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Review

Biocompatible Quantum Dots for Biological Applications

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Semiconductor quantum dots are quickly becoming a critical diagnostic tool for discerning cellular function at the molecular level. Their high brightness, long-lasting, size-tunable, and narrow luminescence set them apart from conventional fluorescence dyes. Quantum dots are being developed for a variety of biologically oriented applications, including fluorescent assays for drug discovery, disease detection, single protein tracking, and intracellular reporting. This review introduces the science behind quantum dots and describes how they are made biologically compatible. Several applications are also included, illustrating strategies toward target specificity, and are followed by a discussion on the limitations of quantum dot approaches. The article is concluded with a look at the future direction of quantum dots.

Introduction

Quantum dots, nanometer-sized crystals composed of semiconductors, are one of the first nanotechnologies to be integrated with the biological sciences. A decade after their introduction to biology (Bruchez et al., 1998; Chan and Nie, 1998), quantum dots are proven powerful probes for fluorescence imaging and are being developed for a range of additional applications including the detection of disease, fluorescent assays for drug discovery, single protein tracking, and intracellular reporting. Quantum dots have five distinct properties that give them their unique capabilities. First, the dots themselves are small, ranging from 4 to 12 nm in diameter. Second, they have size-tunable, narrow, Gaussian emission spectra that can be excited at a single wavelength, enabling multiplexed experiments. Third, they have enormous absorption extinction coefficients and high fluorescent quantum yields, making them exceptionally bright. Indeed, the emission of a single quantum dot can be discerned by eye with a fluorescent microscope. Fourth, they are inorganic and thus photochemically robust. Their resistance to photobleaching enables extended dynamic imaging. Finally, quantum dots "blink." This fluorescence intermittency assures the observation of a single dot event, which translates to the observation of a single protein. These properties are enabling a new generation of fluorescence imaging experiments in biology allowing investigators to unravel biological function at the molecular level.

The synthesis of highly fluorescent, monodisperse quantum dots is sufficiently advanced that they are commercially available (Invitrogen, Evident Technologies, Ocean NanoTech). Biocompatibility and biological targeting is achieved through surface modification and conjugation with antibodies, peptides, or small molecules. Most bioconjugation is straightforward and can be performed in a biology lab; it does not require skilled chemists. The would-be quantum dot user does have to take great care to defeat, or at least minimize, nonspecific binding (NSB) as this can easily lead to the misinterpretation of experimental results. Rigorous controls are a must in quantum dot experiments, and where rigorous controls are absent in the literature the reader should be skeptical. Analytical tools are becoming available online for the analysis of quantum dot single protein tracking experiments, so much is at hand for the investigator considering adding quantum dots to his or her experimental arsenal (Serge et al., 2008; Jaqaman et al., 2008).

This review is aimed at introducing the reader to the field of quantum dots and providing enough information in the text and the references to encourage a new quantum dot user to get started. We answer the question, "What is a quantum dot?" and describe the basic design principles for making biologically compatible quantum dots. Several applications exploiting the properties of quantum dots are presented, and we also discuss some of the limitations of quantum dots. We close with a prospectus of the future for quantum dots.

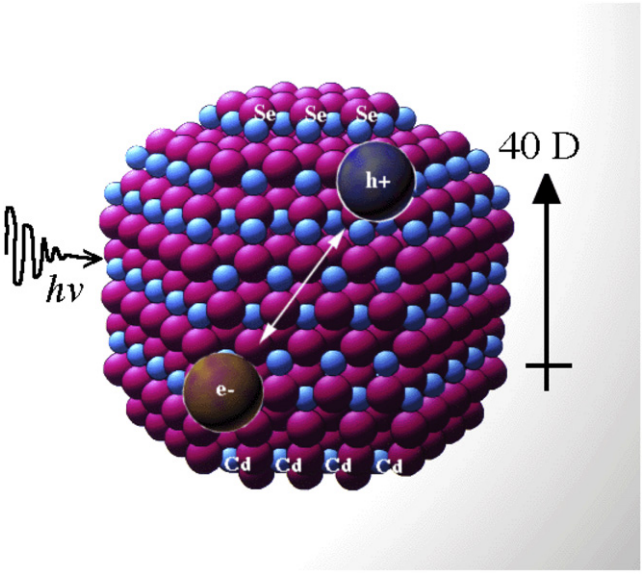
Basic Quantum Dot Design Principles

Quantum dot design comprises three components: synthesizing the quantum dot, modifying its surface so that it is biologically compatible, and then further modifying the surface so that the dot can be directed to a target. There are three primary ways to target a biocompatible quantum dot: with antibodies, with peptides, or with small molecules.

