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Applications of Fluorescent Quantum Dots for Reproductive Medicine and Disease Detection

Sapna Jain, Seong B. Park, Shreekmar R. Pillai,
Peter L. Ryan, Scott T. Willard and Jean M. Feugang

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Abstract

Understanding the mechanisms associated with fertility and disease management in animals remains challenging. Continuing advances in nanotechnology provide new tools and alternative approaches for the investigation of these mechanisms. Fluorescent quantum dot nanoparticles, for example, have unique physicochemical properties, which allow for *in vivo* and *in vitro* imaging in various areas of life sciences. Traditional quantum dots contain heavy metal semiconductor cores, which have raised concern over their potential for toxicity. The majority of available quantum dots today prevent heavy metal release with additional chemical and polymer layers for safe water solubility. In this chapter, the most widely used quantum dots made of cadmium selenide, which possess great potential for real-time imaging in disease detection and reproductive medicine, are discussed.

Keywords: quantum dots, spermatozoa, *in vivo* imaging, real-time imaging, luminescence, fertility

1. Introduction

Since their discovery by Alexie Ekimov and Louis Brus in the 1980s, quantum dot (QD) nanoparticles have been categorized as a novel class of fluorescent particles [1]. Fluorescent nanoparticles exhibit distinct energy levels and size-dependent fluorescent emission [2]. The QD sizes range from 2 to 10 nm (10–50 atoms) in diameter, with the smaller size corresponding to the larger bandgap [3]. Each QD absorbs white light and then reemits a specific color associated with the material's bandgap, from blue to red or near-infrared (NIR) as the QD crystals increase in size [4]. The variety of fluorescence emission is very useful for both *in vitro*

and *in vivo* multiplex bioimaging as multiple QDs can be used in one subject or field of view to image a variety of targets under a single excitation.

QDs have unique advantages over traditional dyes and fluorescent proteins such as a high quantum yield, extreme brightness, tunable emission wavelength, long fluorescence duration, exceptional photostability and resistance to photobleaching [5]. In addition, their high extinction coefficient makes them ideal for optical applications and transport. Since QDs wavelengths are tunable based on size, their conducting properties can be very well controlled to suit various applications. Zinc sulfide (ZnS)-coated cadmium selenide (CdSe) nanocrystals are the most commonly studied QDs for bioapplications due to their wide bandgap and easily tunable emission in the visible range [6]. These qualities make them especially useful for various industrial, agricultural, and biomedical applications [7]. Another reason for the popularity of the CdSe QDs is their well-established synthesis and characterization protocols [8]. In this chapter, the synthesis, toxicity, and surface modification of CdSe QDs in bioanalytics and biomedical diagnostics are discussed.

1.1. Synthesis of QDs

Quantum dots can be prepared by formation of nanosized semiconductor particles through colloidal chemistry or by epitaxial growth and/or nanoscale patterning [9]. Preparation of QDs designed for biological applications has four basic steps: core synthesis, shell growth, aqueous solubilization, and biomolecular conjugation or biofunctionalization.

1.1.1. Core-shell protocol

QDs core is generally made from heavy metal semiconductors of group II–VI (CdSe, CdS, CdTe, HgS, ZnS, ZnSe), III–V (GaAs, GaN, InP, InAs, InGaAs), IV–VI (PbS, PbSe, PbTe, SnTe), and group III–V (InP and InGaP) (**Table 1**). The most common method for preparation of QDs core consists of a rapid injection of semiconductor or organometallic precursors (e.g., Cd precursor and TOPSe) into hot and vigorously stirred specific coordinating solvent (e.g., thiol stabilizers). Coordinating solvents stabilize the bulk semiconductors and avoid aggregation as the QDs grow [10]. Thereafter, the semiconductor core material (e.g., CdSe) must be protected from degradation and oxidation to optimize QDs performance. Hence, an external layer or protective shell (e.g., ZnS) is usually synthesized to cover the QD semiconductor core to enhance stability, while increasing its photoluminescence [11]. Due to their synthesis in nonpolar organic solvents, the inorganic core-shell semiconductor QDs (e.g., CdSe) are typically hydrophobic, which prevents their solubility and enhances the formation of aggregates or precipitates in water-based solutions. This property limits biological applications of core-shell QDs, requiring additional modifications of their surfaces to achieve biocompatibility or solubility in biological or water-based fluids.

