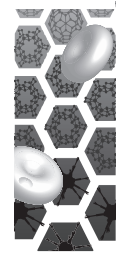
REVIEW

Quantum dots and multifunctional nanoparticles: new contrast agents for tumor imaging



Matthew N Rhyner[1], Andrew M Smith[1], Xiaohu Gao[2], Hui Mao[3], Lily Yang[4] & Shuming Nie[1,5†]

†Author for correspondence
[1]Department of Biomedical Engineering, Chemistry, Hematology and Oncology, and the Winship Cancer Institute, Emory University and Georgia Institute of Technology, 101 Woodruff Circle, Suite 2001, Atlanta, GA 30322, USA
Tel.:+1 404 712 8595; Fax:+1 404 727 3567
E-mail: snie@emory.edu
[2]Department of Bioengineering, University of Washington, Bagley Hall, Room 412 Campus Box 351721, Seattle, WA 98195, USA
[3]Department of Radiology, Emory University, Atlanta, GA 30322, USA
[4]Department of Surgery and Winship Cancer Institute, Emory University, 1365 Clifton Road, Suite C4000, Atlanta, GA 30322, USA
[5]Center for Biotechnology and Bioengineering, Hunan University, Changsha, China

Nanometer-sized particles, such as semiconductor quantum dots and iron oxide nanocrystals, have novel optical, electronic, magnetic or structural properties that are not available from either molecules or bulk solids. When linked with tumor-targeting ligands, such as monoclonal antibodies, peptide fragments of tumor-specific proteins or small molecules, these nanoparticles can be used to target tumor antigens (biomarkers) as well as tumor vasculatures with high affinity and specificity. In the 'mesoscopic' size range of 5–100 nm diameter, quantum dots and related nanoparticles have large surface areas and functional groups that can be linked to multiple diagnostic (e.g., optical, radioisotopic or magnetic) and therapeutic (e.g., anticancer) agents. In this review, we discuss recent advances in the development and applications of bioconjugated quantum dots and multifunctional nanoparticles for *in vivo* tumor imaging and targeting.

The development of high-sensitivity and high-specificity probes is of considerable interest in many areas of cancer research, ranging from basic tumor biology to *in vivo* imaging and early detection. The process to develop new imaging probes, however, remains a slow and expensive undertaking. Faced with this challenge, research on quantum dots (QDs) and self-assembled nanomaterials has attracted much attention because it could lead to a new generation of nanoparticle imaging probes for *in vivo* tumor imaging at high sensitivity and specificity. Indeed, recent advances have led to the development of functional nanoparticles (electronic, optical, magnetic or structural) that are covalently linked to biological molecules, such as peptides, proteins and nucleic acids [1–21]. Owing to their size-dependent properties and dimensional similarities to biomacromolecules, these nanobioconjugates are well suited as contrast agents for biomedical imaging [22,23], as controlled carriers for drug delivery and as structural scaffolds for cellular and tissue engineering [24,25].

In comparison with traditional *in vivo* imaging probes or contrast agents, such as radioactive small molecules in positron emission tomography (PET) and single photo emission computed tomography (SPECT), gadolinium compounds in MRI and labeled antibodies, targeted QDs and other bioengineered nanoparticles provide several unique features and capabilities. First, their size-dependent optical and electronic properties can be tuned continuously by changing the particle size [26]. This 'size effect' provides a broad range of nanoparticles for simultaneous detection of multiple cancer biomarkers. Second, nanoparticles have more surface area to accommodate a large number or different types of functional groups that can be linked with multiple diagnostic (e.g., radioisotopic or magnetic) and therapeutic (e.g., anticancer) agents. This provides the opportunity to design multifunctional 'smart' nanoparticles for multi-modality imaging as well as for integrated imaging and therapy. Third, extensive research has shown that nanoparticles in the size range of 10–100 nm are accumulated preferentially at tumor sites through an effect called enhanced permeability and retention (EPR) [27–29]. This effect is believed to arise from two factors: growing tumors produce vascular endothelial growth factor (VEGF) that promotes angiogenesis and many tumors lack an effective lymphatic drainage system, which leads to subsequent macromolecule or nanoparticle accumulation. This causes tumor-associated neovasculatures to be highly permeable, allowing the leakage of circulating macromolecules and nanoparticles into the tumor tissue.

