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Semiconductor Quantum Dots for Bioimaging and Biodiagnostic Applications

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Abstract

Semiconductor quantum dots (QDs) are light-emitting particles on the nanometer scale that have emerged as a new class of fluorescent labels for chemical analysis, molecular imaging, and biomedical diagnostics. Compared with traditional fluorescent probes, QDs have unique optical and electronic properties such as size-tunable light emission, narrow and symmetric emission spectra, and broad absorption spectra that enable the simultaneous excitation of multiple fluorescence colors. QDs are also considerably brighter and more resistant to photobleaching than are organic dyes and fluorescent proteins. These properties are well suited for dynamic imaging at the single-molecule level and for multiplexed biomedical diagnostics at ultrahigh sensitivity. Here, we discuss the fundamental properties of QDs; the development of next-generation QDs; and their applications in bioanalytical chemistry, dynamic cellular imaging, and medical diagnostics. For in vivo and clinical imaging, the potential toxicity of QDs remains a major concern. However, the toxic nature of cadmium-containing QDs is no longer a factor for in vitro diagnostics, so the use of multicolor QDs for molecular diagnostics and pathology is probably the most important and clinically relevant application for semiconductor QDs in the immediate future.

Keywords

nanotechnology; fluorescence imaging; cellular dynamics; multiplexing; cancer detection; single-cell analysis

1. INTRODUCTION

The development of nanoparticle probes for biomolecular imaging and diagnostics is currently an area of considerable interest (1–10). The basic concept is that nanometer-sized particles have functional and structural properties that are not available from either discrete molecules or bulk materials (1–3). When conjugated with biomolecular affinity ligands,

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DISCLOSURE STATEMENT

B.A.K., A.M.S., and S.M. hold patents related to QD synthesis, coating, and use for diagnostics. The other authors are not aware of any other affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

such as antibodies, peptides, or small molecules, these nanoparticles can be used to detect molecular biomarkers and tumor cells at high sensitivity and specificity (11–13). Nanoparticles also have large surface areas for the attachment of multiple diagnostic (e.g., optical, radioisotopic, or magnetic) and therapeutic (e.g., anticancer) agents. Recent advances have led to the development of biodegradable nanostructures for drug delivery (14–18), iron oxide nanocrystals for magnetic resonance imaging (19, 20), and luminescent quantum dots (QDs) for multiplexed molecular diagnosis and in vivo imaging (21–27).

Semiconductor QDs exhibit novel optical and electronic properties and are emerging as a new class of nanoparticle probes for bioimaging and biondiagnostics (Figure 1). Recent research has generated monodispersed QDs encapsulated in stable polymers with versatile surface chemistries. These nanocrystals are brightly fluorescent, enabling their use as imaging probes both in vitro and in vivo (21–27). Here, we discuss recent developments in the synthesis and modification of QD nanocrystals and their use in dynamic cellular imaging. We also discuss the use of multiplexed QD–antibody conjugates for mapping the molecular, cellular, and glandular heterogeneity of human cancer specimens. For clinical diagnostics, multiplexed QD mapping can provide new molecular and morphological information that is not available from traditional H&E (hematoxylin and eosin) and immunohistochemistry (IHC), especially at complex and suspicious disease foci (25, 26). As discussed in more detail below, these results have raised exciting possibilities for the integration of morphological and molecular biomarker information for cancer diagnosis and treatment selection.

2. QUANTUM DOT PROBE DEVELOPMENT

Extensive research during the past 20 years has led to the development of high-quality and water-soluble QD probes for a broad range of applications in biology and medicine (1–10, 21–27). However, these QDs are still not perfect, mainly because of their large hydrodynamic sizes, a propensity for nonspecific binding to proteins and cellular membranes, poorly controlled conjugation chemistry, and diminished brightness when the crystalline core size is reduced. Often in the size range of 15 to 30 nm, these QDs tend to nonspecifically adhere to cellular membranes and proteins. These interactions cause the adsorption of a protein layer on the nanocrystal surface, which further increases the particle size and induces nonspecific cellular uptake. To mitigate this problem, researchers have coated QDs with neutral hydrophilic polymers such as polyethylene glycol (PEG), yielding reduced nonspecific binding but at the expense of a large increase in the hydrodynamic size (28). Researchers have also used small zwitterionic ligands (such as cysteine) to overcome this problem, resulting in particles that are both small and resistant to nonspecific binding (29–32). However, the resulting QDs frequently suffer from low colloidal stability, photobleaching, or low quantum yields. Bioaffinity ligands are usually attached to QDs through chemical schemes that are inherently stochastic, such that the number and geometric orientation of conjugated molecules vary widely across the nanoparticle population (6). Consequently, some QD bioconjugates have numerous active ligands that can cross-link multiple target molecules (Figure 2). In an attempt to overcome this heterogeneity problem, Ting and colleagues (33, 34) have used monovalent streptavidin to prepare monovalent QD probes, resulting in reduced cross-linking of target proteins. A further problem is that the brightness of QDs quickly diminishes when the crystalline core size is reduced. The reason is that, with the same fluorescence quantum yield, the brightness of single QDs is proportionally related to their molar absorbance, which is scaled approximately to the third power of the particle size (1, 2). Thus, smaller QDs are not as efficiently excited as larger dots and, therefore, are dimmer under the same photon-flux conditions.