Quantum Dot Design

What Is a Quantum Dot?

A quantum dot is a nanometer-sized crystal of inorganic semiconductor, or semiconductor nanocrystal (Figure 1). It has the same arrangement of atoms as in the corresponding bulk material, but many more surface atoms due to three-dimensional truncation. A 2 nm crystal contains approximately 200 atoms, whereas an 8 nm crystal would contain some 10,000 atoms.



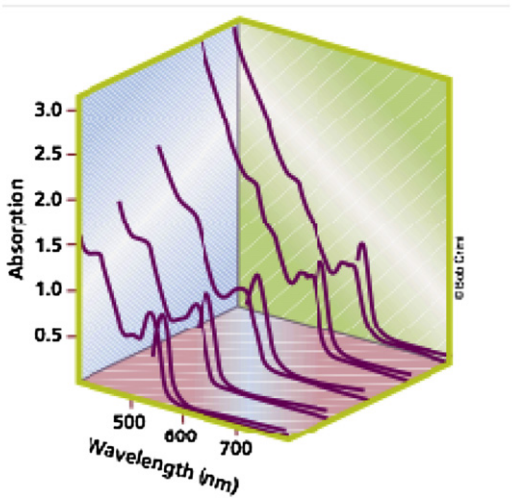


Figure 1. Putative Structure and Size-Dependent Absorption and Emission Spectra of CdSe Nanocrystals

(A) Upon excitation with a photon, an electron-hole pair is created in a nanocrystal. If the electron and hole recombine, fluorescence is given off. If the carriers get trapped to the surface, they are lost. By coating the surface with another semiconductor, material surface loss is eliminated.

(B) Absorption and emission spectra of a series of CdSe nanocrystals. The CdSe core controls the spectral properties of CdSe/CdZnS fluorescent quantum dot. As the size of the core increases, the absorption and emission wavelengths shift to the red. However, as the absorption is continuous above the first excitation peak, one excitation source can be used to excite all sizes of core/shell quantum dots.

Due to the way that the nanocystals are synthesized, they intrinsically have organic ligands capping some of the surface atoms. The name "quantum dot" originates from the fact that the optical properties of the nanocrystal (the "dot") are dictated by quantum mechanics. Here's how it works: when a semiconductor absorbs a photon, an electron is promoted to the conduction band, and a region of positive charge, called the "hole," is left behind in the valence band. The electron and hole can move around in the bulk material, but there is the Coulomb attraction that keeps them together, and the electron orbits the hole at some average distance as in the Bohr model of the atom. As an example, in the semiconductor CdSe the radius of the electron orbit in the bulk material is ~56 Å. So, if you chemically synthesize a CdSe nanocrystal that has a radius smaller than 56 Å, you begin to "squeeze" the electron and hole, and there is confinement energy. The quantum mechanical confinement causes the energy states to shift to higher levels, or blue shift. The smaller you make the nanocrystal, the more you squeeze the electron and the hole, and the higher the energy levels go. So, large CdSe nanocrystals absorb and emit in the red, small nanocrystals absorb and emit in the blue, and the wavelength of emission can be tuned by size (Figure 1B). Changing the nanocrystal composition will change the wavelength range over which the optical properties can be tuned. For example switching from CdSe to ZnSe will lead to bluer nanocrystals, whereas CdTe nanocrystals have energy levels corresponding to near-infrared (NIR) emission in the water window. The lowest wavelength at which the nanocrystal will absorb and emit is dictated by the bulk band gap of the semiconductor. The quantum mechanics behind the size-tunable optical properties of quantum dots are very similar to the "particle-in-a-box" model taught in undergraduate chemistry and physics (Brus, 1984; Kippeny et al., 2002). The smaller the box, the higher the energy levels, following $1/L^2$ where L is the length of the box.

