Chapter 3
Quantum Dots as Biomarker

Michel Boissiere

Abstract Quantum dots (QDs) are semiconductor nanocrystals with unique optical and electronic properties. They have distinct advantages over traditional fluorescent organic dyes in chemical and biological studies in terms of tunable emission spectra, signal brightness, photostability, and can be conjugated to a wide range of biological targets, including proteins, antibodies, and nucleic acid probes. Currently, the major type of QDs is the heavy metal containing II-VI, IV–IV, or III-V QDs. The new generations of QDs, have far-reaching potential for the study of intracellular processes at the single-molecule level, high resolution cellular imaging, long-term in vivo observation of cell trafficking, tumor targeting, and diagnostics. However, with respect to medical applications, caution must be exercised with QDs due to their toxic components. Development of suitable health and safety regulations is necessary for commercialization. Despite of these difficulties, QDs appear to be too valuable to nanomedecine to dismiss, and will eventually come essential into practical use.

3.1 Introduction

Among various nanomaterials, quantum dots (QDs) distinguish themselves in their far-reaching possibilities in many avenues of biomedicine. QDs are nanometer-sized semiconductor crystals with unique photochemical and photophysical properties that are not available from either isolated molecules or bulk solids.

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QD research started in 1982 with the realization that the optical and electric properties of small semiconductor particles were heavily dependent on particle size due to quantum confinement of the charge carriers in small spaces. During the next two decades, extensive research was carried out for potential applications in optoelectronic devices, QD lasers and high-density memory. In 1998, two seminal reports simultaneously demonstrated that QDs could be made water soluble and could be conjugated to biological molecules, providing the first glimpse of the vast potential of QDs as probes for studying biological systems [1, 2]. In comparison with organic dyes and fluorescent proteins, QDs have the advantages of improved brightness, resistance against photobleaching, and multicolor fluorescent emission. These properties could improve the sensitivity of biological detection and imaging by at least one to two orders of magnitude. Significant improvements have been made in the synthesis, surface modification, and biofunctionalization of QDs in the following years, and indeed the current literature is rife with examples of QDs used in various biomedical applications. It can, now, be said with confidence that QDs have completed the transition from a once curious demonstration of quantum confinement in semiconductors to ubiquitous fluorophores providing unique insights into biological investigations [3].

In this chapter, an attempt will be made to provide a comprehensive, state-of-the-art overview of QD applications in biology imaging and diagnostic. Following, a brief introduction describes the photophysics and chemistry properties of QDs and provides a clear understanding of the merits of using QDs in bio-imaging and diagnostic, as well as the requirements and challenges in the synthesis, surface modification, and bioconjugation of QDs in order to make them amenable to bioapplications. The following section describes QDs cytotoxicity because to assess their biomedical application promising, it is important to characterize their behavior in vivo. Next, some recent advances in the use of QDs in various biological applications for detection and diagnosis of different diseases are detailed, both in vivo and in vitro. The literature cited in this chapter is confined to reports, representative, and which are innovative studies.

3.2 Optical and Spectroscopic Properties of Quantum Dots

QDs are colloidal semiconductor nanocrystals that exhibit unique electro-chemiluminescent properties, strong light absorbance, bright fluorescence, size-tunable (2–10 nm) narrow emission spectra, and provide excellent fluorescence quantum yields.

QDs are composed of elements from groups II–VI, III–V, or IV–IV of the periodic table. In comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties such as size and composition-tunable light emission, improved signal brightness, resistance to photobleaching which makes them useful for continuous monitoring of biological phenomena, and simultaneous excitation of multiple fluorescence colors (Fig. 3.1).

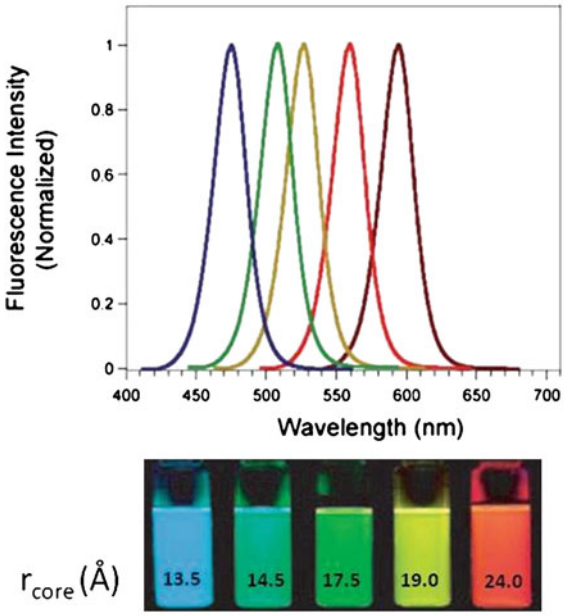

Fig. 3.1 Illustration of size-tunable (CdSe)ZnS QDs and their fluorescence spectra

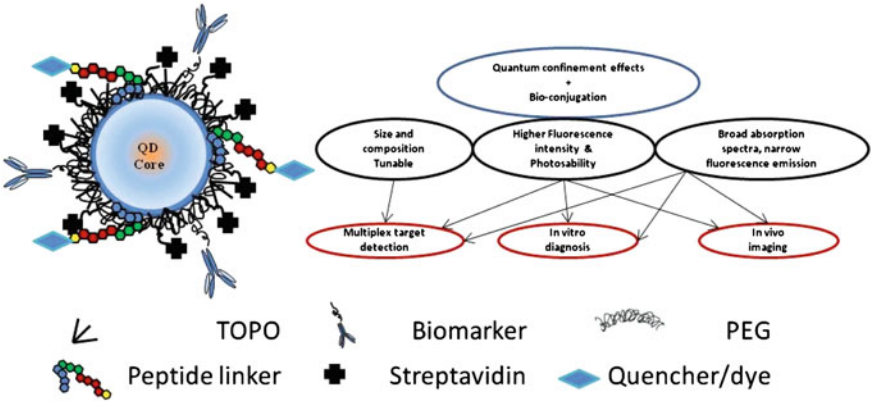

Fig. 3.2 Properties of decorated quantum dots

Also, the long luminescent lifetime (30–100 ns) of QDs diminishes interference, from background autofluorescence in live cell imaging. In addition, different colors of QDs can be simultaneously excited with a single light source, with minimal spectral overlapping, which provides significant advantages for multiplexed detection of target molecules [4–9] (Fig. 3.2). Usually, the core of QDs may be composed of cadmium selenide (CdSe), cadmium telluride (CdTe), or