These novel properties have opened exciting avenues for developing new and advanced nanoparticle probes for biomedical imaging, especially for tumor imaging and targeting. Here, we briefly discuss the novel properties of semiconductor QDs and dual-modality imaging probes and their applications in tumor imaging.

Keywords: contrast agents, fluorescence, magnetic resonance imaging , nanoparticles, positron emission tomography , quantum dots, tumor imaging

future medicine

Novel properties of QDs

QDs are crystalline semiconductors typically less than 10 nm in diameter that have been studied for over 20 years, only recently having made the jump into biomedicine [2,3]. The moniker 'quantum dot' refers to the quantum confinement of charge carriers within this small size range. Researchers have developed many different types of 'quantum' materials, including quantum wells, holes and rods, each with different states of quantum confinement. Several production methods are available, from photolithography to wet chemical synthesis. The QDs produced in colloidal solutions are the most useful for biomedical applications because high-quality nanocrystals can be prepared in large quantities at low costs.

The highest quality QDs are composed of II-VI, IV-VI or III-V semiconductors [30–32]. The most common QD structure is a CdSe core with a thin, protective shell of ZnS (both II-VI materials). Colloidal QDs are produced using surfactant micelles, coprecipitation or high temperature organic solvent synthesis. The latter produces the highest quality materials [3,33,34]. This synthesis leaves QDs with a surface monolayer coating of nonpolar coordinating ligands; as a result, the final QDs are highly hydrophobic and must be transferred into aqueous solutions before biological use. This is accomplished through the use of a bifunctional ligand to replace the hydrophobic surface molecule or by using a polymer coating to protect the hydrophobic surface from the external aqueous environment.

In comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties. First, QDs have molar extinction coefficients that are 10–50-times larger than that of organic dyes, which make them much brighter in photon-limited *in vivo* conditions. Further, QD emission wavelengths are size-tunable. For example, CdSe/Zns QDs of approximately 2 nm in diameter produce a blue emission, whereas QDs approximately 7 nm in diameter emit red light [35]. In recent work, researchers have even pushed the emission wavelength into the near infrared (650 nm to 950 nm) to take advantage of improved tissue penetration depth and reduced background fluorescence at these wavelengths [36]. A key property for *in vivo* imaging is the broad QD Stokes shift, which can be as large as 300–400 nm, depending on the wavelength of the excitation light [37]. In conjunction with the broadband absorption and narrow emission peaks of QDs, this property allows multiplexed imaging applications in which one light source is used to simultaneously excite multicolor QDs without the need for complicated instrumentation. Another important feature is the long-term photostability of QD imaging probes, which opens the possibility of investigating the dynamics of cellular processes over time, such as continuously tracking cell migration, differentiation and metastasis. These properties have made QDs a topic of intensive research in cancer imaging, molecular profiling and cancer biology.

