Recent advances in nanomaterials have produced a new class of fluorescent labels by conjugating semiconductor quantum dots with biorecognition molecules. These nanometer-sized conjugates are water-soluble and biocompatible, and provide important advantages over organic dyes and lanthanide probes. In particular, the emission wavelength of quantum-dot nanocrystals can be continuously tuned by changing the particle size, and a single light source can be used for simultaneous excitation of all different-sized dots. High-quality dots are also highly stable against photobleaching and have narrow, symmetric emission spectra. These novel optical properties render quantum dots ideal fluorophores for ultrasensitive, multicolor, and multiplexing applications in molecular biotechnology and bioengineering.

Introduction

Metal and semiconductor nanoparticles in the 2–6 nm size range are of considerable current interest, not only because of their unique size-dependent properties but also because of their dimensional similarities with biological macromolecules (e.g. nucleic acids and proteins) [1–4]. These similarities could allow an integration of nanotechnology and biology, leading to major advances in medical diagnostics, targeted therapeutics, and imaging. These similarities with biological macromolecules give rise to unique optical and electronic properties that are not available in either discrete atoms or in bulk solids. Extensive research in the past 20 years has focused on the synthesis of highly luminescent QDs in large quantities [19••], a reasonable understanding of the surface chemistry, the preparation of water-soluble and biocompatible nanocrystals [7••,8••], and the incorporation of multicolor QDs into microbeads and nanobeads for multiplexed optical encoding of biomolecules [20••].

Synthesis

Both group II-VI (e.g. CdSe, CdTe, CdS, and ZnSe) and group III-V (e.g. InP and InAs) nanocrystals have been synthesized and studied extensively in the past [1–4]. Before 1993, QDs were mainly prepared in aqueous solution with added stabilizing agents (e.g. thioglycerol or polyphosphate). This procedure yielded low-quality QDs with poor fluorescence efficiencies and large size variations (relative standard deviation [RSD] > 15%). In 1993, Bawendi and coworkers [21] synthesized highly luminescent CdSe QDs by using a high-temperature organometallic procedure. The nanocrystals had nearly perfect crystal structures and narrow size variations (RSD < 5%), but the fluorescence quantum yields were still relatively low (~10%).

The deposition of a surface-capping layer such as ZnS or CdS was found to dramatically increase the quantum yields of CdSe nanocrystals [22–24]. ZnS has a wider bandgap (energy difference between the valence band and the conduction band) than CdSe, but the Zn–S bond length is similar to that of Cd–Se. This property allows the epitaxial growth of a thin ZnS layer on the CdSe core. In practice, the Zn(CH3)2S solution is added slowly in small aliquots to a CdSe/tri-n-octylphosphine oxide (TOPO, a high boiling point and coordinating solvent) solution to prevent ZnS nucleation. The quantum yields of the capped CdSe nanocrystals are about 40–50% at room temperature.
temperature. Most recently, Peng and Peng [19••] have pushed the art of QD synthesis one step further by demonstrating that alternative precursor materials (such as CdO) can be used to prepare high-quality CdS, CdSe, and CdTe nanocrystals. In contrast to traditional core/shell nanocrystals, the QDs synthesized show excellent quantum yields without an inorganic capping layer. The nanocrystal size can be controlled by several procedures; for example, nucleated CdSe QDs can be grown at high temperatures (300°C or higher) for an extended period of time (ranging from minutes to hours, depending on the desired particle size) [25]. In this process (known as Ostwald ripening), smaller nanocrystals are broken down, and the dissolved atoms are transferred to larger crystals. The rate of this ‘ripening’ process is dependent on both the temperature and the amount of limiting reagent [25,26]. Continuous injection of precursor solutions into the CdSe reaction mixture (in TOPO) at 300°C also produces larger nanocrystals. Figure 1 shows a set of different sized ZnS-capped CdSe QDs excited with a handheld near-UV lamp.