Nanocrystal Synthesis and "Core/Shell" Nanocrystals

As quantum dots are commercially available, it is no longer necessary to be a chemist or to collaborate with a chemist in order to utilize quantum dots in biology, so we will not belabor quantum dot synthetic methodology details here (for extensive review, see Rosenthal et al., 2007). However, nanocrystal synthesis dictates properties, so it is important for the end-user to know some details. Although introduced by Brus in 1984, the nanocrystal field did not really take off until 1993, when Murray and Bawendi developed a synthetic methodology to make large quantities of monodisperse nanocrystals (Brus, 1984; Murray et al., 1993). This method uses the injection of the Cd and Se precursors into super-hot surfactant. This nucleates small nanocrystals; then the temperature is dropped, and the nanocrystals are allowed to grow. The reaction is stopped when the desired size is achieved. The separation of nucleation and growth steps allows for maintaining a good size distribution, and much study of the size-dependent properties of nanocrystals commenced (Alivisatos, 1996a, 1996b). The organic surfactants in which the nanocrystals are grown ultimately become the ligands on the surface of the nanocrystal. Nanocrystals grown this way are not particularly fluorescent (10% quantum yield). The problem is that not all of the surface atoms are bound to ligands—some of the surface atoms are bare. The dangling bonds on these surface atoms are traps. If an electron or hole gets stuck on a trap then they lose energy, and some cannot recombine with each other to give off light. In 1996, Hines and Guyot-Sionnest had a very clever idea to get around this problem: wrap the core of one semiconductor in the shell of another before capping it with the surface ligands (Hines and Guyot-Sionnest, 1996). If the shell material has a wider band gap than the core material, then energetically the electron and hole cannot get into the shell and will have no choice but to recombine and give off light. Also,