Table 3.1 Application of different bioconjugated QDs for cancer diagnosis

Cancer type	Biomarker detected	Detection limit	Description
Proteolytic activity for some cancer types [72]	Proteolytic enzymes	200 nM	Nanoscale sensing assemblies (FRET) consisting of QD–peptide conjugates that are capable of specifically detecting the activity of proteolytic enzymes.
Epithelial cancer [13]	Mucin 1	250 nM	Aptamer-based Quantitative detection system with QD labeling
Breast carcinoma cells [73]	Cancer stem cell (CSC) markers CD44v6 + and CD24-	nc	QD conjugated antibodies
Human ovarian cancer [74]	HER2/neu oncomarker	nc	QD conjugated anti-HER2/neu4D5
Lung carcinoma cells (A549) [75]	Folate receptors	nc	Folate-conjugated QDs
J4656-FR mouse lymphoma cells [76]	Folate receptors	nc	Folate-conjugated QDs-8
Human nasopharyngeal epidermal carcinoma cell line (KB) and a human lung carcinoma cell line (A549) [42]	Folate receptors	nc	Folate-conjugated InP-ZnS QDs
Mouse myeloma cells and human cancer cell lines: breast MCF7, prostate LNCaP, lymphoma SKW 6 [77]	Small bivalent antibody fragments, cys-diabodies	nc	Bioconjugated CdSe/ZnS Qdots
Hepatoma detection in vivo [78]	AFP an important marker for hepatocellular carcinoma cell lines	nc	Bioconjugated quantum dots to AFP (alpha-fetoprotein)
In vitro dual color fluorescence imaging some cancer cell lines [79]	Aptamer (nucleolin), integrin $\alpha_v\beta_3$)	nc	QDs conjugated by the AS1411 and arg–gly–asp acid
Microfluidic protein chip for an ultrasensitive and multiplexed assay of cancer biomarkers [80]	Cancer biomarker CEA and AFP (serum)	250 fM	Multicolor imaging and multiplexed bioassay using bioconjugated secondary antibodies (goat anti-mouse IgG) QDs

(continued)

Table 3.1 (continued)

Cancer type	Biomarker detected	Detection limit	Description
Detection of protein lung cancer biomarker [81]	Nitrated ceruloplasmin	8 ng/mL	Using a portable fluorescence biosensor based on quantum dots antinitrotyrosine conjugate and a lateral flow test strip
Determinations of cancer biomarkers [69]	CEA, cancer antigen 125, and HER-2/Neu	0.02 ng/mL in serum and saliva	QD labeled antibodies
Prostate cancer biomarker [82]	PSA	nc	PSA antibodies conjugated QDs
Protein microarrays and quantum dot probes for early cancer detection [83]	Detect six differents cytokines TNF-α, IL-8, IL-6, MIP-1β, IL-13 and IL-1β	pM	Two different models of quantum dot probes: antibody specific to the selected marker—IL-10, and the second by use of streptavidin coated quantum dots and biotinylated detector antibody
Breast cancer [84]	Human epidermal growth factor receptor 2 (HER2)	nc	QDs linked to immunoglobulin G (IgG) and streptavidin
Breast cancer invasion [85]	Human epidermal growth factor receptor 2 (HER2)	nc	Conjugated QDs two primary antibodies from two species (e.g. mouse and rabbit)
Immunosensor for the detection of prostate cancer biomarker [86]	PSA	nc	QD–functionalized graphene sheets (GS) as labels for the secondary antibody (Ab2) GS as labels for signal amplification

indium phosphide (InP), among other combinations. The most common QDs used in biomedical applications are those with a CdSe core surrounded by a semiconductor shell, e.g., zinc sulfide (ZnS) epitaxial grown around the core [10–14]. The function of the ZnS shell is to reduce the oxidation of the core and leaching of metal ions from the core. By passivating the core, the shell also increases the photoluminescence quantum yield [15]. However, as QDs are hydrophobic by nature, it is necessary to solubilize QDs before application by surface modification with biofunctional molecules [16], because QDs have large surface areas for the attachment of such molecules. When conjugated with diagnostic (e.g., optical) and therapeutic (e.g., anticancer) agents, QDs can be used for cancer diagnosis and therapy with high specificity [17–19]. Significant research efforts have been

focused on early cancer diagnosis with QDs [3]. As early as 2002, after overcoming the limitation in obtaining biocompatible nanocrystals, Dubertret [20] showed the potential to revolutionize biological imaging. In case of imaging probes, active targeting of cancer antigens (molecular imaging) has become an area of tremendous interest because of the potential to detect early stage cancers and their metastases [21–23]. Major recent developments in this regard are summarized in Table 3.1.