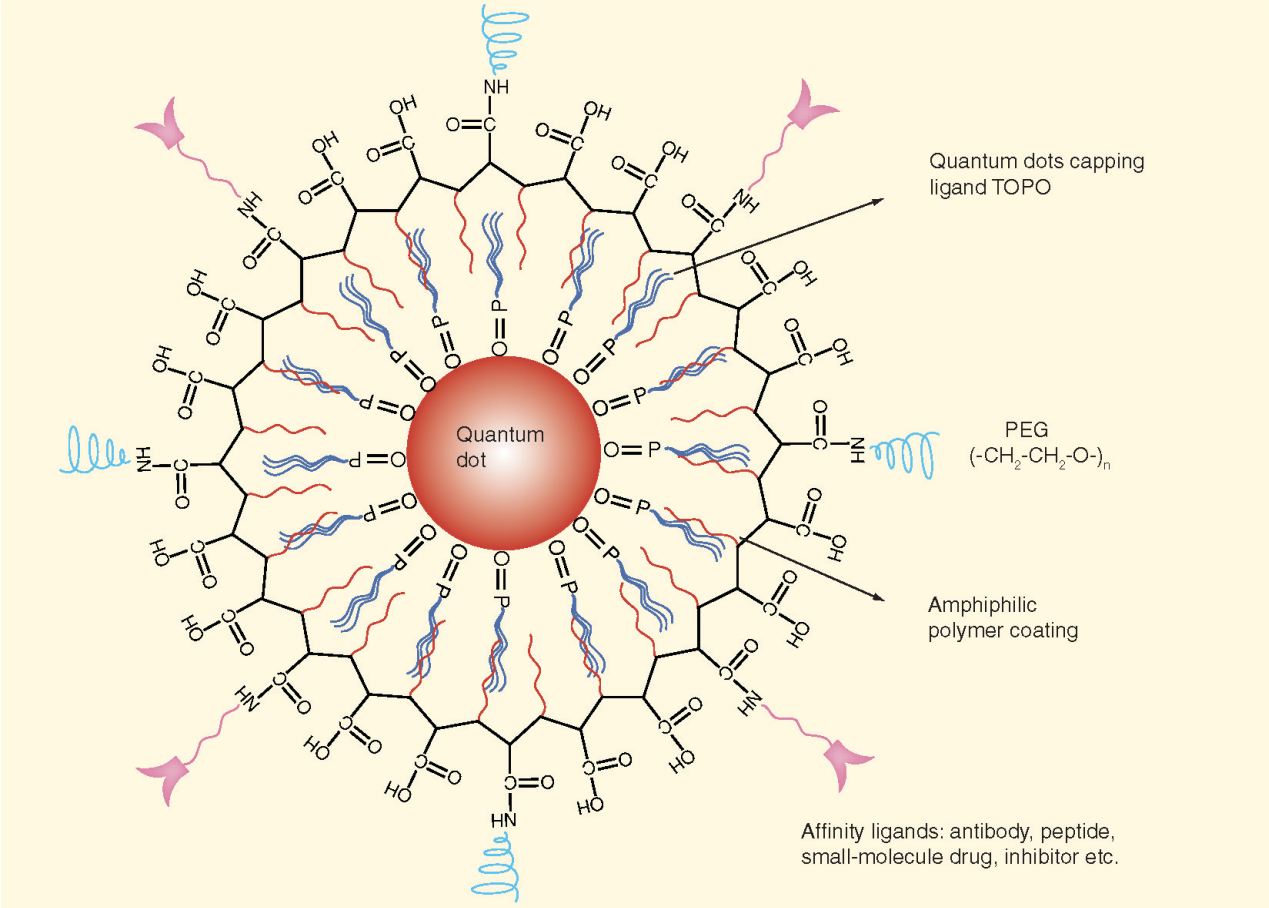
Design of nanoparticle probes

In general, hydrophobically synthesized QDs must be transferred to an aqueous phase before they can be used in biological systems. To accomplish this, the hydrophobic surface ligands can either be exchanged with bifunctional ligands or the entire QD can be coated with an amphiphilic polymer layer. In recent work, Gao and colleagues encapsulated luminescent QDs with a biocompatible copolymer and linked this amphiphilic polymer to tumor-targeting ligands (**Figure 1**) [37]. *In vivo* targeting studies of human prostate cancer in nude mice indicate that this class of QD probes can be delivered to tumor sites by both the EPR effect and by antibody binding to cancer-specific cell-surface markers. For instance, using either subcutaneous injection of QD-tagged cancer cells or systemic injection of multifunctional QD probes, the authors achieved sensitive and multicolor fluorescence imaging of cancer cells under *in vivo* conditions [37]. A whole-body macro-illumination system was also integrated with wavelength-resolved spectral imaging for efficient background removal and precise delineation of weak spectral signatures. With a large number of functional groups on its surface, this encapsulated QD also provides a nanoscale scaffold for linking to additional imaging agents or therapeutics drugs, as discussed in more detail below.

Attachment of biomolecules

This robust design allows the attachment of a wide variety of targeting ligands, such as antibodies, peptides or small molecules. However, the strategy for conjugating ligands to the surface must be considered carefully. For example, the multivalency effect, in which enhanced affinity can be achieved through high numbers and/or special combinations of targeting ligands per QD surface, could be harnessed to achieve superior *in vivo* targeting [38,39]. While the theoretical frame-

Figure 1. Schematic diagram of multifunctional quantum dot probes.