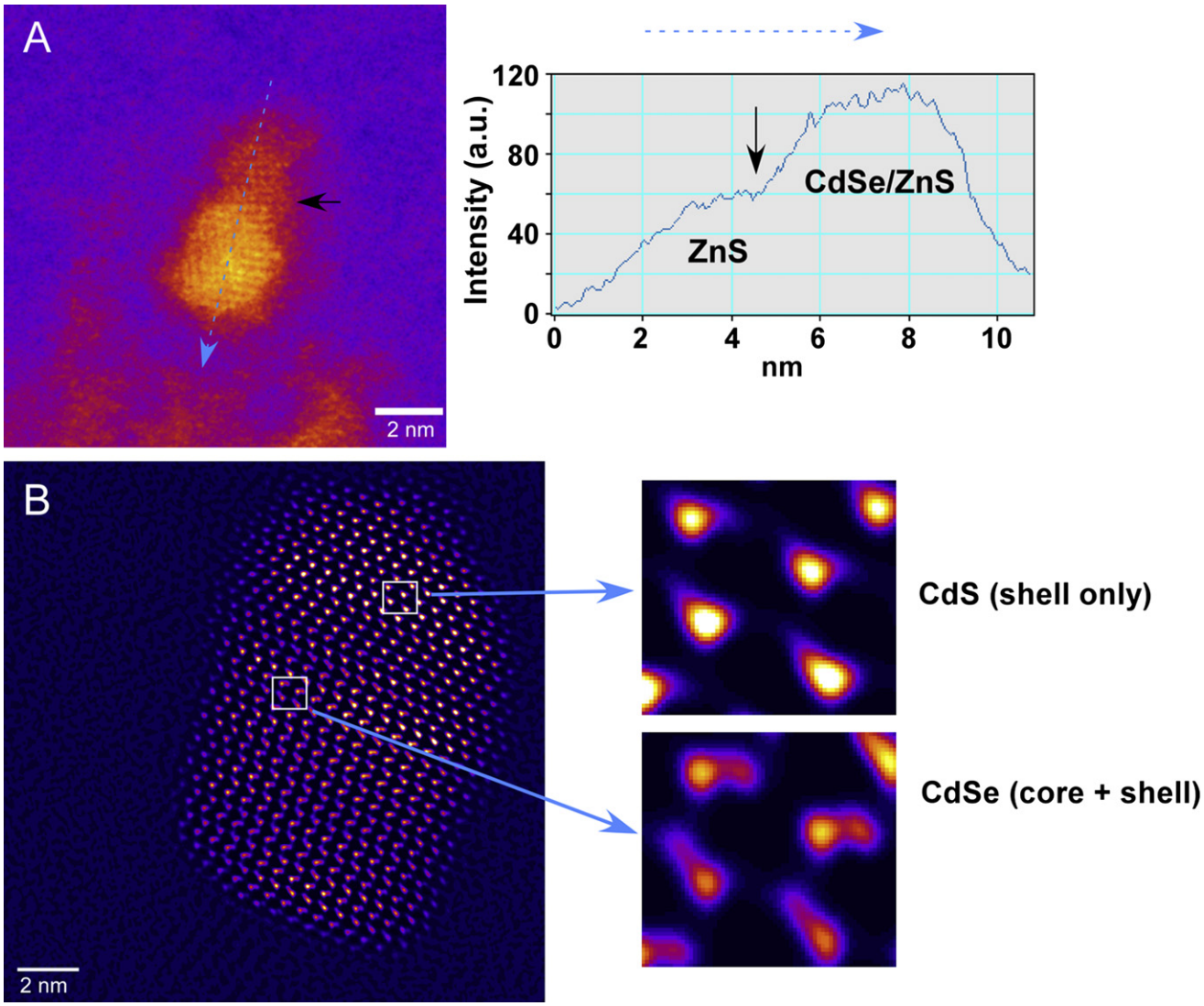


Figure 2. Z-STEM of Quantum Dots
(A) Z-STEM image of a CdSe/ZnS core/shell nanocrystal. The intensity profile clearly indicates the interface between core and shell. The shell appears to be growing primarily off of one facet of the core. (B) Aberration-corrected Z-STEM (Fourier filtered) image of a commercial quantum dot (QD655). The CdS shell can be identified by the loss of the anion atomic dumbbell in the image.

the second semiconductor material will bind up all the surface dangling bonds on the core, so trap states are eliminated. Thus, fluorescent "core/shell" nanocrystals were born with fluorescent quantum yields of up to 35% (Dabbousi et al., 1997).

How to Engineer a Quantum Dot with a 95% Fluorescent Quantum Yield

Although articles reporting the application of fluorescent quantum dots started to appear (Gerion et al., 2002; Dahan et al., 2001; Dubertret et al., 2002; Rosenthal et al., 2002), there were still major inadequacies with the dots. First, the dots were not particularly bright; second, conjugation strategies tended to lower the fluorescence quantum efficiency; third, the dots often lacked good solubility in buffer and tended to aggregate. The first problem, low quantum yield, was solved by a collaborative team of scientists from Quantum Dot Corporation, Vanderbilt University, and Oak Ridge National Laboratory and combined synthetic design with sophisticated aberration-corrected, atomic number-contrast scanning transmission electron microscopy (McBride et al., 2004). Z-STEM imaging of core/shell CdSe/ZnS nanocrystals made then by current methods revealed that shell growth was not uniform; there were patches of the CdSe core that were not passivated by the shell, leading to surface dangling bond trap states as described above, and therefore the fluorescence quantum yield of the dots was low (Figure 2A). The problem was similar to creating heterostructures in thin film surface science. The lattice mismatch between CdSe and ZnS is 14%; that is, the registry of Cd and Se atoms in the CdSe lattice is offset from the arrangement of Zn and S atoms in the ZnS lattice by 14%. As a result, it is difficult to put ZnS down on CdSe, and if a layer of ZnS forms, the next Zn and S atoms will prefer to grow on ZnS instead of CdSe. This problem was solved by adding in some Cd atoms during the growth of the

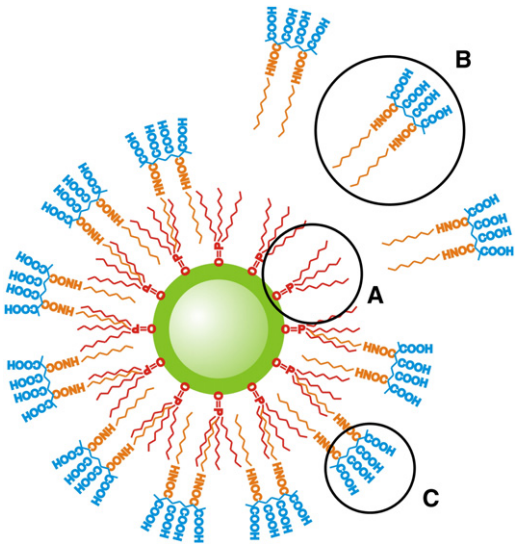


Figure 3. Amphiphilic Polymer Encapsulation Strategy
The original nonpolar ligands on the surface of a quantum dot are left intact (A), and an amphiphilic polymer is used to encapsulate the dot in a water-soluble plastic bag (B). Nonpolar side chains of the polymer intercalate with the nonpolar ligands capping the nanocrystal, and the outer polar, chemically reactive groups of the polymer are used for further conjugation (C).

ZnS shell to help "relax" the lattice. The resulting core/shell nanocrystals had essentially perfect shell growth (Figure 2B) and resulting fluorescent quantum yields that exceeded 95% (McBride et al., 2006). These core/shell nanocrystals are not simple spheres, as one might imagine, but rather have a bullet shape, owing to preferential growth on the Se terminated facets. These are the core/shell quantum dots that are available from Invitrogen.