Also, note that current QDs exhibit rapid on-and-off light emission (known as blinking) when observed individually under a fluorescence microscope (35). This attribute is a mixed blessing because it can be detrimental for single-molecule imaging due to a frequent loss of signal from the molecule being monitored. It can also be beneficial because it is largely a single-particle behavior and can be used to differentiate single probes from aggregates. Recent work by Krauss and colleagues (36) indicates that it is possible to completely eliminate blinking by preparing core/shell particles in which there is a smooth composition gradient from the core to the shell. However, this research is still controversial, and the results have not been independently reproduced.

2.1. Next-Generation Quantum Dots

There has been considerable interest in developing new and improved QDs with optimized brightness, minimized hydrodynamic size, resistance to nonspecific interactions, and site-specific ligand conjugation. In this section, we discuss recent advances both in engineering novel crystalline nanostructures and in developing new surface coatings and molecular tagging strategies. If the hydrodynamic size of QDs is reduced to that of green fluorescent proteins (GFPs), investigators believe that QD-tagged proteins will behave similarly as GFP-tagged proteins inside living cells (6–8). However, the task of developing such protein-sized dots is challenging because small dots often have low optical absorbance and must be coated with a thin polymer layer. As discussed below, novel insights and related results have raised new possibilities in developing the next generation of QDs for molecular and biondiagnostic applications.

2.2. Alloyed Nanocrystals

The most common nanocrystalline cores are composed of CdSe, which allows one to tune the wavelength of fluorescence emission between ~500 and 650 nm by altering the core size (1, 2). However, a major disadvantage of this tuning methodology is that each different color has a different hydrodynamic size and fluorescence brightness, making multicolor comparisons difficult. Recently, several groups have explored the use of ternary alloys in place of CdSe. With alloys such as $\text{CdSe}_x\text{S}_{1-x}$, $\text{CdTe}_x\text{Se}_{1-x}$, $\text{Hg}_x\text{Cd}_{1-x}\text{Te}$, and $\text{Hg}_x\text{Cd}_{1-x}\text{Se}$, the core nanocrystal size can be held constant while the fluorescence wavelength is tuned through chemical composition (37, 38), which normalizes the brightness and size to similar values and widens the spectral range for fluorescence tuning. In particular, Smith & Nie (39) have used cation exchange to partially replace cadmium ions in CdTe nanocrystals with mercury ions, yielding $\text{Hg}_x\text{Cd}_{1-x}\text{Te}$ alloyed nanocrystals in which the size did not change due to a similar bond length between CdTe and HgTe (Figure 3). Because of the large difference in bandgap energy between CdTe and HgTe, these particles can be widely tuned in fluorescence across the near-IR spectrum while maintaining a similarly compact size. Fluorescence emission in the near-IR spectral range is very important for high-sensitivity bioassays due to both the greater penetration of near-IR light in scattering media and the lower autofluorescence background. In contrast to organic dyes and fluorescent proteins that have limited fluorescence emission efficiencies at wavelengths longer than ~700 nm, quantum yields for QDs can approach unity at longer wavelengths, and stable probes composed of nanocrystals such as $\text{Hg}_x\text{Cd}_{1-x}\text{Te}$, InAs, and $\text{Pb}_x\text{Cd}_{1-x}\text{Se}$ can be produced in water with fluorescence emission that is tunable from 700 to 2,000 nm (39–41).

Other nanocrystal architectures under development include heterostructures with mixed dimensionality through seeded growth. A single-component nanocrystal can be used as a seed for the overgrowth of a shell with a different composition and dimensionality (42). For example, the Alivisatos group (43) and the Manna group (44) simultaneously reported the use of CdSe seeds for overgrowth of CdS rods. Thus, the quasi-spherical seeds are buried within the CdS rod. Moreover, Alivisatos and colleagues have reported that tuning the

crystalline structure of the core between hexagonal and cubic causes the rods to grow outward from four facets of the core, yielding a tetrapod structure. Furthermore, a wide variety of compositions have been explored for these materials, opening the door to unique charge-carrier confinement regimes and complex wave function engineering strategies (45). The unique optoelectronic characteristics of these multidimensionality particles has been investigated by the Weller group (46), who demonstrated that an electric field applied along the long axis of a CdSe (coreQD)/CdS (shell rod) nanocrystal alters the fluorescence intensity and wavelength, allowing for electronic modulation of fluorescence resonance energy transfer (FRET) between the nanocrystals and the dye molecules.