Showing the capping ligand trioctylphosphine oxide (TOPO), an encapsulating copolymer layer, tumor-targeting ligands (such as peptides, antibodies, or small-molecule inhibitors) and polyethylene glycol (PEG). Adapted with permission from [37].

work and observations of multivalency in viruses, cell signaling and lymphocyte homing are all promising, several hurdles must be overcome before engineers can make use of this phenomenon. First, current conjugation strategies result in nanoparticle probes with a wide distribution in the number of targeting ligands per particle. In addition, the exact orientation and conformation of the targeting ligand is usually unknown [40], so a certain percentage of the probe is probably nonfunctional. Alivisatos and coworkers used a gold–DNA construct to separate QDs with distinct numbers of double-stranded DNA attached to the surface and observed that large DNA strands (100mer) had much sharper agarose gel separations than small strands (50mer) [41]. Still, it is a challenge to separate populations of QDs with distinct numbers of targeting ligands, which will be important to minimizing probe size and to maintaining binding affinity and specificity [38,42].

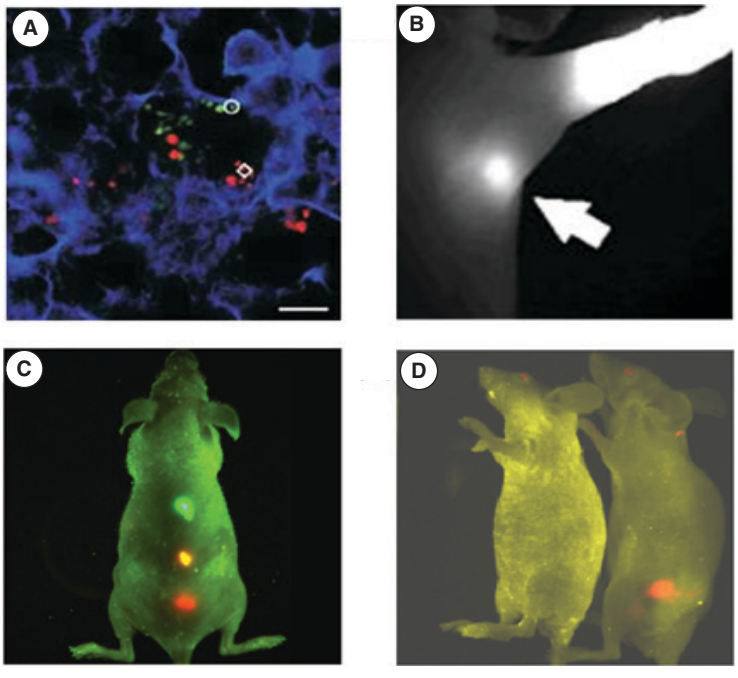
Dual-modality probes

Optical imaging is highly sensitive, but its use *in vivo* and in humans is hampered by a limited penetration depth and the lack of anatomic resolution and spatial information. Although near-infrared wavelengths can be used to improve the penetration depth and 3D fluorescence tomography can be used to provide spatial information [43,44], other imaging modalities, such as MRI, are more suited for tomography and 3D imaging. Thus, there has been considerable interest in developing dual-modality contrast agents for combined optical and MR imaging, which has exceptional tissue contrast and spatial resolution and has been used widely in the clinical setting. For example, by reacting superparamagnetic iron oxide nanoparticles with the fluorescent dye Cy5.5, Josephson and coworkers have developed dual magneto-optical probes that are able to bind to apoptotic cells and are detectable by

fluorescence and MR imaging [45]. Similarly, dual magnetic and optical imaging probes have been used to yield highly detailed anatomical and molecular information in living organisms [46]. These probes are prepared by conjugation of peptides to cross-linked iron oxide amine (amino–CLIO), either by a disulfide linkage or a thioether linker, followed by the attachment of the dye Cy5 or Cy7. Fluorescence quenching of the attached fluorochrome occurs by electronic coupling among the dye chromophores (self-quenching). This class of dual-modality probes provides the basis for 'smart' nanoparticles, which are capable of pinpointing their position through their magnetic properties while providing information on their environment by optical fluorescence signals.