Biological Compatibility and Targeting of Quantum Dots

Introduction of Buffer Compatibility to the Quantum Dot without Quenching

As described above, the synthesis of quantum dots results in an organic ligand on the surface of the nanocrystal. This is true for both simple nanocrystals and core/shell nanocrystals. These organic capping ligands are always nonpolar, which means that another step in the synthesis of biologically useful nanocrystals is to make them soluble in a buffer. An original approach to this problem was to exchange the nonpolar ligands on the surface of the nanocrystal with polar ligands, usually using a thiol functional group for attachment to the surface of the nanocrystal (Chan and Nie, 1998; Pathak et al., 2001). This was problematic. First, thiols tend to quench the fluorescence of quantum dots, and the surface ligand-exchanged dots were not as bright as the original quantum dots with their native, nonpolar surface ligands. Second, the Cd-thiol bonds were not particularly stable, which could affect quantum dot performance over time.

Bruchez solved this problem using an amphiphilic polymer strategy (Figure 3) (Wu et al., 2002). As the native ligands (Figure 3A) provide for the brightest quantum dots, Bruchez left the original nonpolar ligands intact, and, instead of exchanging them, he used an amphiphilic polymer to essentially encapsulate the dot in a "plastic bag" that was water soluble. Nonpolar side chains on the polymer intercalate with the nonpolar ligands capping the nanocrystal (Figure 3B), and then the polymer is crosslinked to encapsulate the nonpolar portion, leaving polar, chemically reactive groups on the outer surface (Figure 3C), yielding bright dots that are soluble in buffer and have a chemical handle for further bioconjugation (Figure 3C). These are the AMP Dots available from Invitrogen. The encapsulated quantum dots depicted in Figure 3 are terminated with carboxylic acid functional groups, but it is possible to utilize amphiphilic polymer strategies to arrive at dots with other terminating functionalities. This is achieved by reacting the carboxylic acid functionalities with crosslinking reagents. Frequently the carboxylic acid functionalities on the AMP surface are reacted with diamino polyethylene glycols, resulting in quantum dots that have amino functionalities on their surfaces. These dots may be conjugated to proteins, peptides, and small molecules via an amide linkage. Alternatively, the amino functionality may be modified further by reacting it with crosslinking reagents such as SMCC (succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate) resulting in quantum dots that have maleimide groups on their surfaces. Maleimide-coated quantum dots may be conjugated to molecules containing free thiols, such as antibodies and antibody fragments. The polymer coating on the core/shell nanocrystals also increases their size; it has been reported that the diameters of nanocrystals encapsulated in poly(maleic anhydride alt-1-tetradecene) increases by 1.2 nm (Pellegrino et al., 2004). Since this polymer has a similar structure to the modified amphiphilic polyacrylamide polymer used to encapsulate the AMP dots, the diameters of these dots are likely to increase by a similar factor. Thus, it is probable that the AMP coating adds 1–2 nm to the AMP dots diameter, resulting in dots with diameters in the range of 5–14 nm. We will discuss newer strategies to obtain buffer solubility and reduce quantum dot size below. For an extensive coverage on alternative quantum dot manufacturing and capping strategies, refer to Medintz et al. (2005); Peng and Peng (2000), and Qu et al. (2001).

Conjugation Chemistries to Attach a Biological Targeting Moiety to Buffer-Compatible Quantum Dots

Various chemistries are available to conjugate biologically active molecules to the surface of nanocrystals. Table 1 lists several possible quantum dot-terminating functional groups, possible terminating groups on the biologically active molecule, and the chemical reagent that is used to link the two. For example, EDAC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) can be used to couple carboxylic acid-terminated dots to biologically active molecules containing amines, whereas crosslinking reagents such as SMCC and N-hydroxysuccinimidyl iodoacetate (SIA) (Orndorff and Rosenthal, 2009) can be used to couple thiols to quantum dots with an amino functionality on their surfaces. Peptides can be coupled to the surface of amine terminated quantum dots using SIA and Traut's reagent (Orndorff et al., 2008). One very convenient, almost universal strategy is to link streptavidin to the surface of the quantum dot, and then use a biotinylated targeting moiety that will link to the streptavidin. Streptavidin can be linked to the surface of quantum dots by utilizing the reagent EDAC to crosslink carboxylic acid groups on the surface of the dot with amino acid side chains on the surface of the streptavidin (Medintz et al., 2005).

