

Quantum-dot nanocrystals for ultrasensitive biological labeling and multicolor optical encoding

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Abstract. Semiconductor nanoparticles in the size range of 2–6 nm are of great current interest, not only because of their size-tunable properties but also because of their dimensional similarity with biological macromolecules (e.g., nucleic acids and proteins). This similarity could allow an integration of nanomaterials with biological molecules, which would have applications in medical diagnostics, targeted therapeutics, and high-throughput drug screening. Here we report new developments in preparing highly luminescent and biocompatible CdSe quantum dots (QDs), and in synthesizing QD-encoded micro- and nano-beads in the size range of 100 nm–10 μ m. We show that the optical properties of ZnS-capped CdSe quantum dots are sensitive to environmental factors such as pH and divalent cations, leading to the potential use of quantum dots in molecular sensing. We also show that chemically modified proteins can be used to coat the surface of water-soluble QDs, to restore their fluorescence, and to provide functional groups for bioconjugation. For multiplexed optical encoding, we have prepared large microbeads with sizes similar to that of mammalian cells, and small nanobeads with sizes similar to that of viruses. © 2002 Society of Photo-Optical Instrumentation Engineers.
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1 Introduction

The integration of nanotechnology with biology and medicine is expected to produce major advances in molecular diagnostics, therapeutics, molecular biology, and bioengineering.^{1–3} 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.^{4–15} Due to their size-dependent properties and dimensional similarities to biomacromolecules, these nanobioconjugates are well suited as contrast agents for *in vivo* magnetic resonance imaging (MRI),^{16–18} as carriers for drug delivery, and as structural scaffolds for tissue engineering.^{19,20} In addition, metal and semiconductor colloidal nanoparticles are under intensive study for potential applications in materials synthesis,^{12–14,21–23} in multiplexed bioassays,^{24,25} and in ultrasensitive optical detection and imaging.^{5–7,26,27}

In this paper, we report the preparation of highly luminescent, stable, and biocompatible quantum dots for biological imaging and sensing. In particular, we show that ZnS-capped CdSe core/shell nanocrystals are sensitive to external factors such as pH and divalent cations, leading to potential applications in optical sensing and homogeneous assays. We also show that chemically modified proteins adsorb spontaneously on the surface of water-soluble quantum dots. The protein-

coated quantum dots are stable for more than 2 yrs in buffer solution, and exhibit excellent spectral widths and quantum yields similar to those of the original dots in chloroform (full width at half maximum=30 nm and quantum yields=40%–50%). Furthermore, the protein layer provides multiple functional groups (amines, carboxylic acids, and cysteine residuals) for covalent conjugation with biological molecules and biocompatible polymers.

In related work, we have demonstrated that quantum dots are ideal fluorophores for multiplexed optical encoding of polymeric microbeads.²⁴ In this paper, we have developed procedures for preparing QD-encoded beads in a broad size range. The smallest encoded beads have a diameter of 150 nm, similar to the sizes of viruses. The largest beads have a diameter of 5–10 μ m, similar to the sizes of mammalian cells. These encoded beads are expected to find use not only in multiplexed, high-throughput bioassays but also in fundamental studies of gene, proteins, and cells.

In comparison with organic dyes and fluorescent proteins, semiconductor quantum dots represent a new class of fluorescent labels with unique advantages and applications. For example, the fluorescence emission spectra of quantum dots can be continuously tuned by changing the particle size, and a single wavelength can be used for simultaneous excitation of all different-sized QDs.²⁴ Also, surface-passivated QDs are highly stable against photobleaching and have narrow, symmetric emission peaks (25–30 nm full width at half maximum). It has been estimated that CdSe quantum dots are

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about 20 times brighter and 100 times more stable than single rhodamine 6G molecules.⁶ These properties offer new possibilities in several areas of research including single-molecule biophysics, multiplexed medical diagnostics, and high-throughput drug screening.