3.3 Cytotoxicity

In spite of the attractive properties and successful applications, it should be realized that the emerging labeling approaches using QDs have limitations as well. Experimental barrier for the large-scale application of the QDs includes the lack of consensus methods for labeling biomolecules with QDs. To this end, organic dyes are advantageous due to the availability of well established labeling protocols, purification, and characterization techniques for dye bioconjugates [24]. Other technical limitations include the lack of reproducibility, limited knowledge on their clearance in living systems, reduced luminescence activity due to their relatively large surface areas and sensitivity to oxidation, and photolysis [25]. Moreover, the cytotoxicity of QDs has been observed in a large number of in vitro studies [26–31], affecting cell growth, and viability [32]. The extent of cytotoxicity has been found to be dependent upon a number of factors including size, capping materials, color, dose of QDs, surface chemistry, coating bioactivity, and processing parameters [31, 33, 34]. Even if not inducing significant alterations in cell physiology, QDs can produce subtle alterations of function which may affect the quality of data derived from their use [30, 35, 36]. The toxic nature of QDs due to the release of free cadmium ions during their degradation poses environmental concerns and generates serious hurdle for diagnostic applications [34, 37, 38]. Examination of QD toxicity in a hepatocyte culture model showed that exposure of core CdSe to an oxidative environment causes decomposition and desorption of Cd^{2+}ions. Such exposure during synthesis and processing played an important role in subsequent toxicity [39]. In addition to the effects of the QD core, ligands added to render the probe biologically active may have toxic effects on cells. Mercaptopropionic acid (MPA) and mercaptoacetic acid, which are commonly used for solubilization, have both been shown to be mildly cytotoxic [28]. 11-mercaptoundecanoic acid (MUA), cysteamine and TOPO have all been shown to have the ability to damage DNA in the absence of the QD core [40]. PEGylated QDs have been shown to have reduced cytotoxicity, but modification of these to produce PEG-amine for biological activity renders them cytotoxic once again [41].

In order to overcome the nanotoxicity and biocompatibility issues of QDs different innovative approaches have been explored. Introduction of functionalized layers, encapsulating shell and capping materials can reduce the sensitivity to oxidative, photolytic, and mechanical degradation that, in turn, abate the QD

toxicity [37]. Groups III–V QDs may provide a more stable alternative to groups II–VI QDs due to the presence of a covalent, rather than an ionic, bond, and have been reported to have lower cytotoxicity [42]. However, these QDs are difficult to prepare on a competitive time scale, and tend to have much lower quantum efficiencies, meaning uptake has been slow.

Combination of semiconductor QDs with plasmonic materials has been found to be effective for partially resolving the nanotoxicity and biocompatibility issues of QDs [43]. Concurrent with the exploration of new effects, further sophisticated applications of quantum particles in medical research are being explored and new avenues for early diagnosis and treatment might soon open up in the imminent future. The multiplexed detection capability and subpicomolar sensitivity can make QDs a good choice for the medical diagnosis once the technical limitations and issues associated with toxicity are surmounted successfully [21, 44, 45]. Recently, in the demand of using biocompatible and nontoxic QDs as nanoprobes, rare earth (RE) elements are used to fabricate a new type of QDs, such as Gd-doped, ZnO QDs. RE-doped QDs have distinct advantages over heavy metal-containing QDs, not only because of avoiding the increase of particle size by polymer or silica coating in synthesis procedure, but also providing a simple, green synthesis method. Liu et al. reported the development of Gd-doped ZnO QDs with enhanced yellow fluorescence, and these QDs can be used as nanoprobes for quick cell detection with very low toxicity [46].

3.4 Biological Applications of QDs

3.4.1 QDs in Molecular Imaging and Cancer Medicine

3.4.1.1 Bioconjugated QDs for In Vitro and In Vivo Imaging

One of the most advancing applications of QDs is in vitro imaging of cancer cells. Soon after the introduction of biocompatible QDs for cell imaging by Chan and Nie [2] and Bruchez et al. [1], many research groups applied QDs for imaging of cancer cells. QDs conjugated with cancer specific ligands/antibodies/peptides were found to be effective for detecting and imaging human cancer cells derived from prostate cancer [47], breast cancer, pancreatic cancer [44], metastatic tumor [48], glioblastoma [49] and cancers of the bone marrow [50], and tongue [51].

Compared to the study of living cells in culture, different challenges arise with the increase in complexity to a multicellular organism, and with the accompanying increase in size. Unlike monolayers of cultured cells and thin tissue sections, tissue thickness becomes a major concern because biological tissue attenuates most signals used for imaging. Optical imaging, especially fluorescence imaging, has been used in living animal models, but it is still limited by the poor transmission of visible light through biological tissue. It has been suggested that there is a near-infrared optical window in most biological tissue that is the key to deep tissue optical imaging [52].

In vivo application of QDs was first tested by Akerman et al. in 2002 [53]. They injected CdSe/ZnS QDs coated with peptides into the tail vein in mouse, and found that the injected QDs preferentially distribute in endothelial cells in the lung blood vessels. Also, based on ex vivo fluorescence microscopic imaging of tissue sections, they found that the QD-peptide conjugates were preferentially bound to tumors.

One of the most immediately successful applications of QDs in vivo has been their use as contrast agents for the two major circulatory systems of mammals, the cardiovascular system, and the lymphatic system. In 2003, Larson et al. demonstrated that green-light emitting QDs remained fluorescent and detectable in capillaries of adipose tissue and skin of a living mouse following intravenous injection [54]. This work was aided by the use of near-infrared two-photon excitation for deeper penetration of excitation light, and by the extremely large two-photon cross-sections of QDs [55]. In other work, Lim et al. used near-infrared QDs to image the coronary vasculature of a rat heart [56], and Smith et al. imaged the blood vessels of chicken embryos with a variety of near-infrared and visible QDs [57]. The later report showed that QDs could be detected with higher sensitivity than traditionally used fluorescein–dextran conjugates, and resulted in a higher uniformity in image contrast across vessel lumena. Jayagopal et al. [58], recently, demonstrated the potential for QDs to serve as molecular imaging agents for vascular imaging. Spectrally distinct QDs were conjugated to three different cell adhesion molecules (CAMs), and intravenously injected in a diabetic rat model. Fluorescence angiography of the retinal vasculature revealed CAM-specific increases in fluorescence, and allowed imaging of the inflammation-specific behavior of individual leukocytes, as they freely floated in the vessels, rolled along the endothelium, and underwent leukostasis. The unique spectral properties of QDs allowed the authors to simultaneously image up to four spectrally distinct QD tags.