2.3. New Surface Coatings to Minimize Hydrodynamic Size and Nonspecific Binding

Recently, Smith & Nie (10, 47) have developed a multifunctional, multidentate polymer ligand for generating highly compact QDs with ultrasmall sizes that preserves the excellent optical properties of the nanocrystals (Figure 4). These multidentate polymers can displace the existing ligands on the QD and tightly bind to the nanocrystal surface in a closed “loops-and-trains” conformation. This unique design eliminates the hydrophobic barrier layer and causes the polymer ligand to tightly conform to the nanocrystal surface, resulting in an exceptionally thin polymer shell and small overall particle size. In contrast to water-soluble QDs with small-molecule ligands, the multidentate binding of the polymer provides excellent colloidal stability, resistance to photo-bleaching, and high quantum yield. Using this strategy, investigators have prepared high-quality QDs with a hydrodynamic size of 4 to 6 nm (47). Importantly, Frangioni and colleagues (31) have demonstrated that QDs with a hydrodynamic diameter of <5.5 nm undergo rapid renal clearance, making these size-minimized nanoparticles ideal for in vivo imaging, wherein the potential toxicity of the heavy metal-containing QD has been a significant impediment to clinical adoption.

In addition to size, another problem is that current QDs are often “sticky” because they have a tendency to bind nonspecifically to proteins, cellular membranes, or extracellular matrixes (48–51). Nonspecific binding reduces the signal-to-noise ratio and limits immunostaining specificity and detection sensitivity. Nonspecific binding can also lead to false-positive staining for biomarkers in fluids, cells, and tissues. In particular, QDs with highly negative or positive surface charges, such as surface coatings containing carboxylic acids or amines, can exhibit strong nonspecific binding to cells and tissues (48–50) as well as to proteins in serum and blood. Because most biomolecules are charged or have charged domains (52), QDs could interact electrostatically with many soluble proteins in solution or with biomolecules on the cell surface and in the cytoplasm, resulting in the commonly observed nonspecific binding.

To reduce nonspecific binding, PEGs are often attached to the organic coating layer of QDs (23, 48). PEGylated QDs have a nearly neutral surface charge and can maintain colloidal stability through steric repulsion between the PEG chains. The reduced charge of a PEG, as well as its conformational flexibility, provides a stable surface coating that can reduce nonspecific binding (Figure 5) in biological environments. Rosenthal and colleagues (48) have shown that PEG chains as short as 550 Da can significantly reduce the nonspecific binding of QDs to cells. As a result, PEGylated QDs have been successfully used for both in vitro (23, 48) and in vivo (27–29) applications. However, PEG-coated QDs have significantly larger hydrodynamic sizes than those of comparable non-PEGylated nanoparticles, often more than doubling the particles’ hydrodynamic size. This increased size can prevent the probes from accessing biological targets deep within complex tissue or cellular structures. Kairdolf et al. (51) reported an alternative approach, in which the QD surface is modified with a small hydroxyl-containing molecule (such as 1,3-diamino-2-propanol), yielding hydroxylated QDs. These particles show a dramatically reduced surface

charge with virtually no nonspecific binding to cells or tissues, while maintaining excellent colloidal stability.