Recent research has shown that QDs can also be linked with Fe_2O_3 or FePt to generate dual functional nanoparticles [47–49]. In principal, these systems should lead to a T2-based 'darkening' contrast offered by iron oxide-based contrast agents. A major hurdle for such probes is the ability to deliver a high concentration of magnetic nanoparticles to an *in vivo* disease site. MRI has fairly low contrast agent sensitivity, especially compared with fluorescence, and, as such, considerably more magnetic nanoparticles must reach the disease site compared with fluorescent probes [23]. To overcome this sensitivity problem and to provide a T1 brightening effect, Mulder and colleagues have recently linked gadolinium to the surfaces of QDs using polymer-conjugated lipids [50]. These polymer-protected QDs offer excellent optical properties (high fluorescence quantum yields, narrow spectral widths and high photostability). By linking targeting ligands through a biocompatible polyethylene glycol (PEG) spacer, these Gd-based dual-modality nanoparticle probes are promising for *in vivo* tumor imaging in animal models. Preliminary work has utilized similar dual modality probes in cells [51], but there is still no published work demonstrating correlated magnetic and optical *in vivo* imaging from a single QD-containing imaging agent.

Figure 2. *In vivo* targeting and imaging with quantum dots.



(A) *Ex vivo* tissue micrograph of quantum dot (QD)-labeled cancer cells trapped in a mouse lung. **(B)** Near infrared fluorescence image of water-soluble Type-II QDs taken up by sentinel lymph nodes [20]. **(C)** *In vivo* image of multicolor QD-encoded microbeads injected into a live mouse [37]. **(D)** Molecular targeting and *in vivo* imaging of a prostate tumor using a QD–antibody conjugate [37].

Integrated therapeutic & diagnostic platforms

Nanoparticles also offer a wide range of surface functional groups to allow chemical conjugation to both diagnostic and therapeutic agents. It is thus possible to design and develop multifunctional nanostructures that could be used for simultaneous tumor imaging and treatment. However, progress has been slow and promising multifunctional platforms, such as dendrimers, liposomes and PEBBLES (probes encapsulated in biologically localized embedding), are still at a 'proof-of-concept' stage using cultured cancer cells, which are not immediately relevant to *in vivo* imaging and the treatment of solid tumors [49,52–55].

In vivo tumor imaging & targeting

Mapping sentinel lymph nodes & tumor angiogenesis

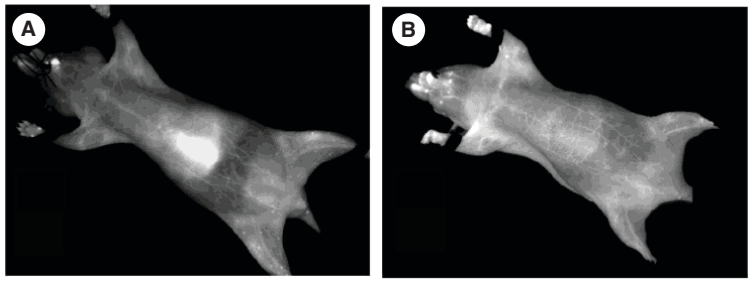
In vivo imaging with QDs has been reported for lymph node mapping, blood pool imaging, angiogenic vessels and cell subtype isolation (**Figure 2**) [56]. For instance, Bhatia and coworkers used peptide-conjugated QDs to target lung tissues, tumor blood vessels and lymphatic conduits [5]. In addition to demonstrating that peptide specificity could be used to target QDs *in vivo*, they also showed the usefulness of attaching a PEG coating to the surface of nanoparticles to reduce uptake by the reticuloendothelial system (RES). In other work, Hoshino and colleagues pre-loaded cells with QDs and detected their fluorescence in the kidney, liver, spleen and lung after resection [57].

Ballou and coworkers injected PEG-coated QDs into the mouse blood stream and studied how the surface coating affects their circulation lifetime (**Figure 3**) [58]. In contrast to small organic dyes (which are eliminated from circulation within minutesthe blood circulation for an extended period of time (half-life of more than 3 h). This long-circulating feature can be explained by the unique structural properties of QD nanoparticles. PEG-coated QDs are in an intermediate size range – they are large enough to avoid renal filtration, but they are small and hydrophilic enough to slow down opsonization (nonspecific binding of proteins) and reticuloendothelial uptake.