1.1.2. Aqueous solubilization

The aqueous dispersal of core-shell QDs is controlled by the chemical nature of their surface coating. Numerous effective methods have been established to create hydrophilic QDs, which can be divided into two main categories [12]. *The first route*, commonly designated as

1																	20											2									
H																	Ca											He									
																		Other nonmetals																			Halogens
3	4																	Alkali metals																			Transition metals
Li	Be																	Alkaline earth metals																			Post-transition metals
																		Noble gases																			Lanthanoids
11	12																	Metalloids																			Actinoids
Na	Mg																																				
19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36																				
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr																				
37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54																				
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe																				
55	56	57-71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86																				
Cs	Ba	La-Lu	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn																				
87	88	89-103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118																				
Fr	Ra	Ac-Lr	Rf	Db	Sg	Bh	Hs	Mt	Ds	Rg	Cn	Uut	Fl	Uup	Lv	Uus	Uuo																				
																		57	58	59	60	61	62	63	64	65	66	67	68	69	70	71					
																		La	Ce	Pr	Nd	Pm	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu					
																		89	90	91	92	93	94	95	96	97	98	99	100	101	102	103					
																		Ac	Th	Pa	U	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Md	No	Lr					

Table 1. Semiconductor elements—groups II to VI—within the periodic table.

“cap exchange procedure,” consists of a complete replacement of the hydrophobic layer of organic solvent by bioactive molecules containing soft acidic and hydrophilic groups pointing outwards, from the QDs surface to surrounding bulk water molecules [13, 14]. This route allows electrostatic stabilization of inorganic core shell of QDs through their interactions with small charged ligands (e.g., amines, cystamine, cysteine, 2-mercaptoethanol, ethylamine, or mercaptopropionate) or charged surfactants to form a new external coating layer that encapsulates QDs. *The second route* allows steric stabilization through modification of the native coordinating organic ligands on the QDs surface with “bulky” uncharged polymeric surface ligands such as the polyethylene glycol or PEG [15, 16]. *Alternatively to electrostatic and steric stabilizations*, bulky and charged ligands (e.g., polyelectrolytes or polyethyleneimine), amphiphilic inorganic shell (e.g., silica added to QDs during polycondensation) or solid lipid nanoparticles composed of high biocompatible lipids of physical and chemical long-term stability have been successfully tested for further stabilization of QDs [17–19]. All aforementioned coating strategies are useful for QDs solubilization while allowing further addition of polymers or bioactive molecules for cell labeling and imaging.

1.1.3. Biofunctionalization

Biofunctionalization refers to the ability to successfully attach or conjugate bioactive molecules (e.g., oligonucleotides, proteins, polysaccharide, and peptides) to water-dispersed QDs. This process can be achieved by binding to polyhistidine tags, electrostatic (e.g., avidin-biotin) or covalent interactions. This later is typically accomplished by activated 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling amine and carboxyl groups and catalyzed maleimide (SMCC) linking amine to sulfhydryl groups [20, 21]. It is important to mention that these processes remain challenging due to surface chemistry of QDs, control of attachment

orientation of biomolecules [19, 21, 22], and determination of conjugation efficacy. QDs have greater surface area-to-volume ratio allowing several types of biomolecules to be attached to a single QD to provide multifunctionality of the conjugate [23]. Only few protocols for biofunctionalization are available, and systematic studies are needed for functional evaluation of conjugated or biofunctionalized QDs [14, 20, 21].