Table 1. Conjugation Strategies to Functionalize Quantum Dots

Quantum Dot Terminating Group	Biomolecule Terminating Group	Linking Agent (Full Name)	Example (References)
Carboxylic Acid	Amine	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide	Tomlinson et al., 2007; Gussin et al., 2006
Amine	Amine	Traut's reagent, N-hydroxysuccinimidyl iodoacetate (SIA)	Orndorff and Rosenthal, 2009
Amine	Thiol	Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP)	Derfus et al., 2007
Avidin/Streptavidin	Biotin		Bakalova et al., 2005; Soman and Giorgio, 2007; Tomlinson et al., 2006; Tomlinson et al., 2005

Streptavidin-terminated quantum dots are also commercially available. The streptavidin-biotin strategy adds substantial bulk to the quantum dots. Streptavidin-coated quantum dots have diameters that are of the order of 15–20 nm.

It is common to incorporate linker arms between the quantum dot and the biological targeting moiety to provide steric freedom. This strategy is especially common if the targeting moiety is a small molecule or a small peptide. The linker arm can serve a dual purpose; for example, polyethylene glycol chains can reduce nonspecific binding, as we will discuss below. Examples of linker arms are given in Figure S1 (available online).

Modifying the Quantum Dot Surface to Direct It to a Target

There are three primary means to direct a quantum dot to label a target: antibodies, peptides, and small molecules. The simplest labeling strategy uses antibodies; the most complicated is that of small molecules, as this approach usually requires more synthetic chemistry. Each approach has its advantages and disadvantages, and no approach is universal for all applications.

Antibody Targeting

If an antibody exists for an extracellular epitope of the target, then the simplest and quickest labeling route is to use an antibody-quantum dot conjugate. Antibody-quantum dot conjugates have been used in a myriad of applications. Examples include using antibodies for F and G proteins on two sizes of quantum dots to detect the presence and follow the progression of respiratory syncytial viral infection in vitro (Bentzen et al., 2005a), using antibody-conjugated quantum dots to target a prostate-specific membrane antigen in tumors in vivo in mice (Gao et al., 2004), and using anti-HER2 quantum dot conjugates to image breast cancer cells in vitro (Wu et al., 2002) and in vivo (Tada et al., 2007). Antibodies can be biotinylated and used with streptavidin-coated quantum dots, or they can be directly conjugated to the quantum dot. A simple kit requiring no prior quantum dot experience for linking antibodies to AMP quantum dots is commercially available from Invitrogen. This kit comprises dithiothreitol (DTT) and an SMCC crosslinker. The DTT is used to reduce disulphide bonds in the antibody resulting in free thiols, and these may then be bound to the AMP surface utilizing the SMCC crosslinker (Pathak et al., 2007). A wide selection of antibody-quantum dot conjugates is also commercially available. Disadvantages to this approach include the availability of antibodies, their selectivity and affinity, and the increased hydrodynamic radius of the quantum dot conjugate. Nonetheless, antibody-quantum dot conjugates are often the method of choice and make up much of the quantum dot-in-biology literature.

Peptide Targeting

When ligand-target interactions are facilitated by a peptide epitope of a protein or a known peptide, it is logical to utilize a peptide-quantum dot nanoconjugate for labeling. Similar to antibody recognition, the strength of peptide-target interaction is highly variable. However, the use of peptides in quantum dot probe architecture leads to significant reduction of expenses associated with synthesis and the ultimate nanoconjugate size. Additionally, covalent conjugation of multiple peptides to one quantum dot may considerably increase the binding capacity of the resulting complexes to the target through multivalent interactions. Covalent conjugation of peptides to quantum dots is typically achieved through the use of water-soluble crosslinking reagents such as EDAC, SIA, and Traut's reagent (Orndorff et al., 2008; Orndorff and Rosenthal, 2009). An alternative route is to utilize streptavidin-biotin noncovalent self-assembly (Chen and Gerion, 2004).

Peptide-functionalized quantum dots have been successfully used for targeting cellular proteins such as growth factor receptors, G protein-coupled receptors, integrins, and ion channels (Vu et al., 2005; Tomlinson et al., 2005b; Cai et al., 2006; Orndorff and Rosenthal, 2009). In particular, Smith et al. conjugated ~30–50 arginine-glycine-aspartatic acid (RGD) peptides to NIR quantum dots to specifically target $\alpha_v\beta_3$ integrins in mouse tumor neovasculature in vivo, while Orndorff et al. relied on high-affinity peptide neurotoxin quantum dot nanoconjugates to image endogenous proteins in living cells and ex vivo tissue (Smith et al., 2008a; Orndorff et al., 2008; Orndorff and Rosenthal, 2009). Additionally, protein transduction domains such as HIV TAT, Pep-1, polyarginine, and SV40 T antigen have been linked to quantum dots to facilitate intracellular delivery (Ruan et al., 2007; Rozenzhak et al., 2005; Gao et al., 2004; Derfus et al., 2004). Overall, peptide-quantum dot nanoconjugates offer distinct advantages over antibody-mediated targeting, and their potential as biological probes is being actively explored.