2.4. Tagging Strategies

The most widely used bioconjugation strategy is covalent attachment of a molecule to the QD coating surface via a functional group. This approach typically involves the formation of an amide bond between a carboxylic acid group on the nanocrystal and an amine group on the affinity molecule by use of carbodiimide chemistry. Other common functional groups for the covalent attachment include a thiol group coupling to maleimide to form a thioether bond (53, 54). This chemistry is particularly useful for conjugating QDs to antibodies, which have thiol groups that can be exposed following reduction of the interchain disulfide bonds and do not disrupt the affinity site. These covalent techniques can also be used to conjugate streptavidin and biotin to QDs and biomolecules (23, 55) to produce versatile reagents that can simply be mixed prior to use to form a targeted probe. Noncovalent interactions have also been employed to attach molecules to the QD surface. Mattoussi et al. (56) have demonstrated that QDs with a highly negative surface charge can be bound to biomolecules through electrostatic interactions by use of a chimeric fusion protein with a positively charged attachment domain. Gao and colleagues (57) have further demonstrated this principle by using positively charged QDs for noncovalent binding of negatively charged small interfering RNA (siRNA). Furthermore, histidine tags have been used to directly couple molecules to the QD surface in a defined orientation for optimal function (58, 59). These tags consist of a polyhistidine peptide, which has strong affinity to charged metal atoms such as Ni^{2+} and Zn^{2+} and can be fused to the termini of recombinant proteins. Size-minimized QDs with thin coatings have accessible surfaces, allowing the polyhistidine peptide to bind directly to the nanocrystal through coordination with the surface metal atoms. Rao and colleagues (60) have described another strategy for site-specific conjugation that uses the covalent coupling of a chloroalkane to Halo Tag proteins. These proteins are haloalkane dehalogenases that have been adapted to cleave the carbon–halogen bond in a chloroalkane molecule to form a stable ester bond. Such a protein, through fusion to a biomolecule of interest, can be coupled to a chloroalkane-containing QD in a highly controlled manner.

3. DYNAMIC CELLULAR IMAGING

Recent research using QD–ligand or QD–antibody conjugates has revealed the complex workings of membrane receptors at high sensitivity and temporal resolution (61, 62). New receptor behaviors, such as motor-driven transport of the epidermal growth factor receptor along cellular outgrowths toward the cell body (63), have been reported. In particular, neurons are known to have richly complex plasma membranes with multiple types of microdomains that form intracellular signaling complexes termed synapses, which exhibit dynamic receptor exchanges. The diffusion of several types of neurotransmitter receptors in and out of the synapse has been studied by use of QDs attached to glycine neurotransmitter and AMPA glutamate receptors; these investigations have revealed rapid fluctuations in diffusion rates in different membrane domains (61, 63). Some receptors, such as the neuronal growth factor (NGF) receptor, become internalized into the cell once they bind to a specific ligand, a process that can now be studied in great detail due to the photostability of QDs (64). QDs conjugated to NGF bind to the NGF receptor in the terminals of neuronal axons (long neurite outgrowths involved in signal transduction), inducing endocytosis of the receptor–ligand pair within vesicular structures. QD imaging revealed that these vesicles usually contain only a single NGF receptor and that they are shuttled great lengths to the cell body along multiple molecular tracks that behave as a multilane highway within the axons. However, these studies were carried out by using conventional QDs with large sizes and multivalent ligand presentation. As discussed above, the large size of conventional QD

probes is a major problem for their application in crowded cell-surface domains, such as the synaptic cleft, an intracellular junction between neurons that is typically only 20 nm wide. Larger QD conjugates have limited access to this region compared with smaller antibody–fluorophore conjugates, which adds some uncertainty to the QD neuronal diffusion studies reported to date (65, 66).

For intracellular transport, Ruan et al. (67) have used peptide-conjugated QDs to examine the complex behavior of nanoparticles in live cells. Dynamic confocal imaging revealed that the peptide-conjugated QDs were internalized by macropinocytosis, a fluid-phase endocytosis process triggered by QD binding to cell membranes. The internalized QDs were tethered to the inner vesicle surfaces and trapped in cytoplasmic organelles. The QD-loaded vesicles were actively transported by molecular machines (such as dyneins) along microtubule tracks (Figure 6). The destination of this active transport process was an asymmetric perinuclear region (outside the cell nucleus) known as the MTOC (microtubule organizing center). Indeed, motor protein translocation proceeds in discrete steps and with a velocity indicative of specific motor protein–filament pairs. QD–kinesin and QD–myosin conjugates delivered to the cellular cytoplasm through osmotic pinosome lysis undergo directed motion that was remarkably similar to that observed in purified filaments (68, 69). The molecular motors were tracked for extended periods of time without loss of signal.

Currently, a major challenge is to deliver freely diffusing and monodispersed QD probes into the cytoplasm of living cells. One effective technique is to directly inject QDs into living cells by using a microneedle. However, this process is rather low throughput because the individual cells must be injected one at a time (70). To achieve higher-throughput delivery of QDs to cell populations, investigators have attempted to temporarily permeabilize the cellular plasma membrane through the formation of microscopic pores, either through the use of bacterial toxins (e.g., streptolysin O) that form well-defined membrane pores or through brief exposure to a pulsed electric field. These mechanisms are promising but have yet to demonstrate homogeneous delivery of free QDs in cells.