2. *In vitro* and *in vivo* toxicity of QDs

Due to their heavy metal semiconductor cores, QDs are considered toxic when the cores are not adequately contained by an outer shell, such as the ZnS shells mentioned above. Without containing the cores, potential damage to biological systems can occur, which composes a challenge to surmount for medical and other *in vivo* applications. The core of the most widely used and studied QDs consists of cadmium selenide (CdSe) or telluride (CdTe) given their quantum confinement region spanning the entire optical spectrum [24]. Cadmium ions (Cd^{2+}) have been identified as the primary cause of QDs cytotoxicity due to their overtime leaking, upon illumination or oxidation [25, 26]. Leaked Cd^{2+} is able to bind to thiol groups of key molecules of mitochondria and cause enough stress and damage leading to cell death [27].

Moreover, the cytotoxicity of QDs appears directly related to the protective inorganic surface layers [25]. Additional surface coatings may be needed to substantially reduce or eliminate the release of Cd^{2+} [26]. The utilization of gelatin during the production of CdTe QDs has resulted in reduced toxicity of particles [28]. In the case of CdSe QDs, it is believed that properly prepared closed (ZnS) or multiple (e.g., ZnS/ SiO_2 , ZnS/PEG hydrophilic coating) shells render cadmium leakage less likely [29, 30]. However, oxidized QDs surface may unintentionally react with intracellular components, causing formation and release of reduced Cd that results in apoptosis within primary hepatocytes isolated from rats [31–33]. In addition, the charge and chemical reactivity of QDs play dominant roles in their biocompatibility, independent of their size [32]. Various studies have demonstrated the crucial roles of multiple positive charges and size-dependent polycationic materials of QDs in cytotoxic mechanisms of nanoparticles [34, 35].

Majority of *in vitro* studies use transformed cell lines to demonstrate the cytotoxicity of QDs that may not fully reflect the response cascade in normal cells [12]. Nonetheless, the use of these cell types allows for many generalizations to be made regarding the toxicity related to specific QDs features (i.e., size, protective shell, and surface chemistry), experimental dosage, and exposure conditions. **Table 2** summarizes few studies exemplifying the complexity of investigating QDs nanotoxicity due to multiple variables such as their size, shell components and surface chemistry that should be taken into account when designing an experiment. The comparable size of QDs with certain cellular components may facilitate their passage through many biological barriers and accumulation in different tissues to cause adverse effects after long-term exposure [36]. At equal concentrations and positive charges, smaller QDs (i.e., 2–3 nm) display high cytotoxicity than larger ones (i.e., >5 nm), with liver and kidneys often being main target organs due to their blood filtering function [37].