Small Molecule Targeting

A method that we have pioneered is the use of small molecules to target quantum dots to cell surface receptors (Rosenthal et al., 2002). Ligands with high affinity and selectivity can target the quantum dot directly to the binding site of the protein of interest. Examples are shown in Figure 4. Labeling the binding site of the protein directly can initiate a dynamic process and enable monitoring, for example, of receptor-mediated endocytosis. Ligand-conjugated quantum dots also enable following the

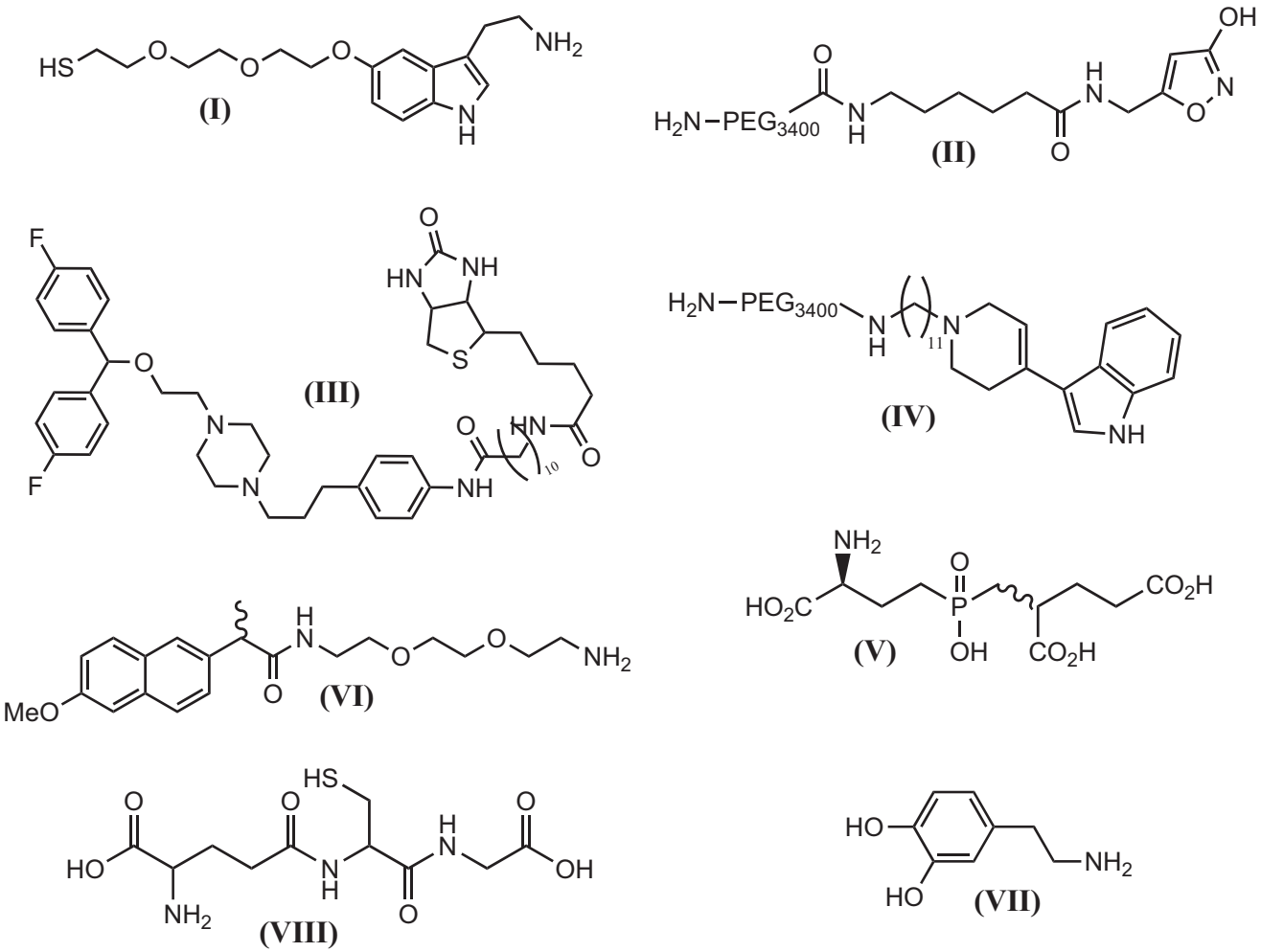


Figure 4. Small Molecule Ligands that Have Been Conjugated to Quantum Dots
(I) A PEGylated serotonin derivative (Rosenthal et al., 2002); (II) a muscimol derivative (Gussin et al., 2006); (III) a dopamine transporter antagonist (Tomlinson et al., 2006); (IV) a serotonin transporter antagonist (Tomlinson et al., 2007); (V) GPI a tumor targeting ligand (Choi et al., 2010); (VI) A derivative of the NSAI D naproxen (Byrne et al., 2007); (VII) dopamine (Clarke et al., 2006); (VIII) glutathione (Tortiglione et al., 2007).

dynamics of the target protein, and can help elucidate factors that regulate protein expression and cell surface mobility. Also, as the quantum dot is tied off at the binding site of the protein, displacement assays are possible. A test compound that targets the binding site will displace the ligand-conjugated quantum dot, enabling a fluorescence-based assay for drug discovery. Ligand-conjugated quantum dots can also be used as probes for allosteric modulation of the binding site. One can envision several ligand-conjugated quantum dots, with each ligand conjugated to a different size (color) quantum dot, allowing a multiplexed fluorescent assay for drug discovery. Based on the structure of the cell surface receptor, the ligand can be tailored with a linker arm that optimizes binding. The one major disadvantage to the ligand-conjugated quantum dot approach, however, is that fairly sophisticated organic chemistry is required to synthesize an optimized ligand.

High-Affinity Fusion Tag Targeting Approaches

To expand the arsenal of tools for site-selective, specific quantum dot labeling of cellular targets, several new approaches based on high-affinity fusion tags have recently been reported. A cellular target of interest is expressed with a genetically engineered high-affinity fusion tag, usually a short peptide sequence with a recognition epitope for complementary binding partner. For instance, Ting and co-workers genetically fused a 15-amino acid acceptor peptide (AP) to the N