With the development of QDs in recent years, many studies explored the potential of QDs in wider fields. Kobayashi reported fluorescence lymphangiography by injecting five QDs with different emission spectra [59]. Through simultaneous injection of five QDs into different sites in the middle of phalanges, the upper extremity, the ears, and the chin, different parts of the mouse body can be identified by certain fluorescence color. This is the first demonstration of simultaneous imaging of trafficking lymph nodes with QDs having different emission spectra in vitro cells imaging. Another study was reported by Kim et al. who used near-infrared QDs for sentinel node mapping in cancer surgery in animals. QDs were injected intradermally in distal extremities and imaging used to track their movement along lymphatic channels, with identification of the sentinel node. Furthermore, these experiments demonstrated high contrast between auto fluorescence and emission signal, allowing minimal surgical incision for removal of positive sentinel node [60]. Other teams [61, 62] have, recently, undertook fluorescent tracking of solubilized near-infrared QDs injected subcutaneously in the anterior paw in mice demonstrating accumulation in regional lymph nodes within 5 min of injection and with a maximum concentration at 4 h which then gradually fell over the next 10 days, with resultant low-level uptake in other organs. Tracking using fluorescent imaging was compared with inductively coupled plasma mass spectroscopy, demonstrating viability of fluorescent imaging (Fig. 3.3).

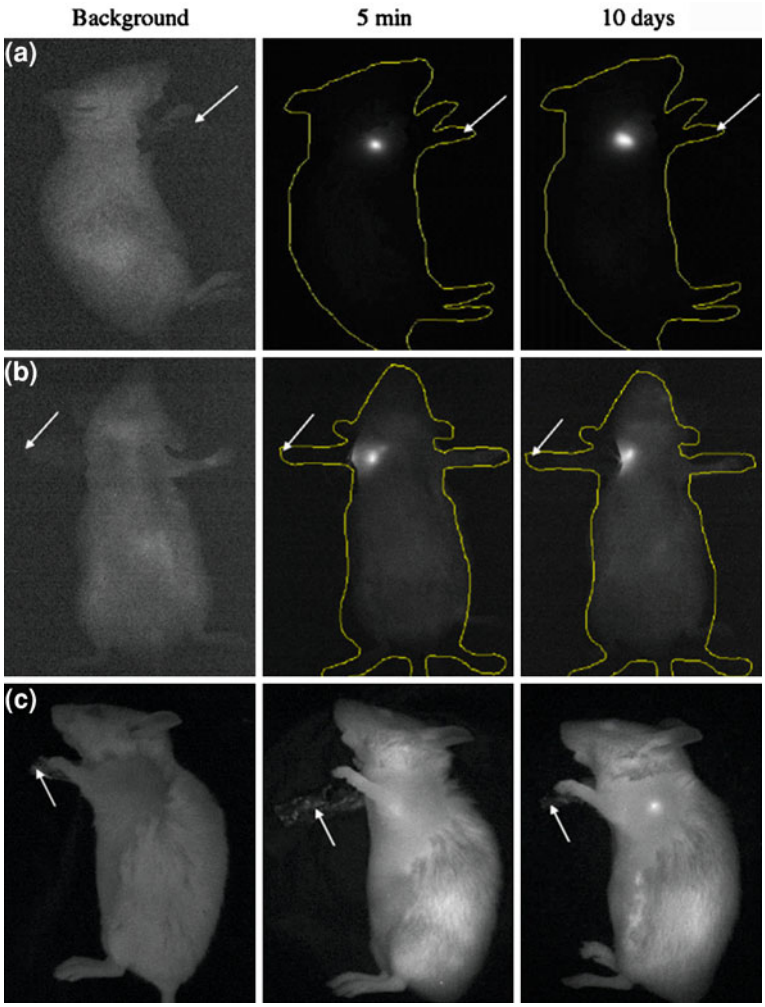

Fig. 3.3 In vivo fluorescence imaging of mice after s.c. injection of 20 pmol of QDs. **a** Images of the *right flank* (visualization of RALN). **b** Images on dorsal decubitus (observation of RLTLN). **c** Images of *left flank* (visualization of LALN). Left column corresponds to background signal, middle and right columns to images at 5 min and 10 days post-injection, respectively. For **a** and **b** images, the exposure time is 10 ms, and for **c** images, the exposure time is 100 ms. The *white arrow* indicates the injection point. With kind permission from Springer Science in Molecular imaging and biology [61]

In another field of application of imaging, QDs have been developed for multimodal imaging. Magnetic resonance imaging (MRI), radiography, and fluorescence imaging are powerful biomedical imaging modalities. Each imaging modality has its merits and demerits and hence cannot achieve comprehensive imaging. Quality imaging requires high spatial and temporal resolutions, 3-D tomography, excellent

signal-to-noise ratio, and noninvasiveness. Individual modalities lack one or more of these qualities and therefore, multimodality has been sought as active imaging technology in basic research and biomedical applications. Independent implementation of imaging probes for different modalities cannot be an ideal solution to achieve multimodal imaging, because different probes often differ in their biodistribution and other pharmacodynamic properties. Thus, grouping the properties for different imaging modalities in the same chemical entity have been sought after. Multimodal imaging probes have components that function synergistically, complementing, and enhancing the functionality of each other. Notably, QDs are promising multimodal probes as it is possible to combine multiple probe characteristics in QDs. For example, fluorescence imaging using QDs can be combined with MRI and radiography imaging if interfaced with molecules/materials having paramagnetism and radioactivity on the surface of QDs [63, 64].

