].

Table 8.3 Tabular representation of mechanisms of action of QDs

QDs	Functionalization	Experimental model	Exposure time	Assay	Cell fate (toxicity)	Mechanisms	Ref.
ZnSe/CdSe QDs	PVP & oleic acid	Cancer cell line (EAC, MCF-7) and HEK-293	24–48 h	MTT	Dose- and time-dependent cytotoxicity	Release of free Cd^{2+} & Se^{2+} ions	[97]
CdSe QDs	MAA	In vivo study in male Balb/c mice	2–7 days	DNA fragmentation and micronucleus assay	Genotoxicity (DNA damage, formation of micronuclei, and DNA adduct)	Induction of oxidative stress	[98]
ZnSe/CdSe	Oleic acid	Human hepatocellular carcinoma (Hep3B and HepG2)	24–48 h	MTT, DNA fragmentation, ROS assay, and intracellular Ca^{2+} level	Apoptosis induced cytotoxicity and also exhibited genotoxicity	Cytotoxicity via ROS generation by ER stress and mitochondrial disruption. Elevated level of Ca^{2+} level and genotoxicity induced by DNA fragmentation.	[99]
CdTe QDs	Glutathione	Bacterial strain (*E. coli* BW25113)	24 days	Growth inhibition assay and intracellular ROS-level assay	Photo-induced toxicity	Photon induced the transfer electrons and generated ROS level and caused oxidative stress inside cells.	[100]
In-based water-soluble QDs	–	Wistar rat model	90 days	Histological assessments, hematological and biochemical markers	Less toxic	Accumulation of QDs was observed maximum in liver, and spleen-indicated indium QDs are degraded by liver.	[101]
CdTe QDs	–	Human hepatic carcinoma (HuH-7 cells)	48 h	MTS and LDH assay, comet assay, oxidative stress	Dose and time exposure cytotoxicity and genotoxicity	Induction of apoptosis facilitated via ROS generation, GSH reduction, and oxidative stress.	[102]
CdSe/ZnSe QDs	Streptavidin	pDNA	1 h	Agarose gel electrophoresis AFM imaging and DNA damage assays	Photo-induced toxicity	DNA strand breakages and nucleobase damages in pDNA are correlated with photo-induced production of ROI.	[103]

Gradient-alloyed QDs	MPA and PEG	Cervical cancer cell (HeLa-cells)	24 h	MTT, oxidative stress, lysosomal and autophagy markers	Autophagy-mediated cytotoxicity	MPA-QDs induced autophagy by decreasing ROS generation while PEG-QDs induced elevate ROS generation and autophagy blockage via lysosomal impairment.	[104]
$CuInS_2$/ZnS QDs	PEGylated	In vivo model of Balb/c mice	1–90 days	Histology, ICP-MS, serum analyses	Low cytotoxicity	PEG modification can be $CuInS_2$ QDs in order to achieve low toxicity.	[105]
CdS/ZnS	mAbs (trastuzumab)	Human breast cancer cell line (SK-BR-3 and BT-474)	24–72 h	MTT assay	Exhibited low cytotoxicity and high biocompatibility	Provide targeted delivery to HER2 overexpressing cancer cells.	[106]

DNA, deoxyribonucleic acid; *EAC*, Ehrlich-Lettre ascites carcinoma; *ER*, endoplasmic reticulum; *ICP-MS* inductively coupled plasma mass spectrometry; *LDH*, lactate dehydrogenase; *MAA*, mercaptoacetic acid; *MPA*, mercaptopropionic acid; *MTT*, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; *PEG*, polyethylene glycol; *PVP*, polyvinylpyrrolidone; *ROS*, reactive oxygen species.

8.7.2 Release of Cadmium from Cadmium-Containing Quantum Dots

Release of Cd^{2+} ions from QDs is one of the proposed mechanisms of toxicity induced by QDs. Once QDs are internalized in the cell, they are degraded by the acidic environment of lysosomes, the Cd^{2+} ions so released can attach to the thiol groups of the intracellular proteins that can disrupt their structure and function [107]. Wang et al. analyzed the histopathological changes that occurred in mice liver and kidney based upon time-dependent toxicity of CdTe QDs. The toxicity was possibly induced by the elevated levels of Cd^{2+} ions and OH^{-} ions, and they used metallothionein (MT) as a biomarker to measure the increased Cd^{2+} ions in the tissues. This study concluded that elevated MT levels can interact with Cd in the cytosol and reduce its bioavailability to vital organelles [108]. In addition to this, QDs also have tendency to destroy the structure and function of macromolecular substances. One such study performed by Sun et al. found that QDs can bind to Cu/Zn superoxide dismutase (SOD) under the influence of hydrophobic forces, which causes alteration in the tyrosine kinase and secondary structure of the protein. The activity of SOD is inhibited causing oxidative damage to the cell [108, 109].