QDs (core and protective layers)	Concentration	Exposure	Toxic effect	References
CdSe/ZnS-SSA	0.1–0.4 mg/mL	0–24 h	0.1 mg/mL altered cell growth; most cells nonviable at 0.4 mg/mL	[38]
CdSe/ZnS-SSA	0.1 mg/mL QDs per 5×10^7 cells	2 h to 7 days	No toxicity in mice <i>in vivo</i>	[38]
CdSe/ZnS conjugates: NH ₂ , OH, OH/COOH, NH ₂ /OH, COOH	1–2 μ M	12 h	2- μ M QD-COOH-induced DNA damage upon 2 h of exposure	[39]
CdSe/ZnS/MUA	0–0.4 mg/mL	24 h	0.2 mg/mL, Vero; 0.1 mg/mL, HeLa; 0.1 mg/mL, hepatocytes	[40]
CdTe	0.01–100 μ g/mL	2–24 h	10 μ g/mL cytotoxic	[41]
CdSe-MAA, TOPO QDs	62.5–1000 μ g/mL	1–8 h	62.5 μ g/mL cytotoxic under oxidative/photolytic conditions No toxicity on addition of ZnS cap	[25]
QD micelles: CdSe/ZnS QDs in (PEG-PE) and phosphatidylcholine	1.5–3 nL of 2.3- μ M QDs injected, approx. 2.1×10^9 to 4.2×10^9 QDs/cell	Days	5×10^9 QDs/cell: cell abnormalities, altered viability and motility No toxicity at 2×10^9 QDs/cells	[42]
CdSe/ZnS amp-QDs and mPEG QDs	Injections, approx. 180-nm QD, approx. 20-pmol QD/g animal weight	15-min cells incubation, 1–133 days <i>in vivo</i>	No signs of localized necrosis at the sites of deposition	[43]
CdSe/ZnS-DHLA	400–600 nM	45–60 min	No effect on cell growth	[44]
Avidin-conjugated CdSe/ZnS QDs	0.5–1.0 μ M	15 min	No effect on cell growth and development	[44]
CdSe/ZnS-amphiphilic micelle	60- μ M QD/g animal weight, 1- μ M and 20-nM final QD concentration	Information not provided	Mice showed no noticeable ill effects after imaging	[45]
CdSe/ZnS-DHLA QDs	100 μ L of B16F10 cells (approx. 2×10^5 to 4×10^5) used for tail vein injection	4–6 h cell incubation, mice sacrificed at 1–6 h	No toxicity observed in cells or mice	[46]
CdSe/ZnS-MUA QDs; QD-SSA complexes	0.24 mg/mL	2 h	0.4 mg/mL MUA/SSA-QD complexes did not affect viability Vero cells	[47]
CdSe/ZnS	10-pmol QDs/ 1×10^5 cells (approx. 10 nM)	10 days (cell culture)	10 nM QD had minimal impact on cell survival	[48]
CdTe aqQDs	300–600 nM	3 days	Nearly completely inhibited cell growth even from the very beginning	[49]
CdTe-gelatinized/nongelatinized	1–100 nM	72 h	At 1 nM, did not initiate any detrimental effects; at 100 nM, resulted in the death of all PC12 cells	[50]

QDs (core and protective layers)	Concentration	Exposure	Toxic effect	References
CdTe, CdTe/CdS, CdTe/CdS/ZnS	0.2–0.3 μ M	0–48 h	Cells treated with CdTe and CdTe/CdS QDs were mostly nonviable by 48 h (for all concentrations tested)	[51]
CdSe/ZnS-PEG (EviTag T1 490 QD)	0.84–105 μ M	0–24 h	Commercially available QDs demonstrated low cytotoxicity but induced cell detachment	[52]
CdSe	1, 10, and 20 nM	24 h	1 nM QD for 24 h showed no decreased in cell viability; in contrast, cells treated with 10 and 20 nM QDs for 24 h showed decreases in cell viability in the order of 20 and 30%	[53]

Table 2. Inconsistent considerations on QDs toxicity evaluation (modified from [3]).

Due to the complexity to characterize the cytotoxicity of nanoparticles, the US National Cancer Institute and several other US health agencies have created the Nanotechnology Characterization Laboratory (NCL) for efficacy and toxicity testing of nanoparticles, including fluorescent QDs. As part of the process, the NCL will describe physical attributes of nanoparticles, their *in vitro* biological properties and their *in vivo* biocompatibility.

3. Bioapplications

Despite the reported and controversial cytotoxicity, QD nanoparticles remain excellent candidates for numerous bioapplications. Compared to organic dyes, QDs display narrow, symmetrical, and tunable emission spectra and contingency for their size and material composition [54]. Various QD sizes have closer but nonoverlapping emission wavelengths [20], which excitation through a single light source leads to a photostable and broad absorption spectra [52].