In vivo studies, in particular for cancer imaging and therapy, have been limited owing to the poor stability or short systemic circulation times in living animals. Aiming to this problem, Park et al. [65] described tumor targeting, long circulating, micellar hybrid NPs (MHNs) that contain Magnetic Nanoparticles (MNs), QDs, and the anti-cancer drug Dox within a single poly(ethylene glycol)-phospholipid micelle modified with F3 peptide, and provide the first example of simultaneous targeted drug delivery and dual-mode NIR fluorescence imaging and MRI of diseased tissue in vitro and in vivo. The PEG coating of micelles prevented them from recognition and endocytosis by reticuloendothelial system, and prolonged the circulation and targeting time, which was a key factor for the successful application in vivo.

3.4.1.2 Bioconjugated QDs for Cancer Diagnosis

Biomarker assays may be useful for the screening, diagnosis and prognosis of disease, monitoring the effect of treatment and detecting cancer if a set of molecular markers can be quantified and statistically differentiated between cancerous cells and healthy cells. In early stage of cancer, biomarkers are often present at very low concentrations, so methods capable of low detection limits are required. QDs are emerging as promising probes for ultrasensitive detection of cancer biomarkers. QDs attached to antibodies, aptamers, oligonucleotides, proteins, or peptides can be used to target cancer markers. Their fluorescent properties have enabled QDs to be used as labels for in vitro assays to quantify cancer biomarkers, and they have been investigated as in vivo imaging agents [47, 66–68]. Antibodies are proteins that are capable of specific recognition of an antigen, and have been used in QD assays to detect carcinoembryonic antigen (CEA) [69] and prostate-specific antigen (PSA) [70]. Aptamers are synthetic single-stranded DNA or RNA that are selected for their high binding affinity from a random library of 10^{13} to 10^{15} oligonucleotides in an in vitro process termed “systematic evolution of ligands by exponential enrichment” (SELEX) [71].

These nanometer-sized semiconductor particles bind to target biomolecules through electrostatic interaction, covalent cross linking, or via the implication of specific tagging molecules. QDs have been used as biological probes for the simultaneous detection of multiple biomarkers directly from physiological components [25]. During the past two decades several groups have reported use of QDs for detection of different types of cancers (Table 3.1).

Wu et al. [84] explored a new technology to label HER2 (human epidermal growth factor receptor 2) on breast cancer cell membrane, which is known as c-erbB-2/HER2/neu and overexpressed in approximately 25–30 % invasive breast cancer and plays an important role in breast cancer prognosis and treatment selection. This study reported the multiplexed detection of breast cancer markers using semiconductor QDs as immunofluorescent probes. Simultaneous detection of HER2 and other cellular targets was performed using QDs with different emission spectra conjugated to IgG and streptavidin. This study testified the efficiency of QDs for simultaneous labeling of multiple molecular targets at a subcellular level. After that, several studies on the detection of HER2 for breast cancer diagnosis with QDs have completed [85, 87, 88]. Yezhelyev et al. [89] reported the use of multicolor QDs for quantitative and simultaneous profiling of multiple biomarkers using intact breast cancer cells and clinical specimens and the comparison between the new QDs-based molecular profiling technology with standard western blotting and Fluorescence In Situ Hybridization (FISH). The multicolor bioconjugates were used for simultaneous detection of the five clinically significant tumor markers, including HER2 (QD-HER2), Estrogen Receptor (QD-ER), Progesterone Receptor PR (QD-PR), Epithelial Growth Factor Receptor (QD-EGFR), and mammalian Target Of Rapamycin (QD-mTOR), in breast cancer cells MCF-7 and BT474.

Multiplexed detection of cancer biomarkers, CEA and alpha-fetoprotein (AFP), directly from crude serum using a QD-based microfluidic protein chip was recently reported [78]. In this study, they designed a versatile fluorescent probe by conjugating secondary antibodies (goat anti-mouse IgG), QDs, and found that the QD-based protein chip could rapidly detect CEA and AFP with high sensitivity and selectivity, even in human serum and in the format of both sandwich immunoassay, and reverse phase immunoassay. Multicolor imaging and multiplexed bioassay using QDs directly prepared in aqueous phase CdTe/CdS with different emission wavelengths were also developed. QD-antibody conjugates are also well suited for the multiplexed detection of low abundance cancer biomarkers directly on human tissue biopsies [90, 91]. Use of multicolor and multiplexing capabilities of semiconductor QDs, enabled the authors to detect four protein biomarkers. First is CD15 a transmembrane protein expressed in malignant Hodgkin and Reed-Sternberg (HRS) cells and certain types of epithelial cells. And the last three are CD30 cytokine receptor belonging to the tumor necrosis factor (TNF), CD45, and Pax5. This last used to detect the malignant HRS cells from infiltrating immune cells such as T and B lymphocytes of Hodgkin's lymphoma from lymphoma tissues (Fig. 3.4 [90]).