**Optical properties**

The optical properties of semiconductor nanoclusters arise from interactions between electrons, holes, and their local environments. Semiconductor QDs absorb photons when the excitation energy exceeds the bandgap. During this process, electrons are promoted from the valence band to the conduction band. Measurements of UV–visible spectra reveal a large number of energy states in QDs. The lowest excited energy state is shown by the first observable peak, known as the quantum-confinement peak. Excitation at shorter wavelengths is possible because multiple electronic states are present at higher energy levels. In fact, the molar extinction coefficient gradually increases towards shorter wavelengths. This is an important feature for biological applications because it allows simultaneous excitation of multicolor QDs with a single light source (Figure 2).

Light emission arises from the recombination of mobile or trapped charge carriers (Figure 2b). The emission from mobile carriers is called excitonic fluorescence and is observed as a sharp peak. The emission spectra of single ZnS-capped CdSe QDs are as narrow as 13 nm (full-width at half maximum; FWHM) at room temperature [8••]. The excited-state lifetimes of nanocrystals contain three exponential components. In bulk measurements, the lifetimes are 5 ns, 20–30 ns, and 80–200 ns, with 20–30 ns dominating [27,28]. These excited-state decay rates are slightly slower than those of organic dyes (1–5 ns), but much faster than those of lanthanide probes (1 μs–1 ms). Single-dot measurement further reveals that the excited-state lifetimes are dependent on the emission intensity, but the exact origins of this multi-exponential behavior remain unclear.

By varying the size and composition of QDs, the emission wavelength can be tuned from the blue to the near infrared. For example, CdS and ZnSe dots emit blue to near-UV light [29], different sized CdSe dots emit light across the visible spectrum, and InP and InAs QDs emit in the far-red and near-infrared [30–32]. We also note that elongated QDs (quantum rods) show linearly polarized emission [33], whereas the fluorescence emission from spherical CdSe dots is circularly polarized or non-polarized [34,35].

In comparison to organic dyes such as rhodamine 6G and fluorescein, CdSe nanocrystals show similar or slightly lower quantum yields at room temperature, but the lower quantum yields are compensated by their larger absorption cross-sections and much reduced photobleaching rates. Bawendi and coworkers [21,22] estimated that the molar extinction coefficients of CdSe QDs are about $10^5$–$10^6$ M$^{-1}$ cm$^{-1}$, depending on the particle size and the excitation wavelength. These values are 10–100 times larger than those of organic dyes, but are similar to the absorption cross-sections of phycoerytherin, a multichromophore fluorescent protein. Chan and Nie [8••] estimated that single ZnS-capped CdSe QDs are $\sim20$ times brighter and $\sim100$–200 times more stable than single rhodamine 6G molecules.

**Surface chemistry**

The complex surface chemistry of nanocrystals has been studied by NMR spectroscopy and X-ray photoemission
Morphologically, QDs are not smooth spherical particles, but are faceted with many planes and edges. They are generally considered to be negatively charged owing to molecules adsorbed on the surface [38]. TOPO strongly coordinates to the surface metal atoms, whereas tri-n-octylphosphine (TOP) or tributylphosphine (TBP) are only weakly bound. The surface properties (such as TOPO molecules interacting with each other and their dimerization) [39] improve the long-term solubility of QDs in organic solvents.

Two methods have been developed to prepare water-soluble QDs. Alivisatos and coworkers [7••,40] reported the use of a silica/siloxane coating for creating water-soluble ZnS-capped CdSe QDs. In this procedure, 3-(mercaptopropyl) trimethoxysilane (MPS) is directly adsorbed onto the nanocrystals and TOPO molecules are displaced. A silica/siloxane shell is formed on the surface by the introduction of a base and hydrolysis of the silanol groups. Polymerizing silanol groups help stabilize the nanocrystals against flocculation. The QDs become soluble in intermediate polar solvents, such as methanol and dimethyl sulfoxide. Further reaction with bifunctional methoxy compounds, such as aminopropyl trimethoxysilane or trimethoxysilyl propyl urea, renders the particles soluble in aqueous buffer. The second method to prepare water-soluble QDs involves the direct adsorption of bifunctional ligands such as mercaptocacetic acid or dithiothreitol to the QD surface [8••]. Here, a mercapto compound and an organic base are added together to TOPO-capped QDs dissolved in chloroform. The base deprotonates both the thiol and the carboxylic group, leading to favorable electrostatic binding between negatively charged sulfur atoms and surface Cd²⁺ or Zn²⁺ ions. The semiconductor QDs precipitate out of the organic solution, but can be redissolved in aqueous solution.