An alternative and promising approach is the controlled disruption of endosomal vesicles. Cells naturally engulf their surrounding environment through various processes that yield intracellular vesicles containing extracellular fluid. This mechanism is a convenient way to enable entry of QD probes into cells, but the particles remain trapped and therefore are not free to interact with target molecules, so it is necessary to have a strategy for QD release or endosomal escape. One method is to use osmosis for swelling and bursting the endosomes (68). This process can be performed by allowing cells to engulf QDs during a brief exposure to a hypertonic medium (prepared by adding sucrose or other solutes), which leads to the rapid formation of pinocytic vesicles that bud off of the plasma membrane due to water moving out of the cells (efflux). In the second step, a brief and well-controlled exposure of these cells to a hypotonic solution containing a low solute concentration causes water to rush into the solute-rich vesicles, inducing osmotic lysis and allowing the QDs to be dispersed into the cytoplasm.

Recent research has further shown that QDs coated with proton-sponge polymers can escape from endosomes after cellular internalization (56). The proton sponge effect arises from numerous weak conjugate bases (such as carboxylic acid and tertiary amine, with buffering capabilities at pH 5–6), leading to proton absorption in acid organelles and an osmotic pressure buildup across the organelle membrane (71). This osmotic pressure causes swelling and/or rupture of the acidic endosomes and a release of the trapped QDs into the cytoplasm. Alternatively, QDs can be encapsulated in proton-sponge polymer beads, which are broken down into proton-absorbing units in the lysosomes, thereby releasing the QD cargo into the cytoplasm (72).

4. BIOMEDICAL DIAGNOSTICS

In contrast to in vivo imaging, in which the potential toxicity of QDs remains a major concern (73–75), analyses of cells and tissues as well as solution-based biomarkers are performed on in vitro or ex vivo clinical patient samples. Because toxicity is of no concern when analyzing these specimens, the use of multiplexed QDs as ultrasensitive probes for in vitro biodiagnostics is probably the most important and clinically relevant application of QDs (22–26). The unique optical properties of QDs can significantly enhance the sensitivity of biodiagnostic assays such as IHC, fluorescence in situ hybridization (FISH), flow cytometry, and biochips and can provide new capabilities to extend the utility of biodiagnostic assays in the clinic. In particular, the multiplexing capability of QDs can be used to quantitatively measure a panel of molecular biomarkers, enabling personalized diagnostics and treatment.

4.1. Multiplexed Immunostaining

One of the most widely explored clinical applications for QDs is in multiplexed immunostaining of formalin-fixed paraffin-embedded (FFPE) tissue specimens (Figure 7a). IHC was first reported for protein marker detection and localization in tissue specimens 70 years ago (76–78) and has been extensively used in anatomic pathology since the development of robust staining methods (79–81). IHC is especially useful for clinical biomarker detection because it preserves the morphology of the tissue, which is critical for many diagnoses. Despite its ubiquitous use, IHC for diagnostics has seen only minor improvements in the past 50 years; the most notable innovations have involved the development of companion diagnostics, such as PATHWAY (<http://www.ventana.com/product/98?type=93>) and HercepTest™ (82), to predict response to a specific therapy. Nie and colleagues (26) have recently described detailed methods for the preparation, staining, and analysis of clinical tissue specimens by using QDs in both direct and indirect procedures, laying the foundation for multiplexed and quantitative QD-based clinical IHC assays. Research by several other groups (83–87) has shown that multiplexed QD staining for biomarkers in clinical tissue specimens enhances the diagnostic potential of IHC and enables the detection of multiple disease markers within a single slide. Paired with analytical hardware and software tools, QD-based methods have transformed immunostaining into a powerful diagnostic tool for high-throughput analysis of disease markers in clinical samples, including minute specimens such as needle aspirates. These methods are expected to play a key role in medical diagnostics as pathology continues to progress, particularly with the transition to digital pathology (88).

4.2. Cancer Diagnostics

One of the most common biomarker panels employed by oncologists and pathologists is ER/PR/Her2. This biomarker panel is used to diagnose breast cancer and to determine the most effective treatment strategy for breast cancer patients (89–91). These markers are currently measured individually with immunoassays such as HercepTest and traditional IHC techniques (92, 93), all of which rely on the subjective assessment of protein marker expression visualized by standard chromagens. Yezhelyev et al. (25) have demonstrated the simultaneous staining and measurement of these biomarkers in both cultured human breast cancer cells and in fixed (FFPE) clinical tissue specimens by using multiplexed QDs. These authors have shown that the QD-based methods for the quantification of ER/PR/Her2 proteins on a single slide correlate closely with the results achieved from traditional IHC, Western blot analysis, and FISH. Also, they used five QD colors simultaneously on a single clinical tissue specimen to detect five unique markers (ER/mTOR/PR/EGFR/Her2), further demonstrating the molecular profiling potential of these nanoparticles in complex tissue samples.