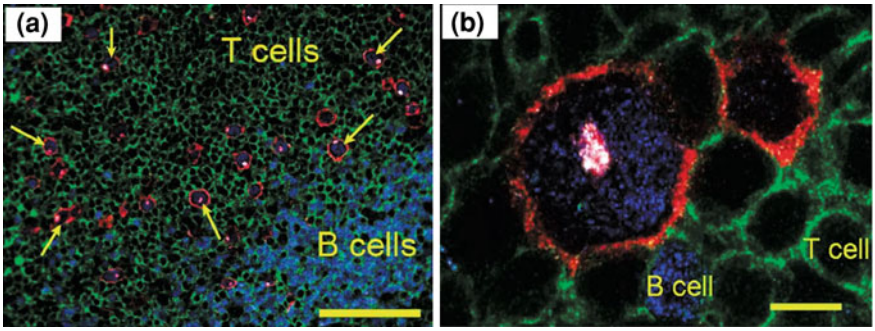

Fig. 3.4 Multiplexed QD staining images of HRS malignant cells and infiltrating immune cells on lymph node tissue specimens of a Hodgkin's lymphoma patient. **a** Malignant HRS cells (*red* membrane, *blue* nuclear, and *red/whitish* Golgi) are identified by a unique multiplexed staining pattern of CD30 positive (membrane staining), CD15 positive (Golgi staining), Pax5 positive (nuclear staining), and CD45 negative. They are differentiated from infiltrating B cells (*blue* nuclear staining) and T cells (*green* membrane staining). A few prominent HRS cells are indicated with arrows. Scale bar: 100 μm. **b** Detailed view showing the distinct staining patterns of HRS cells, B cells, and T cells. Scale bar: 10 μm. Reprinted with permission from Analytical Chemistry, Vol. 82, N°14, july 15, 2010. Copyright (2011) American chemical Society [90]

Simultaneous visualization of multiple biomarkers using multiplexed QD staining was beneficial for the selective identification of rare HRS cells, a primary diagnostic target for Hodgkin's disease, which was not achievable using traditional immunohistochemistry (IHC) assays.

In another studies, Shi et al. [92] showed the superior quality of QDs, in comparison to IHC, for the detection of androgen receptor (AR) and PSA in prostate cancer cells. With QDs probes conjugated to a PSMA monoclonal antibody (Ab), another marker for prostate cancer diagnosis and therapy, Gao et al. [47] have achieved sensitive and multicolor fluorescence imaging of cancer cells under in vivo conditions (Fig. 3.5). Both of those two studies, showing the potential ability of QDs as a diagnosis technology, are good examples to demonstrate why QDs are promising nanoparticles for diagnostic applications. In a second study, Gao et al. [93] demonstrated the potential of QDs as a new diagnosis technology for metastasis prostate cancer.

Usually, antibodies conjugated to QDs are full-length antibody, which leads to dramatically reduced binding activities. Recently, a study demonstrated that the use of single-chain antibody fragments (scFvs) conjugated with QDs appears to have a number of advantages, in terms of solubility, activity, ease of preparation and ease of structure-based genetic engineering, which were approved by detecting prostate cancer cells [94]. In another study, CdSe/CdS/ZnS QDs were used for improved photoluminescence efficiency and stability as optical agent for imaging pancreatic cancer cells using transferrin and anti-Claudin-4. Pancreatic cancer specific uptake is also demonstrated using the monoclonal antibody anti-Claudin-4. This targeted QDs platform will be further modified to develop early detection imaging tool for pancreatic cancer [95]. One of the greatest challenges in preparing highly efficient

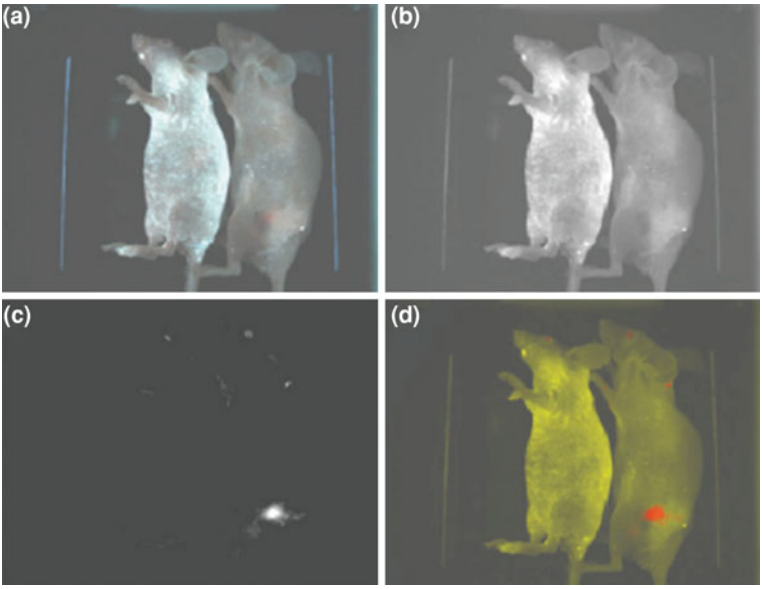

Fig. 3.5 Spectral imaging of QD-PSMA Ab conjugates in live animals harboring C4-2 tumor xenografts. *Orange-red* fluorescence signals indicate a prostate tumor growing in a live mouse (*right*). Control studies using a healthy mouse (no tumor) and the same amount of QD injection showed no localized fluorescence signals (*left*). **a** Original image; **b** Unmixed autofluorescence image; **c** Unmixed QD image; and **d** Super-imposed image. After in vivo imaging, histological and immunocytochemical examinations confirmed that the QD signals came from an underlying tumor. Note that QDs in deep organs such as liver and spleen were not detected because of the limited penetration depth of visible light. REPRINTED BY PERMISSION FROM Macmillan Publishers Ltd on behalf of Cancer Research UK: [Nature biotechnologies] [47], copyright (2004)

QDs involves the selection of QDs core, biocompatible, and nontoxic. For imaging live pancreatic cancer cells Yong et al. [96] used noncadmium-based InP/ZnS QDs conjugated with pancreatic cancer specific monoclonal antibodies, such as anti-Claudin-4 which allow specific in vitro targeting of pancreatic cancer cell line. The receptor mediated delivery of the bioconjugates was further confirmed by the observation of poor in vitro targeting in nonpancreatic cancer cell lines without Claudin-4 receptor. These observations suggest the immense potential of InP/ZnS QDs as noncadmium based safe and efficient optical imaging nanoprobes in diagnostic imaging.