3.1. QDs labeling

The brightness of CdSe QDs fluorescence has made them the widely labeled nanoparticles for various biosensing (e.g., oligonucleotides, organic dyes) and single or multiplex labeling (e.g., antibodies, peptides) [22, 23, 31, 55–57]. Yet, the localization (intracellular or extracellular), expression level, and environment (oxidizing or reducing) of the target molecule should be considered during the QDs labeling protocol. For example, the intracellular targeting may pose additional challenges requiring the need of cell-penetrating peptides (e.g., polyarginine, polylysine) for effective intracellular delivery of QDs conjugates, while maintaining the homeostasis and osmotic balance of cells. Reproductive studies have shown the ability of porcine gametes to interact with self-illuminated CdSe/ZnS QDs [58, 59] with the necessity to determine the suitable sperm-to-QDs ratio, avoiding or limiting QDs toxicity to sperm function, as observed in previous studies using various nanoparticles [58–65]. *In vitro* matured

oocytes appeared to accumulate higher levels of QDs compared to spermatozoa, which, instead, exhibited stronger membrane labeling (**Figures 1** and **2**). Reduced QDs internalization within the spermatozoa was attributed to sperm membrane specificities, whereas the limitations of QDs as compared to organic dyes may not be ruled out [66]. The conjugation of self-illuminated CdSe/ZnS QDs with anti-plasminogen antibody (for specific targeting) revealed stronger signals within the porcine oocyte than the nonconjugated QDs, applied for plain targeting (**Figure 2**). The use of these CdSe/ZnS QDs also provided opportunity for *ex vivo* imaging of cultured porcine ovarian follicles (**Figure 3**), which would, in the near future, permit real-time monitoring of key molecules having role(s) during folliculogenesis.

3.1.1. QDs labeling for cell imaging and disease detection

Effective labeling of fluorescent QDs is crucial for extracellular and intracellular tracking of target molecules in their native environment. QDs functionalized with antibody are optimal for extracellular targeting of cell-surface membrane proteins (e.g., receptors) and subsequent targeted imaging [20, 22, 31], which practice will create opportunities for precise assessments of cellular and molecular mechanisms of diseases (e.g., cancer) and their treatments. Near-infrared QDs (e.g., CdSe, CdTe) emit in the wavelength range of 650–900 nm to overcome the optical property variations and endogenous autofluorescence of tissues under *in vivo* conditions [67], permitting tumor localization and visualization while offering a new mean for cancer prevention and treatment.

Figure 1. Confocal microscope imaging of mature boar spermatozoa labeled with CdSe QDs 655 nm. Labeled spermatozoa revealed major localizations of QDs (red spots) in the head and mid-piece regions. Sperm nuclei are counterstained in blue with DAPI. Micrograph A = overlay of 3 lights (visible, blue DAPI and red QDs 655 nm); Micrograph B = overlay of DAPI and QD 655 nm.

Figure 2. Confocal microscope imaging of porcine oocytes matured in the presence of QDs 655 nm. Cumulus-oocyte complexes were matured in the presence of QD alone (plain targeting; micrographs A/B/C) or QD conjugated with anti-plasminogen antibody (specific targeting; micrographs D/E/F). Micrograph NC = Control without QDs; A and D = Overlays of visible light and QDs 655 nm filter; B and E = overlays of DAPI and QDs 655 nm, and C and F = QDs 655 nm filter alone. The stronger and differential distribution of the red signal can be seen following QDs conjugation.

Figure 3. Confocal microscope imaging of porcine follicles microinjected with nonconjugated QDs 655 nm. Dissected antral follicles were microinjected, cultured for 1 (A) or 3 (B) days, then prepared for histology slides and imaging. QDs (red spots) are mainly visible within the layer of granulosa cells (GC) after 1 day of culture (A) and then throughout the theca interna (TI) and externa (TE) after 3 days of culture (B).

The intracellular localization of selective biomolecules for targeting presents additional challenges associated with QDs conjugates delivery within the cells. Available methods for QDs delivery are composed of, but not limited to, positively charged peptides or cell-penetrating peptides on QDs, microinjection, electroporation, or nonspecific or receptor-mediated endocytosis

[57, 67–69]. Electroporation technique has shown robust and highly efficient delivery of both monomer and aggregate QDs to the cells due to induced electrical pulses that temporarily permeabilize the plasma membrane [70]. It has been used for *in vivo* imaging of cancer cells through active intracellular delivery of QDs [67]. Electroporation of QDs in lung (NCI-H460) and ovary (SK-OV-3) cancer cells revealed high and longer (over a month) QDs retention inside the cells, allowing observation of the entire process of subcutaneous tumor growth and cancer cell dissemination at late stages of metastasis in a natural tissue environment [67]. It is important to mention that biofunctionalized QDs have been used for imaging in many other diseases, including the brain tissue [71, 72].