Investigations into the effectiveness of QD immunostaining and comparisons with current clinical methods have been reported by Chen et al. (94), who used lung cancer tissue microarrays to detect caveolin-1 and PCNA (proliferating cell nuclear antigen). These authors reported that QD-based immunostaining methods have a higher detection sensitivity in comparison to conventional clinical techniques. Increased accuracy and sensitivity were independently demonstrated by Li and colleagues (85), who performed a detailed examination of QD staining for the Her2 protein. Marker detection using QDs, compared with conventional IHC and FISH, was more sensitive and accurate than the standard techniques, particularly in cases with moderate marker expression, in which subjective assessment using conventional methods are often problematic and can introduce error or bias.

On the basis of these findings, further studies were conducted to determine the full diagnostic potential of QD-based methods. By combining the increased accuracy and sensitivity of QD-based immunostaining with another key parameter (total tumor size), Li and colleagues (95) introduced a new indicator (total Her2 load) to assess prognosis in breast cancer patients. This indicator identified more patients in the poor prognosis group than did Her2 gene amplification. The total Her2 load parameter identified a distinct subgroup of patients with particularly poor 5-year disease-free survival who were not differentiated with other methods. These results are especially promising for improving breast cancer diagnosis, and demonstrate the potential for individualized diagnostics and patient classification using QD-based immunostaining assays.

4.3. Single-Cell Analysis

Because of the high sensitivity and multiplexing capabilities of QD probes, biodiagnostic assays with these nanomaterials can even be used to analyze single rare cells, such as circulating tumor cells or isolated malignant cells, within the complex microenvironments of heterogeneous tumor tissue or in biological fluids (Figure 7 *b*). Such biodiagnostic analysis also leaves the specimens structurally intact, preserving important morphological information to correlate with the molecular profiling data. Morphology and biomarker expression data cannot be integrated with conventional molecular profiling or analytical methods such as gene chips, protein microarrays, or polymerase chain reaction (PCR). Liu et al. (26) have demonstrated that a panel of four biomarkers (E-cadherin/HML cytokeratin/p63/AMACR) can be simultaneously measured with QD probes and used to detect and characterize individual cells on prostatectomy and needle aspirate specimens. The QD-based molecular profiling technique enabled the mapping of molecular and cellular variations within a heterogeneous tissue specimen and enabled the identification of isolated malignant cells within predominantly benign prostate glands. This technique is a major improvement over other methods to analyze distinct regions (such as laser capture microdissection) because it allows molecular and morphological data to be digitally extracted from single cells, cell clusters, or glands without physically removing the regions of interest from the section. Using this method, Liu et al. identified prostate glands with only a single cancerous cell as the gland began its malignant transformation.

The rapid identification of low-abundance cancer cells has also been reported for the diagnosis of Hodgkin's lymphoma (96). The presence of Reed–Sternberg (RS) cells is a hallmark of the disease, but these cells constitute only ~1% of the total infiltrating cells in the lymph node specimens. Using multiplexed QD probes, Liu et al. (96) characterized individual RS cells with a panel of four biomarkers (CD15/CD30/CD45/Pax5) and distinguished them from other immune cells in clinical specimens. To further evaluate the diagnostic potential of QDs, they also compared QD staining analysis with previously determined pathological examination results. The QD-based methods rapidly identified all

patients with confirmed disease and showed the presence of disease in two patients who were classified as suspected of having the disease. The abundance of RS cells in these “suspicious” specimens was extremely low, which probably caused the ambiguous results originally obtained from standard pathological examination. Specimens from patients with reactive lymph nodes (but not Hodgkin’s lymphoma) showed a complete absence of RS cells. The results from these studies clearly illustrate the detection sensitivity of QD probes and show that biodiagnostic assays with multiplexed QDs can be used to diagnose patients at a much earlier stage than is achievable with conventional diagnostic methods, possibly improving the therapeutic success rate for patients.

4.4. Solution-Based Diagnostics

Although the use of QDs for immunostaining in tissue has been the focus of recent research, solution-based biodiagnostic assays are another area in which the unique properties of QDs can be exploited. Numerous assays using QDs as ultrasensitive and multiplexed probes for analyte detection have been developed. In particular, polychromatic flow cytometry is a technique that dramatically benefits from the superior signal brightness and multiplexing capabilities of these nanomaterials. Immunophenotyping using flow cytometry is a powerful tool for the detection, identification, and characterization of many cell types and has broad applications in diagnostic medicine. Using a combination of organic fluorophores and multiplexed QDs, Roederer and coworkers (97) demonstrated the simultaneous quantification of 17 unique markers with flow cytometry; this result is a dramatic increase over techniques using organic fluorophores alone. The increase in multiplexing capability has significant implications for the use of flow cytometry in the characterization of cellular immune responses; the diagnosis of complex diseases such as cancer; and the identification of T cells for HIV characterization, which exhibit a surprisingly high degree of heterogeneity.