2 Experiment

2.1 Materials

All chemicals and biochemicals used in this work were obtained from commercial sources. The dimethylcadmium $\text{Cd}(\text{CH}_3)_2$ and tributylphosphine were purchased from Strem Chemicals (Newburyport, MA). Selenium (Se), tri-n-octylphosphine oxide (TOPO), dimethylzinc $\text{Zn}(\text{CH}_3)_2$, and hexamethyldisilathiane were purchased from Aldrich (Milwaukee, WI). Methanol, chloroform, and hydrogen chloride (HCl) were purchased from EM Sciences (Itasca, IL). Acetone, mercaptoacetic acid, phosphate buffer saline (PBS), poly-lysine, sodium hydroxide (NaOH), and rhodamine 6G were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol was purchased from Aaper Alcohol (Shelbyville, KY). Microscope coverslips (0.13 mm thick) were purchased from Fisher Scientific (Pittsburgh, PA). All experiments were done with ultrapure Millipore water (Millipore, Bedford, MA).

2.2 Methods

ZnS-capped CdSe quantum dots were synthesized according to published procedures.^{28–33} Water-soluble quantum dots were prepared by using mercaptoacetic acid, as described previously.⁶ The dots were characterized by UV-vis absorption, fluorescence, and transmission electron microscopy (TEM). For pH dependence studies, water-soluble quantum dots were aliquoted into 1.0 mL vials. To each vial, 30% acetone was added to precipitate the quantum dots, and this solution was centrifuged (1000 × RPM, Fisher Scientific, Pittsburgh, PA). The supernatant was decanted and the samples were dried in air overnight. These samples were stored as a dried powder until use. Each vial was redissolved in doubly distilled water (pH=7.0). Fluorescence and absorbance spectra were obtained and were referred to as the “original” spectra. Then, a specific amount of 1.0 M HCl was added, and the solution was incubated for 1 min. Fluorescence and absorbance measurements were repeated. Spectra obtained from the second measurement were normalized to the original spectra. To investigate the effects of base or metal ions on fluorescence, sodium hydroxide or cadmium chloride was used.

Chemically reduced bovine serum albumin (BSA) was prepared by treating commercial BSA samples (Sigma, St. Louis, MO) with 1 mM sodium borohydride at 60–80 °C in water solution. Under these conditions, BSA was denatured and most of its disulfide bonds were converted to sulfhydryl groups (–SH). Excess borohydride was removed by spontaneous decomposition upon heating. In another procedure, the primary amine groups in BSA were converted to carboxylic acids by using succinic anhydride, and the product was purified by dialysis against PBS buffer.

Quantum dots solubilized with mercaptoacetic acid were coated with reduced BSA by incubation in 1 mg/mL BSA solution for 2–5 days. During this process, the QD fluores-

cence intensity gradually increased to ~97% of the original value (from 10% to 50% at room temperature). This intensity increase was used as an indicator of coating success. BSA-coated dots were purified by 3–4 rounds of ultracentrifugation, and were then covalently linked to biomolecular probes using standard cross-linking procedures.⁶ Dynamic light scattering measurements showed a particle size increase of 2–5 nm after BSA coating, but we did not observe a significant reduction in the kinetic rates and binding affinities of antibody molecules that were linked to quantum dots via BSA.

Polymer beads in the size range of 0.1–10 μm were synthesized by emulsion polymerization of styrene (98% vol/vol), divinylbenzene (1% vol/vol), and acrylic acid (1% vol/vol) at 70 °C. The amounts of different monomers were changed in order to control the bead size. Polymerization reaction was initiated by a common initiator and was allowed to proceed for 10 h. Transmission electron microscopy revealed that the beads are uniform with a standard deviation of 2%–10% in diameter (depending on the bead size). Incorporation of QDs was achieved by swelling the beads in a solvent mixture containing 5% chloroform and 95% (vol/vol) propanol or butanol, and by adding a controlled amount of ZnS-capped CdSe QDs to the mixture. The embedding process was complete within 30 min at room temperature. The encoded beads were then protected by using 3-mercaptopropyl trimethoxysilane, which polymerized inside the pores upon addition of a trace amount of water.