An exciting advance was the demonstration of near-infrared QDs as intrasurgery guides for sentinel lymph node resection both in small mice and larger pigs [59–61]. Kim and colleagues demonstrated rapid uptake of nontargeted QDs into lymph nodes, after intradermal injection, allowing clear imaging and delineation of involved sentinel nodes (which are often removed surgically in patients diagnosed with cancer). This work highlights the possibility that QD probes could be used for real-time intra-operative optical imaging, providing an *in situ* visual guide so that a surgeon could quickly and accurately locate and remove sentinel nodes or even small lesions (e.g., metastatic tumors) that may be difficult to identify without imaging guidance [59].

Using two photon excitation and 550 nm dots, QDs circulating in blood vessels were imaged up to 100 µm deep [9]. The two-photon absorption cross-sections of QDs are 2–3-orders of magnitude larger than those of traditional organic fluorophores. Also, multiphoton excitation studies showed that metastatic tumor cell extravasation could be tracked by using QDs and fluorescence emission-scanning microscopy after tissue resection [56]. The results indicated that QDs could be used to track a single metastatic cell from its originating tumor site through its localization to a new site. In addition, Jain and coworkers used a PEG-conjugated lipid encapsulation method to distinguish tumor blood vessels from both perivascular cells and extracellular matrix while studying the size-dependent access of these probes to the tumors [20]. The results demonstrated a much clearer boundary between blood vessels and cells than that achieved by using traditional high-molecular weight dextran agents. Most recently, So and coworkers created a self-illuminating QD probe based on bioluminescence resonance energy transfer [62]. After activation by the target enzyme, the luciferase-conjugated QDs were detected *in vivo* with no excitation light. This result points to enhanced, site-specific targeting with 'on or off' emission from QDs.

Figure 3. Noninvasive imaging of polyethylene glycol-coated 645 nm quantum dots circulating in mice 10 min after injection.

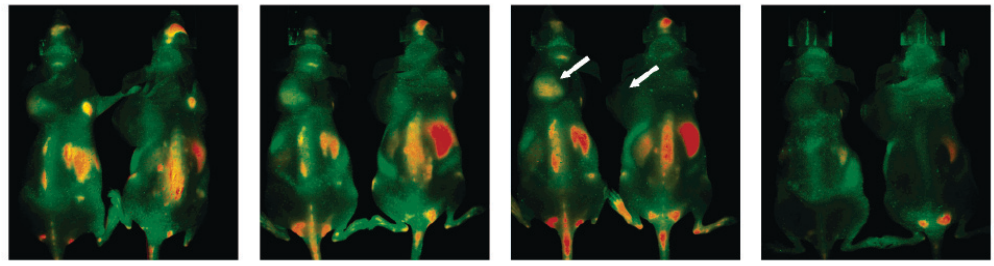


(A) Significant liver uptake is observed after injection with quantum dots coated with a 750-Dalton polyethylene glycol chain. **(B)** A larger PEG coating (5000 Daltons) slows uptake by the reticuloendothelial system, allowing the nanoparticles to remain distributed evenly throughout the circulatory vessels [58].

Tumor targeting and imaging

In the first report of targeted delivery of QDs in animals, Akerman and colleagues used QDs conjugated to peptides with affinity for tumor vasculatures, but the QD probes were not detected in living animals [5]. Nonetheless, their *in vitro* histological results revealed that QDs homed to tumor vessels guided by the peptides and were able to escape clearance by the RES. In 2004, We used a decorated triblock polymer coating to both passively and actively target tumors in a whole mouse [37]. For active tumor targeting, QDs were conjugated to an antibody for a prostate-specific membrane antigen (PSMA) (**Figure 2D**). The accumulation and retention of this PSMA antibody at the site of tumor growth is the basis of radioimmunoscintigraphic scanning (e.g., ProstaScint scan) and targeted therapy for human prostate cancer metastasis [63]. Most recently, Cai and colleagues used QDs conjugated to arginine–lysine–aspartic acid (RGD) peptides to target glioblastoma tumor vasculature in mice (**Figure 4**) [64]. The RGD peptide has specific affinity for the angiogenic factor integrin $\alpha_v\beta_3$, which is upregulated in growing tumors. As in previous work, this study demonstrated high specificity with peptide targeting, but spectral processing was needed to separate the QD signal from the tissue autofluorescence. In the future, near infrared

Figure 4. *In vivo* near-infrared fluorescence imaging of U87MG tumor-bearing mice (left shoulder, pointed by white arrows) injected with 200 picomoles of 705-RGD (left) and QD705 (right) probes, respectively.