QDs have also been used in a microfluidic instrument for the detection of single intact viruses in solution. Agrawal et al. (98) developed a dual-color method using red and green QD probes for identifying respiratory syncytial virus, a primary cause of lower respiratory tract disease in infants and young children and an important pathogen of the elderly and immune-compromised individuals. By targeting the probes to two different antigens on the virus surface (F and G proteins), these authors used photon coincidence to distinguish the signals of QDs bound to the virus particles from unbound QDs in the solution. QDs are ideal for this application because of their large Stokes shift and broad excitation profiles, which allow multiple colors to be excited simultaneously using a single high-energy excitation source. Strong coincidence signals were observed from samples containing the virus, whereas control samples showed little to no signal. This method also distinguished variations in the relative expression of viral surface proteins to determine virulence in a sensitive manner in real time (99).

In addition to protein analysis, the analysis of genes and genetic defects is a vital tool for disease diagnosis and is the major application of many molecular profiling tools such as gene chips and PCR. The exceptional optical properties of QDs make them ideal probes for use in these applications and provide unique capabilities that are not available with existing technologies. Han et al. (100) were the first to report a novel bar-coding technology using QD-tagged microbeads for the optical coding of biomolecules. With the use of six different QD colors with 10 intensity levels, one million unique combinations can theoretically be obtained. By coupling the microbeads to a unique DNA-recognition sequence, the authors easily detected and identified the target molecules. Hybridization studies (100) have shown that coding and target signals can be read at the single-bead level, demonstrating the utility of QD bar coding in the rapid analysis of DNA. Single-QD nanoparticles are also useful for

DNA analysis, and probes have been developed for the ultrasensitive detection of DNA and genetic mutations (101, 102).

Gerion et al. (101) have reported QD–DNA conjugates for the detection of single-nucleotide polymorphisms (SNPs), in which a sequence varies by a single base. These probes can detect both SNPs and single-base deletions in minutes at room temperature with high specificity. More recently, Wang and colleagues (102) developed a DNA nanosensor system by using single QDs with a bioconjugated capture sequence and a separate dye-conjugated reporter sequence (Figure 8). Following binding of the target DNA sequence to the QD sensor, the reporter sequence binds the target in a sandwich assay, bringing the reporter dye in close proximity to the nanocrystal and forming a FRET donor–acceptor pair for target detection at femtomolar (10^{-15} M) sensitivity. This process enables analyte detection without amplification, dramatically reducing the time and cost of gene analysis, which typically requires amplification with currently used technologies.

5. CONCLUDING REMARKS

Looking into the future, we expect major advances in both fundamental studies and practical applications for semiconductor nanocrystals. For fundamental research, the synthesis of new nanocrystals with unusual structures and properties is a boundless frontier and will continue to yield surprises such as doped and strain-tuned QDs. There are a wide variety of new nanocrystalline materials available with a diverse range of chemical, electronic, and optical properties. In particular, oxide materials such as ZnO would be an exceptional shell material for nanocrystal capping because of their wide bandgap and resistance to oxidative degradation; IV–VI semiconductors have uniquely positive deformation potentials; and mercury-based II–VI materials may allow for the continuous tuning of bandgaps through spontaneous cation-exchange reactions. For biomedical applications, it is important to minimize the overall size of bioconjugated nanocrystals, to reduce steric hindrance and nonspecific protein adsorption, to develop chemically activatable or photoswitchable nanocrystals for multicolor superresolution optical microscopy, and to understand the potential toxic effects of semiconductor materials (6). A major long-term goal is the development of QD probes that are simultaneously monovalent, free from nonspecific adsorption, compact in size, and still bright for single-molecule imaging. Reaching this goal will require innovations not only in developing novel crystalline nanostructures but also in developing new surface-coating, molecular tagging, and cellular delivery strategies.

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Glossary

IHC	immunohistochemistry
PEG	polyethylene glycol
GFP	green fluorescent protein
FRET	fluorescence resonance energy transfer
FISH	fluorescence in situ hybridization

FFPE	formalin-fixed paraffin-embedded
ER/mTOR/PR/ EGFR/Her2	a breast cancer protein biomarker panel consisting of estrogen receptor (ER), mammalian target of rapamycin (mTOR), progesterone receptor (PR), epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (Her2)
Caveolin-1	scaffolding protein located in the caveolae of the cell membrane
E-cadherin/HML cytokeratin/p63/ AMACR	a prostate cancer protein biomarker panel consisting of epithelial (E-)cadherin, high-molecular weight (HML) cytokeratin, transformation-related protein 63 (p63), and α -methylacyl-CoA racemase (AMACR)
RS cell	Reed–Sternberg cell
CD15/CD30/CD45/ Pax5	a Hodgkin’s lymphoma biomarker panel consisting of cluster of differentiation molecules (CDs) and a paired box protein (PAX)

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Figure 1.