Fluorescence emission spectra were recorded by using a SPEX Fluoromax-2 spectrometer (Edison, NJ). Single-particle imaging was achieved by using an Olympus IX70 inverted microscope equipped with an oil-immersion objective (PlanApo 100×, NA=1.4), a 100 W mercury lamp, and a high-resolution charge coupled device camera (Sensys, Photometrics, Tucson, AZ). After obtaining an initial image, a drop of NaOH (pH=12.0), HCl (pH=4.0), water (pH=7.0), or CdCl_2 (1 mM) was added to the sample, and another fluorescence image was acquired. True-color fluorescence images were obtained with a digital color camera (Nikon DI) and broadband excitation in the near-UV (330–385 nm). A longpass dichroic filter (DM 400, Chroma Technologies, Brattleboro, VT) was used to reject the scattered light and to pass the Stokes-shifted fluorescence signals.

3 Results and Discussion

3.1 Effects of pH and Metal Ions on Photoluminescence

For quantum dots to be broadly useful in biology and medicine, it is important to understand the factors that affect their optical properties such as emission wavelength and quantum yields. For quantum dots passivated with an inorganic capping layer, it was believed that the quantum-dot core was isolated from the outside environment.^{28–30} As a result, the optical properties should not be affected by external factors. However, the results in Figure 1 reveal that the quantum yields of mercaptoacetic-acid solubilized CdSe/ZnS quantum dots are highly sensitive to pH and external cadmium ions.

Under acidic conditions (pH=2–4), the fluorescence intensity shows a decrease of ~80% from the original value (measured at neutral pH). In basic solutions (pH=10–12), the fluorescence intensity is increased by nearly threefold. Furthermore, the addition of both cadmium and hydroxide

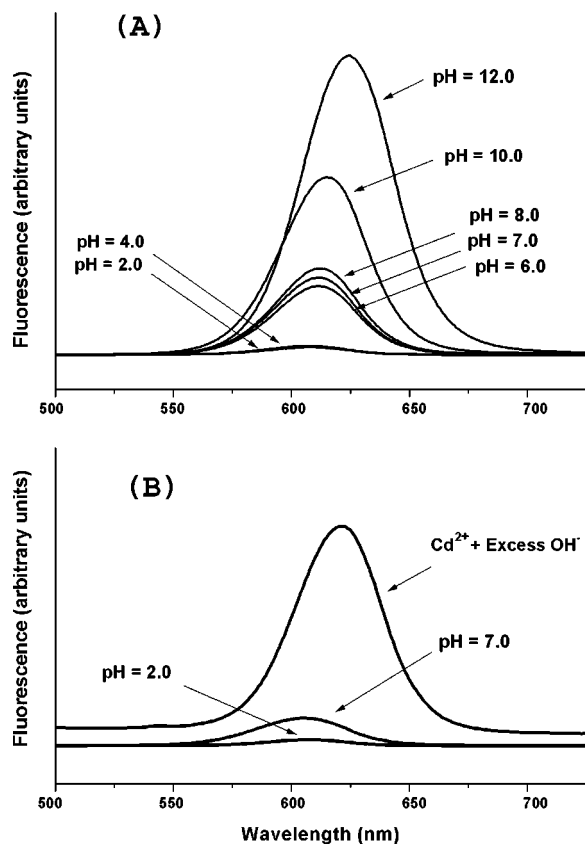


Fig. 1 Effects of pH and Cd^{2+} ions on the photoluminescence of water-soluble ZnS-capped CdSe quantum dots. (A) Fluorescence spectra obtained in the pH range of 2–12. (B) Fluorescence spectra obtained with 1.0 mM excess Cd^{2+} ions.

ions results in a fivefold enhancement in the fluorescence intensity (in comparison to that at $pH=7.0$). Figure 2 shows a direct comparison of single-dot fluorescence signals before and after the addition of 1 mM cadmium ion solution. This finding is similar to that reported by Henglein and Co-workers,^{34,35} who showed that the fluorescence of simple CdS quantum dots was dramatically activated by excess cadmium ions at basic pH s.