3.1.2. QDs labeling for cell imaging in reproductive biology

The small size (2–10 nm in diameter) and unique physicochemical properties of QDs, especially their tunable size-dependent fluorescence emission, make them excellent candidates for applications in the reproductive field. For example, the multicolor detection of various QDs permits spectral multiplexing for simultaneous detection and quantification of different biomolecules in *in vitro* bioassays [23, 54, 73–75], which may be crucial in understanding the complexity of mammalian gamete maturation in their native environment.

Additionally, signal amplification of enzymatic reactions could be achieved through QDs emitting localized and bright fluorescence in bioassays. This later could be illustrated by the novel QD-BRET (Bioluminescent Resonance Energy Transfer), a luciferase-doped QDs which enzymatic reaction with its substrate (Luciferin or coelenterazine) produces energy (480 nm) that is immediately absorbed by the CdSe QDs to emit brighter and long-lasting NIR fluorescence [76]. Numerous *in vivo*, *in vitro*, and *ex situ* studies have successfully applied the QD-BRET for imaging of somatic and reproductive (mammalian gametes and ovarian follicle; **Figures 1–3**) cells [58, 59, 77].

The use of spectral multiplexing and signal amplification in reproductive biology has potential for fast diagnostics of gamete quality through direct (e.g., fluoroimmunoassays) or indirect (lab-on-chip arrays) evaluations. The proposed arrays should contain various QDs sizes that are biofunctionalized to target various key biomarkers of reproductive cells.

In addition to above-mentioned applications, there is potential to use QDs conjugates for targeted labeling, tracking, and imaging of ovarian follicle cells (during folliculogenesis) or spermatozoa (during intrauterine migration). Moreover, a recent study using amphibians reported the ability of living tadpoles to accumulate QD (655 nm) nanoparticles, likely impeding with their development [78].

4. Future outlook

QDs applications have been explored for molecular and pharmaceutical fields, but are rapidly expanding to other research areas. It is expected that QDs will be used for (1) categorizing various types of biological processes, (2) localizing and identifying molecular mechanisms of disease, (3) developing novel drug-action mechanisms, (4) applications in intracellular and extracellular compartments and (5) innovative approaches for biochemical assays. Ventana

Medical Systems has just begun publicizing their QDs Map family of immunohistochemistry reagent kits for automated slide processing and fluorescent detection of fixed specimens (www.ventanadiscovery.com). The increased commercial offering of QDs products reflects the desirability of QDs photophysical properties, namely, photostability, single source excitation, narrow emission, multiplexing capabilities, and high quantum yield.

Unfortunately, the lack of reliable and reproducible techniques to conjugate a variety of biomolecules such as antibodies, protein markers, DNA, and RNA to QDs in a methodical way with control over their ratio, orientation, and avidity remains to hinder their ongoing use in clinical diagnostics [3]. In the future, it is to be expected that more commercial products integrating QDs for clinical, diagnostic, and research purposes will be released for public use and manipulation, which will likely give rise to more reliable conjugation techniques as they are further investigated. The outcomes of current research combining nanotechnology and reproductive biology clearly indicate nanoparticles as promising tools for both basic and applied research in animal reproduction [79–84].

Author details

Sapna Jain¹, Seong B. Park², Shreekmar R. Pillai¹, Peter L. Ryan², Scott T. Willard² and Jean M. Feugang^{2*}

*Address all correspondence to: jn181@ads.msstate.edu

1 Alabama State University, Alabama, USA

2 Mississippi State University, Mississippi, USA

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