AFP is an important tumor marker for hepatocellular carcinoma (HCC). In a prior study, Liang Chen [97] used CdSe/ZnS QDs with emission wavelength of 590 nm (QDs 590) linked to AFP monoclonal antibody (Ab) as a probe for fluorescence spectral analysis of HCC. In another study [98], they tested the biocompatibility, hemodynamics, tissues distribution of the QDs-AFP-Ab probes, and studied the imaging of HCC and its metastasis in vitro and in vivo. Their results indicate that such QDs-based probes have good stability, specificity, and biocompatibility for ultrasensitive fluorescence imaging of molecular targets in their liver cancer model system.

There are evidences supporting the application of QD-conjugated protein microarrays for the detection of cancer biomarkers [82]. Use of QD-conjugated PSA anti-bodies for fabrication of protein biochip allowed selective detection of the target protein PSA and effectively minimized nonspecific binding. High specificity, reduction of nonspecific binding, and elimination of need of any blocking or additional biotin–streptavidin interactions have been achieved by QD-conjugated protein microarray, which are improvement over the conventional microarray approaches.

Apart from classical QDs, rod shaped nanocrystals known as quantum rods (QRs) are also attractive probes for cancer detection and imaging [44]. Authors have employed CdSe/CdS/ZnS QRs bioconjugated with lysine and transferrin (Tf) for specific targeted bioimaging. The internalization of QR-Tf bioconjugates in human cancer cell line (HeLa) which overexpress the transferrin receptor (TfR) was demonstrated by confocal and two-photon imaging. The application of QRs as biological probes has started recently and few initial studies have suggested that they can act as brighter single molecule probes than QDs and exhibit good potential for biomedical applications [99].

In summary, the use of QDs in cancer investigations has increased dramatically due to their unique size-dependent optical properties. Trends in the applications of bioconjugated QDs in cancer research clearly show that QDs can provide powerful tools for cancer management. There is still scope for improvement in terms of developing QD probes with improved target specificity, signal intensity, multimodality, and therapeutic potentials. However, toxicological and pharmacological issues remain in the advancement of QD technology towards the diagnosis and therapy of cancer and other diseases. This advancement is setback due to mismatch between rapid developments of QD bioprobes and fewer studies on the toxicology [100].

3.4.2 Bioconjugated QDs Used as Biosensors

Fluorescence resonance energy transfer (FRET) involves the transfer of fluorescence energy from a donor particle to an acceptor particle whenever the distance between the donor and the acceptor is smaller than a critical radius, known as the Förster radius [101]. This leads to a reduction in the donor's emission and excited state lifetime, and an increase in the acceptor's emission intensity. FRET is suited to measuring changes in distance, rather than absolute distances, making it appropriate for measuring protein conformational changes [102], monitoring protein interactions [103], and assaying of enzyme activity [104]. Several groups have attempted to use QDs in FRET technologies [105], particularly when conjugated to biological molecules [106], including antibodies [107], for use in immunoassays. QDs can be used as donors in assays involving FRET [13, 14, 72, 108, 109], or as acceptors in bioluminescence resonance energy transfer (BRET) [110, 111]. In a recent study, quantitative maltose sensing has provided an example of how QDs might play a role in enzyme assays. In this study, QDs

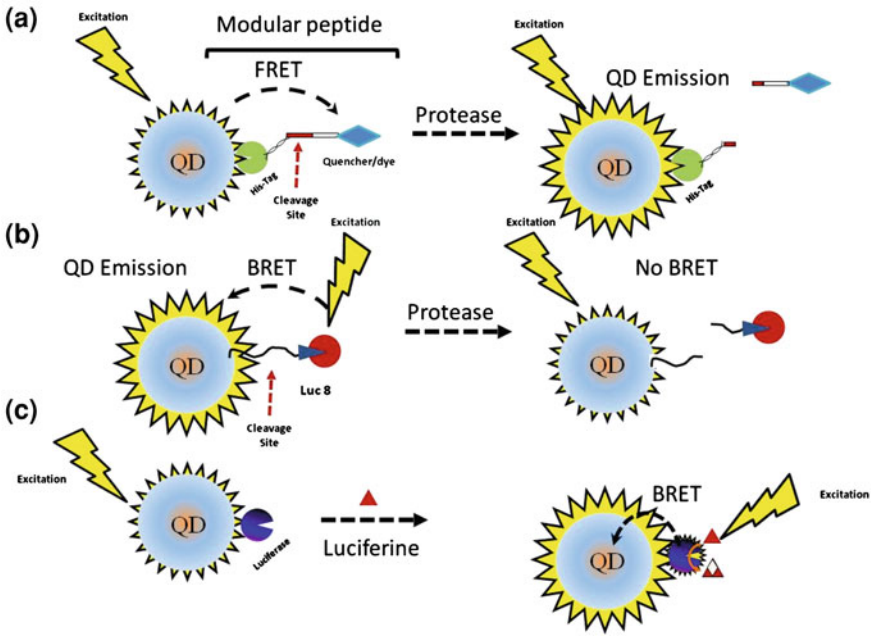

Fig. 3.6 Schematics diagrams showing biosensing for enzymes. **a** QD base protease activity sensors. Quencher conjugated protease substrate peptides bind the QD through a His-tag. Where QD fluorescence is quenched through FRET. Upon proteases mediated substrate cleavage the QD fluorescence is recovered [14, 72]. **b** A BRET strategy for analysis of protease, in the absence of protease, the peptide linker holds Luc8 in close proximity to the QD, and transfer of bioluminescence energy to the QD occurs. In presence of an active protease, the peptide is cleaved, and BRET is disrupted. Reproduced from [110] with permission of ACS. **c** QD is covalently coupled to a BRET donor, Luc8. The bioluminescence energy of Luc8-catalysed oxidation of Luciferine is transferred to QD, resulting in quantum dot emission [111]