All images were acquired under the same instrumental conditions. The mouse autofluorescence is color-coded green while the unmixed quantum dot (QD) signal is color-coded red. Prominent uptake in the liver, bone marrow and lymph nodes is visible. Long-term studies could yield valuable information about the pharmacodynamics of QDs and other nanoparticles *in vivo* [64].

QDs may eliminate the need for spectral processing, making *in vivo* QD targeting more attractive as an experimental technique.

Future perspective: prospects for clinical imaging

Cellular and solution-based diagnostic QD assays may appear in clinics in the next few years [8,11,21,56,65–67]. However, studies using animal models have raised new possibilities for *in vivo* tumor imaging, paving the way for the further development of targeted tumor imaging in cancer patients. For clinical applications, the current QD probes encounter several challenges, such as limited tissue penetration, lack of spatial resolution in tumor depth and potential toxicity concerns. To address these needs, it is first important to develop broadly tunable, high-efficiency, near-infrared-emitting QDs to improve tissue penetration depth. It is also important to develop new surface coatings that can mitigate the cytotoxic problems of QDs. While recent work has made clear strides towards near infrared QDs, the design of novel surface coatings is a challenging task. Work to date has shown that the type and size of QDs, their preparation methods and surface coatings all determine their toxicity profile [68–70]. The observed cytotoxicity probably arises from the release of cadmium ions, photochemical generation of free radicals, such as triplet oxygen, and nanoparticle aggregation on the cell surface [71,72]. The energy of UV-irradiation is close to that of a covalent chemical bond and can dissolve the semiconductor particles by photolysis, which releases toxic cadmium ions into the culture medium. It is also important to note that QDs with a stable polymer coating have been found to be essentially nontoxic to cells and animals, with no effect on cell division or ATP production (D Stuart, X Gao, S Nie, unpublished data). For clinical imaging applications, however, systematic studies are needed to understand the mechanisms of cadmium release, tissue and organ clearance and *in vivo* degradation of QD-based probes.

Concluding remarks

QDs have already fulfilled some of their promise as a new class of molecular probes for cancer research, while their applications in the clinical diagnosis and management of cancer patients are just beginning to be explored. With a versatile polymer coating, QDs have also provided a 'building block' for the assembly of multifunctional nanostructures and nanodevices. In particular, dual-modality imaging probes have been prepared by integrating QDs with paramagnetic or superparamagnetic agents. By correlating the deep imaging capabilities of MRI with sensitive optical imaging, a surgeon could visually identify tiny tumors or other small lesions during an operation and remove the diseased cells and tissue completely. Another desired multifunctional device would be the combination of a QD imaging agent with a therapeutic agent. Not only would this allow tracking of pharmacokinetics, but diseased tissues could be treated and monitored simultaneously in real time. Practical applications of these multifunctional nanodevices will only come with careful research, but the multi-

disciplinary nature of nanotechnology may expedite these goals by combining the great minds of many different fields.

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Executive summary

- Nanometer-sized particles have novel optical, electronic, magnetic or structural properties and are currently under intense development for applications in molecular and cellular imaging.
- Quantum dots are one type of nanoparticle with novel optical properties, such as size-tunable emission, improved signal brightness, resistance against photobleaching and simultaneous excitation of multiple fluorescence colors.
- Quantum dot probes have already fulfilled some of their promise as a new class of imaging probes for noninvasive tumor imaging *in vivo*, and have provided new opportunities for ultrasensitive and multicolor imaging at the molecular and cellular levels.
- Dual-modality and multifunctional probes can be developed by attaching molecular moieties with imaging, therapeutic and targeting functions to quantum dot-based nanometer-scaled scaffolds. These integrated nanoparticle probes may allow simultaneous imaging and therapy of tumors in live animal models.
- • Future work needs to address the potential long-term toxicity, degradation and metabolism of nanoparticle agents, to identify and develop new biomarker-probe systems and to develop multifunctional nanoscale platforms for integrated imaging, detection and therapy.

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