Unique optical properties of quantum dots (QDs). (a) Fluorescence image of vials containing QDs of increasing size (*left to right*). The size-dependent properties of nanocrystals allow for the synthesis of fluorescent probes with emissions covering the entire visible-to-near-IR wavelength range. (b) (*Top*) Fluorescence and (*bottom*) absorbance spectra of green and red QDs. Narrow and symmetric fluorescence spectra enable accurate modeling for deconvolution and spectral unmixing to differentiate probes with significant emission overlaps. The absorbance spectra show a broad absorption profile, which enables a wide wavelength range for excitation and a single excitation source for multiple QD colors.

Figure 2.

Current and next-generation quantum dots (QDs.) (a) Current QDs are often large and elongated nanocrystals with a thick and sticky micellar bilayer and randomly linked bioaffinity molecules. (b) Next-generation QDs will have compact and spherical cores with a thin, inert monolayer coating conjugated to a single biomolecule through a site-specific, high-affinity attachment. Adapted with permission from Reference 10.

Figure 3. Structure and energy-band diagrams of CdTe and cation-exchanged $\text{Hg}_x\text{Cd}_{1-x}\text{Te}$ quantum dots (QDs). (a) Cd^{2+} replacement by Hg^{2+} . (b) The potential energy wells (gray lines), quantum-confined kinetic energy levels (blue lines), and wave functions (red) of electrons and holes in CdTe and $\text{Hg}_x\text{Cd}_{1-x}\text{Te}$ QDs, as calculated using the effective mass approximation. Reproduced with permission from Reference 39.

Figure 4.

Quantum dot (QD) size minimization by use of multidentate ligands that contain a mixture of amine and thiol functional groups. (a) The multidentate ligands can wrap around the QD in a closed monolayer conformation, in contrast to the bulky bilayer structure of amphiphilic polymers. (b) A balance of thiol (–SH) and amine (–NH²) functional groups is needed for stable multidentate ligand binding. Abbreviations: DIC, diisopropylcarbodiimide; DMSO, dimethylsulfoxide; FMOC, fluorenylmethyloxycarbonyl; NHS, *N*-hydroxysuccinimide. Adapted with permission from Reference 47.

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Figure 6.

Direct observation of active transport of endocytosed Tat quantum dots (Tat-QDs) inside living cells. (*a, b*) Directed motion from the cell periphery to an intracellular region adjacent to the cell nucleus. The boxed red area in panel *a* is magnified in panel *b*. The white line represents the trajectory of the Tat-QD vesicle indicated by the red arrow. The green line shows the plasma membrane boundary of the cell. (*c, d*) Directed motion along cell-peripheral tracks. The boxed red area of panel *c* is magnified in panel *d*. The green line indicates the plasma membrane of the cell. Reproduced with permission from Reference 67.

Figure 7.

Immunoprofiling in complex tissues for the identification and characterization of rare cells. (a) Multiplexed quantum dot (QD) imaging in prostate biopsy specimens allows for the differentiation of a benign prostate gland (*left*) from a gland with a single malignant cell (*right*), as determined by positive AMACR staining (*arrowhead*). (b) A four-biomarker panel was used to identify (i) low-abundance Reed–Sternberg cells, (ii) B cells, and (iii) T cells in a heterogeneous lymph node specimen for the diagnosis of Hodgkin’s lymphoma (*left*). By use of wavelength-resolved imaging, the QD staining pattern can be analyzed to determine the biomarker expression profile of single cells within the specimen (*right*), enabling accurate differentiation. Adapted with permission from References 26 and 96.

Figure 8.

Quantum dot (QD) nanosensor for the detection of DNA. (a) A QD nanosensor with bioconjugated capture sequences, bound to target DNA and a dye-conjugated reporter sequence in a sandwich assay. The reporter sequence brings the dye into close proximity to the nanocrystal and is excited by fluorescence resonance energy transfer (FRET) between the dye acceptor (Cy5) and the QD donor. (b) Experimental flow setup for the detection of QDs and dye signal. In the presence of the target sequence, coincident fluorescence signals are measured in both the donor (c) and acceptor (e) detectors. In the absence of the target sequence, signal is detected only from the QD donor (d) and is not observed on the acceptor detector (f). Adapted from Reference 102.