conjugated to maltose binding protein (MBP) allowed binding of either maltose or a quenching molecule [11]. The quenching molecule, with a binding affinity similar to that of maltose, was readily displaced on addition of maltose, and a concentration-dependent increase in luminescence was observed. Several studies have exploited QD-FRET for imaging activity of proteases [112–115]. For this application, a QD-probe conjugate is bound to a quencher probe by a peptide sequence which is recognized by a protease, in which state the fluorophore is quenched. On cleavage of the two molecules by a protease, emission is restored, allowing its activity to be visualized (Fig. 3.6a). QDs gave an increased luminescence compared to previous results using organic fluorophores [116, 117]. In addition to FRET, QDs can be involved in another nonradiative energy transfer process known as BRET [118, 119]. The mechanism is similar, but the source of energy does not come from an external light source [119]. The BRET process involves a donor with intrinsic fluorescent properties, for example a light-emitting protein, and QDs are energy acceptors. The method is advantageous because the

possibility of direct excitation of the acceptor molecule by external light is eliminated. Therefore, the background signal is lower [110]. A BRET-based sensor has been developed to detect the activity of matrix metalloproteinases MMP-2, MMP-7, and urokinase-type plasminogen activator (uPA) [110]. Luciferase-catalyzed substrate oxidation produced luminescence, which was the source of energy in the system. The QD and luciferase were held in close proximity by a peptide linker, and cleavage by a specific protease disrupted BRET (Fig. 3.6b). The protease concentration was determined from the change in BRET ratio, as an increase in protease concentration caused a decreased BRET ratio (QD emission decreased, and luciferase bioluminescence increased). The reliability of the assay is enhanced because rather than the change in acceptor emission, the change in the ratio of emission from donor and acceptor is measured [110]. Another representative BRET study using QD acceptors was reported by Rao et al. [111]. They selected an optimized eight-mutation variant of luciferase (Luc8) with improved catalytic efficiency to facilitate BRET; an average of six copies of Luc8 were coupled, via EDC condensation, to carboxyl-modified 655 nm emitting QDs. Upon addition of coelenterazine substrate to the complex, a strong emission peak from the QDs was detected in addition to the 480 nm Luc8 donor emission (Fig. 3.6c). To estimate the efficiency of these interactions, a BRET ratio (similar to FRET efficiency) defined as QD acceptor (A) emission-to-Luc8/donor (D) emission was used. By using different emission/colors of QDs and varying the center-to-center separation distance, the authors found that the BRET ratio was sensitive to both changes in D-A separation distance and the overall "spectral overlap" between Luc8 emission and QD absorption, with redder emitting QDs providing more efficient energy transfer. Furthermore, the authors coupled Luc8 to QDs with emissions ranging from 605 to 800 nm and observed distinct emissions from each or all when mixed together in a multiplex format. Testing these conjugates in cell lines and in vivo within mice tissues, they showed that after addition of the substrate complex, luminescence spectra characteristic of the QD combination used could be collected and deconvolution of each QD color can be performed. Additionally, enhanced sensitivity and high signal to background ratios were measured when performing in vivo imaging in mice for comparatively small amounts of QD Luc8 conjugates.

3.5 Conclusion

QDs have drawn interests due to their unique and advantageous optical properties, which include broad absorption, narrow size-tunable photoluminescence spectra, and superior resistance to photobleaching [120]. They have appeared as a new promising class of fluorescent probes for biomolecular detection, cell-based application, and in vivo animal imaging. QDs' size-tunable properties enable them to solve the problems of spectral overlap in conventional organic labels. Moreover, QDs can be immobilized on solid surfaces or embedded in microbeads to realize multiplexed detection. At the same time, QDs optical properties such as

photostability and narrow emission spectra also make them robust labels in cell-based applications. Besides these regular advantages, QDs exceed fluorescent dyes in in vivo imaging because of their fluorescence in NIR region, which offers low tissue absorption. However, the use of QDs in biological systems, especially in in vivo application, suffers from their toxicity. Due to their chemical composition of toxic metal atoms (e.g. Cd, Hg, Pb, As), hindrance exists when QDs are applied in living cells and animals [121]. Although metal ions like divalent cadmium today are covered with inert zinc sulfide and encapsulated within a stable polymer, they might still be toxic in living bodies. Moreover, QDs could aggregate or bind nonspecifically to cellular membranes and intracellular proteins [121]. It has been reported that concentration of cadmium in the liver and kidneys could gradually increase after intravenous administration of cadmium-based QDs [122]. Former studies have shown that QDs' size, shape, and surface coating all could affect their toxicity [121]. Overall, the unique optical properties of QDs and the modulation of those properties have provided researchers versatile toolkit for bioanalysis [123]. Importantly, multiplexed detection is possible as a new type of simple, flexible method for novel diagnostic technologies, and intracellular probes [123]. Both quantum efficiency and sensitivity should be increased in the future [124]. As for in vivo applications, new types of QDs exempt from heavy metal atoms should be developed [125, 126]. Moreover, nanoparticle distribution, excretion, metabolism, pharmacokinetics, and pharmacodynamics should be included in nanotoxicology studies in animal models in vivo [121]. The next challenge associated with QD-based medical applications is the commercialization of the products and development of the appropriate regulations. Undoubtedly, biologists will catch on to these exciting developments and will find as yet unforeseen applications for this new toolkit, thus enhancing and complementing their existing arsenal of bioimaging tools.

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