The observed spectral changes include both intensities and wavelength shifts. While the fundamental mechanisms of these changes are still not clear, it is clear that the fluorescence signals of core/shell quantum dots are sensitive to external factors. As a result, small-molecule ligands and biorecognition molecules might be designed to modulate the optical properties of quantum dots, which would allow quantum dots to be used in optical sensing applications.³⁶

3.2 Surface Passivation and Bioconjugation

For fluorescent tagging or labeling, it is desirable to have water-soluble quantum dots with stable optical properties that are not affected by environmental factors. High-quality QDs are often synthesized under high-temperature organometallic conditions,^{28–33} and are not compatible with biological systems. Two methods have been developed to solve this problem, one based on the direct adsorption of bifunctional ligands on the nanocrystal surface,⁶ and the other based on surface

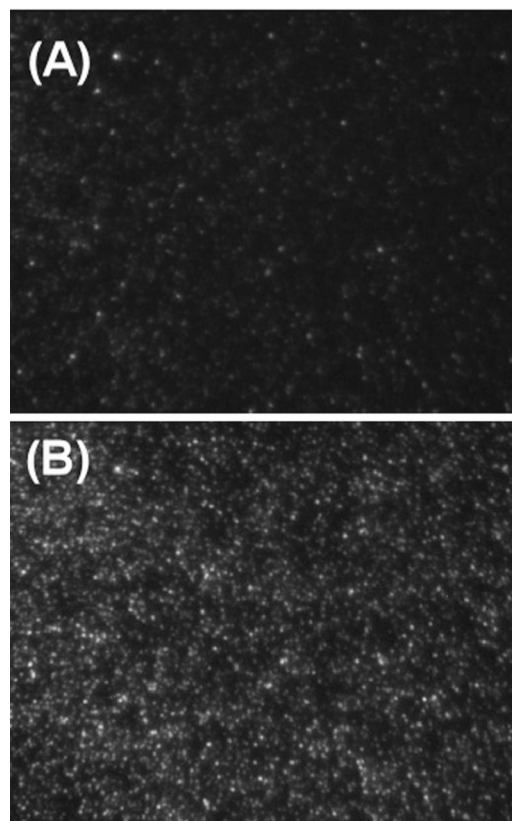


Fig. 2 Fluorescence images of single CdSe quantum dots (A) before and (B) after the addition of 1 mM cadmium ions. Quantum dots were spread and immobilized on a polylysine-coated glass surface. The same surface area was imaged in (A) and (B); that is, the same quantum dots were imaged before and after the addition of cadmium ions. The intensity changes were due to an activation effect of cadmium ions, not due to quantum-dot aggregation or precipitation.

coating with a silica layer.⁵ The first procedure uses mercaptoacetic acid to make QDs water soluble, because the mercapto group has a large affinity to Zn atoms, while the carboxylic acid group is hydrophilic and reactive to biomolecules. However, the fluorescence quantum yields have been reported to drop below 10% after solubilization,³⁷ and slow desorption of mercaptoacetic acid molecules often leads to aggregation and precipitation of the solubilized dots. This procedure has recently been modified by attaching engineered proteins to QDs through electrostatic interactions,⁷ and by using dithiothreitol for nanocrystal stabilization and bioconjugation.⁹

In the second procedure, 3-(mercaptopropyl) trimethoxysilane is directly adsorbed onto the nanocrystals in which TOPO molecules are displaced. A silica/siloxane shell is formed on the surface by introduction of a base and hydrolysis of the silanol groups. Polymerization of silanol groups helps stabilize the nanocrystals against flocculation. The quantum dots 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 solution. In comparison with the mercaptoacetic acid method, polymerized siloxane-coated