Polymerized siloxane-coated QDs are highly stable against flocculation, but only small amounts (milligram quantities) can be prepared per batch. Also, residual silanol groups on the nanocystal surface often lead to precipitation and gel formation at neutral pH. In comparison, the direct adsorption procedure yields gram quantities of water-soluble QDs, but the mercapto ligands are not completely stable. Slow desorption of mercaptocacetic acid molecules often results in aggregation and precipitation of the solubilized QDs. This problem has recently been solved by using chemically modified proteins to coat and ‘passivate’ the QD surface (XH Gao, SM Nie, unpublished results). Preliminary results indicate that the protein-coated QDs are stable indefinitely (for more than 2 years) in buffer solution, exhibit excellent spectral widths, and have quantum yields similar to those of the original QDs in chloroform (FWHM = 25 nm and quantum yield = 40–50%). Furthermore, the protein layer provides multiple functional groups (amines, carboxylic acids, and cysteine residues) for covalent conjugation with a variety of biological molecules and bioincompatible polymers.

### Bioconjugation and applications

Reactive functional groups include primary amines, carboxylic acids, alcohols, and thiols. Primary amines react with carboxylic acids, catalyzed with a carbodiimide or sulfo N-hydroxysuccinimide ester, to form a stable amide bond. A thioether bond is formed when sulfhydryl groups react with maleimides. Another approach for linking biomolecules to nanocrystals is to use a thiol-exchange reaction. Here, mercapto-coated QDs (prepared by direct adsorption) are mixed with thiolated biomolecules (such as oligonucleotides or proteins) [10,41]. After overnight incubation at room temperature, a chemical equilibrium is reached between the adsorbed thiols and the free thiols.
A similar approach has recently been used by Mattoussi and coworkers [9] in which engineered proteins with a linear polylysine chain are directly adsorbed onto negatively charged nanocrystals through electrostatic interactions. Current bioconjugation methods are schematically illustrated in Figure 3. It should be noted that the surface area of a single QD is large enough for linking to multiple biomolecules. Two to five protein molecules and 50 or more small molecules (such as oligonucleotides or peptides) may be conjugated to a single 4 nm QD.

Bioconjugated QDs have been used in DNA hybridization [10], immunoassay [8••], receptor-mediated endocytosis [8••], and time-gated fluorescence imaging of tissue sections [28]. Nanocrystals are also emerging as a new class of fluorescent labels for in vivo cellular imaging. An important advantage is that the extremely high photostability of QDs allows real-time monitoring or tracking of intracellular processes over long periods of time (minutes to hours). Another advantage is the ability to use multicolor nanocrystals to simultaneously image multiple targets inside living cells or on the cell surface. Furthermore, with an inert layer of surface coating, the nanocrystals are believed to be less toxic than organic dyes. In preliminary studies, we have conjugated luminescent QDs to transferrin (an iron transport protein), antibodies that recognize cancer biomarkers, and folic acid (a small vitamin molecule recognized by many cancer cells). In each case, we found that receptor-mediated endocytosis occurred and the nanocrystals were transported into the cell. Single QDs as well as clusters of dots trapped in vesicles were clearly visible inside living cells (Figure 4).

Another promising area of application is the use of near-infrared (NIR) QDs for in vivo molecular imaging. With long-wavelength organic dyes, Weissleder and coworkers [42,43•] have developed bioconjugated probes for tumor imaging in live animals. Recent research in our group has synthesized novel NIR fluorescent nanocrystals with emission wavelengths as long as 850 nm and quantum yields as high as 50% at room temperature (RE Bailey, JB Strausburg J, SM Nie, unpublished results). Similar to the CdSe nanocrystals, the NIR QDs can be made water-soluble and biocompatible. In comparison with...
carbocyanine and related organic dyes, our NIR dots are much brighter (higher quantum yields and larger absorption cross-sections) and are much more stable against photobleaching. These NIR nanocrystals could provide new opportunities for \textit{in vivo} imaging of gene expression and enzyme activities.