8.7.3 Elevated Intracellular Ca^{2+} Levels

Apart from the above-mentioned mechanisms, dysregulation of cellular calcium homeostasis also plays a pivotal role in QDs toxicity. Ca^{2+} are the most significant second messenger molecules in eukaryotic cells which are responsible for the regulation of cellular function by activating different protein kinases involved in biological processes such as ROS generation, cellular proliferation, differentiation, and apoptosis [109]. It was reported that CdSe QDs triggered elevation of cytoplasmic calcium levels in primary cultured hippocampal neurons, which impaired the function of voltage-gated sodium ion channels that ultimately disrupted the electrical activity and augmented neuro-toxicological damage [110]. Richter et al. showed that increase in intracellular calcium levels impaired mitochondrial membrane permeability (MMP), caused the release of free cytochrome c, and initiated apoptosis [109]. Moreover, it has been studied that CdTe QDs induced a 7.4-fold elevation in Ca^{2+} levels that was facilitated by ROS generation. Meantime, high concentration of calcium further boosts ROS production and depletes oxidative stress [111]. Analogously, CdSe/ZnS QDs led to increased calcium levels in L02 cells, which mediated mitochondrial ROS generation and triggered formation of NLRP3 inflammasomes [112].

8.8 Absorption, Distribution, Metabolism, and Excretion of QDs In Vivo

Pharmacokinetics helps to assess the disposition of the drug within an organism by the four phases it traverses: absorption, distribution, metabolism, and excretion (ADME). Literature suggests that QDs can be systemically circulated and may accumulate in the tissues and organs. The ADME of QDs is highly variable owing to the extensive variation in their physicochemical properties. The stability, size, surface charge, concentration, and the shell/outer coating and its bioactivity, each contributes to the possible toxicity of the QDs and also to their ADME characteristics. Physicochemical properties in conjunction with microenvironmental factors and QD stability (oxidative and photolytic lability) together make a paradigm shift in the way ADME characteristics of QDs behave as it can be highly variable and extremely difficult to predict.

Elimination of QDs from the system largely depends on the excretory mechanisms and the metabolic processes involved, along with the in vivo bioactivity, all of which are poorly understood and need to be evaluated. In vivo data suggests that, irrespective of the specificity of QDs, they are recognized as foreign by the vertebrate system and are eliminated via the primary excretory organs/systems: mainly liver, lymphatic systems, and spleen. However, this is a gross generalization, and plenty of inconsistencies exist in the published literature, such as subcutaneous injection of CdSe/ZnS-PEG-coated QDs in mice showed clearance of the QDs from the site of injection, with accumulation of QDs in lymph nodes [113]. Distribution kinetics of QDs can be significantly affected by size alone, and the surface coating can further influence the serum half-life and the pattern of accumulation. Since studies are limited, the tissue/organ distribution of QDs appears to be multifactorial, depending on size, core-shell components, and the bioactivity of conjugated or other incorporated functional groups. Nonspecific QDs, especially without specific functionalized groups, are internalized via the endocytic mechanisms by various types of cells, both in vivo and in vitro. Paradoxically, QDs with natural ligands that are specific for certain cell surface receptors and membrane proteins are specific only for that given cell membrane proteins or type of receptor. Nonspecific QDs tend to adhere to cell surfaces, probably via interactions of QD with glycolipids and glycoproteins present in cell membranes. The exact mechanism of toxicity needs elucidation, although innumerable reports indicate the intracellular vesicular trafficking and accumulation of QDs. Variable subcellular localization and systemic distribution exist based on the unique physicochemical properties' dependent on type of QDs. This will definitely prove extremely relevant for developing screening protocols for evaluating QD toxicity based on their characterization, including the size nonuniformity, biofunctional coatings, core-shell conjugates, and surface coating oxidative and photolytic stability [76, 114–116].

8.9 Conclusion and Future Perspectives

Over the last 20 years, emergence of nanotoxicology has attracted attention to address the main issues and to intricately understand the mechanisms of nanomaterials toxicity. The prevalence of QD-related products is increasing rapidly in the market; these products also require further evaluation of toxicity under toxicity standards with safer range for sustainable application. It is essential to thoroughly characterize the QDs with respect to size, activity, and cross-reactivity with various biological biomolecules. The physiochemical properties of QDs such as shape, size, composition, and surface coating after solubilized in suitable biological buffer must be well understood during biocompatibility evaluation. In addition, there is first step to select appropriate test model system, period of exposure, and relevant assay that are also important factors in order to study the engineered QDs. Therefore, all the toxicity protocols should be validated by alternative methods to assess the accuracy of the experimental outcomes.

The major finding reported in literature described in this chapter highlights concerns toward the genotoxicity, cytotoxicity, and photo-induced toxicity, but this is not limited to QDs size, charge, morphology, and dissolution. There is a need to study the intrusion between QDs and experimental components that may affect the actual toxicity. The key points to summarize are that all QDs are not alike and engineered QDs cannot be considered as a uniform group of nanomaterials. The ADME and toxicity of QDs depend on multiple factors resulting from both the inherent physicochemical properties and the microenvironment. The size, surface charge, concentration, surface coating bioactivity (capping material and functional groups), and mechanical, oxidative, and photolytic stability have been implicated as the critical factors in QD toxicity. Therefore, it is likely that QDs may be grouped or classified as per their possible toxicities depending on the size or other physicochemical properties. We conclude that under certain conditions, QDs may pose hazardous to human health as observed in rodent animal models as well as in in vitro cell cultures.

A few techniques based on predictive computational models, mechanism-centered high-throughput testing, genome arrays, and high-throughput screening are being extensively explored but have not been established in toxicity assay approval. Therefore, in order to prevent and challenge the hazard and risks associated with experiments and associated activities, the regulatory and safety precautions must be implemented. The researchers should harness the full potential of nanotechnology by bridging the gap of nanomedicine, medical science, biomedical engineering, and toxicology for betterment of human beings.

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