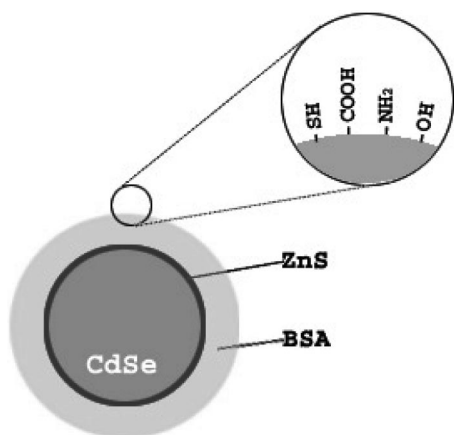


Fig. 3 Schematic illustration of BSA-coated CdSe/ZnS quantum dots. The use of chemically modified BSA was found to restore the fluorescence of water-soluble quantum dots and to provide functional groups such as amines and thiols for biomolecular conjugation.

quantum dots are highly stable against flocculation, but only small amounts (milligrams) can be prepared per batch. At the present, this procedure is not well defined and not reproducible because the residual silanol groups on the nanocrystal surface often lead to precipitation and gel formation at neutral pH.

We have developed a new method for surface passivation and bioconjugation of quantum dots by using chemically modified proteins such as denatured BSA (Figure 3). This simple procedure not only yields highly stable QDs, but also restores the fluorescence quantum yields to the original values (as measured in chloroform). Furthermore, the BSA layer contains functional groups for covalent conjugation to other biomolecules. Figure 4 shows the fluorescence spectra of water-soluble QDs stabilized with various forms of BSA, and Figure 5 shows the kinetics of fluorescence recovery. The results reveal that chemically reduced BSA is most effective in fluorescence restoration, in agreement with the report of Van Or-

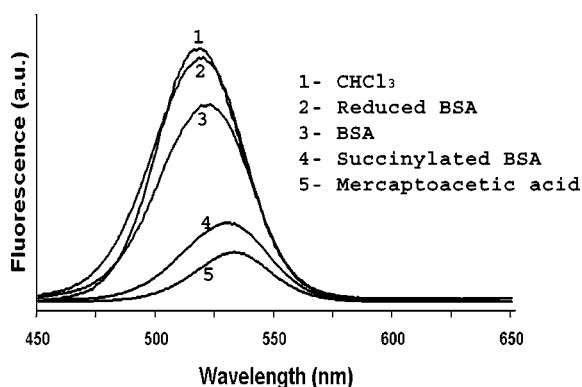


Fig. 4 Fluorescence spectra of water-soluble CdSe/ZnS quantum dots coated with chemically reduced BSA (curve 2), standard BSA (curve 3), and succinylated BSA (curve 4). Without BSA treatment, the fluorescence spectra of water-soluble dots (curve 5) showed a 10 nm redshift in wavelength and were only 1/5 as bright as that of the original dots dissolved in chloroform (curve 1). With reduced BSA layer, both the fluorescence intensity and the spectral wavelength were restored to the original values.

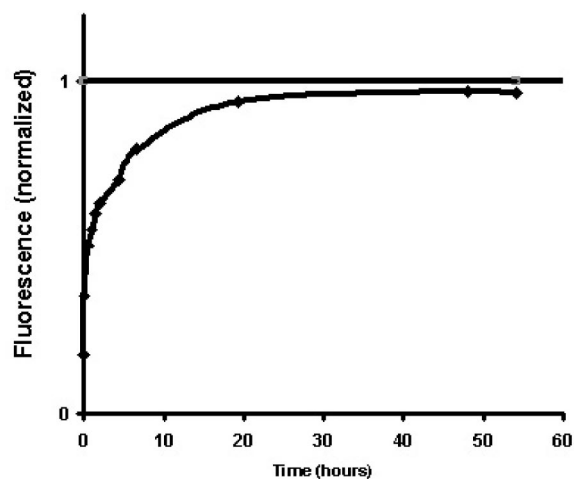


Fig. 5 Plot of fluorescence recovery as a function of time when water-soluble quantum dots were treated with chemically reduced BSA. The fluorescence intensity was normalized by using that of the original dots in chloroform.