A further application of QDs is the multiplexed optical encoding and high-throughput analysis of genes and proteins, as reported by Nie and coworkers [20**]. Polystyrene beads are embedded with multicolor CdSe QDs at various color and intensity combinations (Figure 5). The use of six colors and 10 intensity levels can theoretically encode one million protein or nucleic acid sequences. Specific capturing molecules such as peptides, proteins, and oligonucleotides are covalently linked to the beads and are encoded by the bead’s spectroscopic signature. A single light source is sufficient for reading all the QD-encoded beads. To determine whether an unknown analyte is captured or not, conventional assay methodologies (similar to direct or sandwich immunoassay) can be applied. This so-called ‘barcoding’ technology can be used for gene profiling and high-throughput drug and disease screening. Based on entirely different principles, Natan and coworkers [44**] reported a metallic nanobarcoding technology for multiplexed bioassays. Together with QD-encoded beads, these ‘barcoding’ technologies offer significant advantages over planar chip devices (e.g. improved binding kinetics and dynamic range), and are likely to find use in various biotechnological applications.

Conclusions
A pervasive trend in biotechnology is the development of ultrasensitive and high-throughput technologies for the rapid detection and quantification of genes, proteins, and cells. The ability to quickly screen a large number of biomolecules is important in several research areas, such as drug discovery and medical diagnostics. In the next 10 years, we envision that novel platforms based on multicolor QDs will be developed for massive parallel biosensing and analytical detection. These multiplexing technologies will probably combine the advantages of QDs with those of microfluidics and microarrays. In addition, non-invasive molecular imaging technologies could be developed using luminescent QDs. This should allow viral particles to be followed \textit{in vivo}, drug molecules to be analyzed in biological systems, and tumor cells to be tracked in real time.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
* of special interest
** of outstanding interest
This is a timely review article discussing recent progress and future prospects in combining nanomaterials and biomolecules. With a comprehensive reference
based on the novel optical properties of semiconductor QDs. For both color
This paper reports the development of a multiplexed coding technology
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20. Han MY, Gao X, Su JZ, Nie SM: Selection of peptides with semiconductor binding specificity for directed
This is an outstanding paper that reports the synthesis and use of water-soluble QDs for biological applications. The key feature of this is a silane/silica layer that coats the QD surface. This coating layer is hydrophilic and contains functional groups for covalent conjugation. In practice, however, this procedure is complicated, not well-defined and often not reproducible.
This is a breakthrough paper that reports a simple procedure for preparing water-soluble and bioconjugatable QDs, based on the use of bifunctional organic compounds such as mercaptoacetate acid. The main problems with this procedure are that the solubilized QDs are only stable for a short period of time and that fluorescence yields are lower than those of the original dots in chloroform. These problems have recently been solved by using chemically denatured proteins to coat and passivate the QD surface. The protein coating also provides many functional groups for stable conjugation to other molecules.
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This is a news feature article on the biological applications of luminescent QDs. It is clearly written and is intended for people who are interested in new technologies.
18. Mitchell P: Turning the spotlight on cellular imaging, Nat Biotechnol •• 2001, 19:1013-1017. This is a news feature article with special focus on using luminescent QDs for cellular imaging.
This is an excellent contribution that describes the large-scale synthesis of high-quality QDs using cheap starting materials. The authors have also developed procedures for controlling the surface structure of QDs and for producing stable dots with red emissions. However, it is still unclear whether these uncapped nanocrystals could maintain their surface structure and optical properties when they are made water-soluble and are conjugated to biomolecules.
This paper reports the development of a multiplexed coding technology based on the novel optical properties of semiconductor QDs. For both color and intensity multiplexing, multicolor QDs are embedded into polymeric microbeads at precisely controlled ratios. A surprising finding is that the developed QDs are spatially separated from each other and do not undergo fluorescence resonance energy transfer within the beads.


This is an excellent paper on the use of NIR fluorescence for molecular imaging in live animals.


This paper reports the development of metallic nanobarcodes for multiplexed bioanalysis. Fluorescence is still used for target detection, but the barcodes are read out by measuring the reflectivity differences between different metals. It is still unclear how similar technologies, such as encoded nanobars and QD-tagged microbeads and encodable radiofrequency devices, would compete in genomic, proteomic, and clinical diagnostic applications.