den et al.³⁸ However, it is surprising that little or no fluorescence increase is observed when the amine ($-\text{NH}_2$) groups are removed by succinylation. This finding suggests that the amine groups are either directly involved in restoring the fluorescence signals (through nitrogen-cadmium bonding) or (and) are responsible for BSA adsorption. It is possible that the succinylated BSA molecules do not adsorb on water-soluble quantum dots because of repulsive electrostatic interactions. Particle size measurements before and after BSA incubation will provide a definite answer to this question.

3.3 QD-Encoded Microbeads and Nanobeads

Recent research has shown that multicolor quantum dots are ideal fluorophores for multiplexed optical coding of biomolecules. The basic concept is that multicolor QDs can be incorporated into polymeric microbeads at precisely controlled ratios. The use of six colors and ten 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 beads spectroscopic signature. A single light source is sufficient for reading all the quantum-dot-encoded beads. This optical coding technology is expected to open new opportunities in gene expression studies, high-throughput screening, and clinical diagnosis.

In order to realize the potential of this barcoding technology, it is essential to prepare QD embedded beads in a broad size range. A key question is how to change the bead size, while still preserving the porous internal bead structure required for efficient QD incorporation. We have solved this problem by carefully controlling the monomer, the initiator and the stabilizer concentrations in the polymerization mixture. We have succeeded in preparing a series of QD-encoded beads in the size range of 0.1–10 μm diameter. Figure 6 shows both fluorescence and TEM images of the QD-encoded beads at two sizes (3.5 and 0.15 μm diameter). As determined by TEM and single-bead fluorescence measurements, these beads are highly uniform and should be suitable for encoded bead assays.

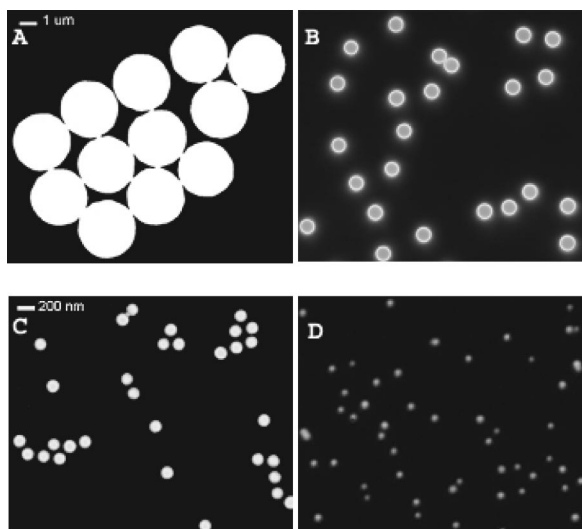


Fig. 6 Transmission electron micrographs (left panels) and color fluorescence images (right panels) of QD-encoded microbeads (A and B, $d = 3.5 \mu\text{m}$, $\lambda_{\text{em}} = 575 \text{ nm}$) and nanobeads (C and D, $d = 150 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$). The fluorescent images were obtained with an inverted Olympus microscope (IX-70) and a Nikon D1 digital camera. In image (D), the variations in signal intensities were caused by beads that were out of focus.

In conclusion, we have shown that water-soluble CdSe/ZnS core/shell quantum dots are sensitive to environmental factors, which could have implications in using quantum dots for chemical and biochemical sensing. Our results also establish chemically reduced BSA as a versatile biopolymer for passivating water-soluble QDs, for restoring their fluorescence quantum yields, and for covalent conjugation to other biomolecules such as peptides, proteins, and oligonucleotides. Together with QD-encoded micro- and nanobeads, we anticipate that bioconjugated quantum dots and optically encoded beads will find broad applications in biotechnology, bioengineering, and clinical medicine.

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