or C terminus of AMPA receptors in hippocampal neurons (Howarth et al., 2005; Howarth and Ting, 2008). The AP sequence was then biotinylated by biotin ligase, followed by incubation with streptavidin-quantum dots. In another example, Dahan and colleagues fused a decahistidine tag to the N-terminus of type-1 interferon receptor subunit, and quantum dot targeting was achieved through the use of tris-nitriloacetic acid, which has a subnanomolar affinity for linear polyhistidine motifs (Roullier et al., 2009). Other fusion tags used in conjunction with quantum dots include CrAsH (Genin et al., 2008) and Halotag (So et al., 2008).

Nonspecific Binding

Depending on the type of surface functional group, quantum dots may display nonspecific cellular binding that negatively

impacts experimental results. Nonspecific binding of quantum dots has been observed in several systems and has been attributed primarily to electrostatic interactions between quantum dot surface groups (e.g., carboxyl) and the target cell surface (Gerion et al., 2002; Bentzen et al., 2005b). In particular, Bentzen et al. reported nonspecific cellular binding of carboxyl-terminated AMP Dots in a cell type-dependent manner (Figure S2) (Bentzen et al., 2005b). According to Warnement et al., quantum dots can also be subject to nonspecific protein adsorption to the quantum dot surface, specifically adsorption of a 66kDa serum protein albumin present in standard blocking solutions (Figure S3) (Warnement et al., 2008). Therefore, the would-be quantum user must carefully examine selected quantum dot probes for nonspecific interactions in the experimental system and choose a surface functionalization approach that will ultimately result in minimal nonspecific binding.

Since nonspecific cellular binding of quantum dots has a negative impact on the experiment, several approaches to address the issue of nonspecific binding have been reported. In 2003, Ballou et al. reported that quantum dots conjugated to methoxy-terminated PEG^{5000} (molecular wt 5000 g) displayed considerably reduced nonspecific binding in mouse animal models (Ballou et al., 2003). In 2005, Bentzen et al. demonstrated that a small number of methoxy PEG^{2000} conjugated to the surface of AMP Dots resulted in greatly reduced nonspecific binding in six different mammalian cell lines (Figure S2) (Bentzen et al., 2005b). As a consequence, quantum dot PEGylation currently constitutes the most popular approach to combat the issue of nonspecific cellular binding. In an alternative approach, Kairdolf et al. used 1,3-diamino-2-propanol (DAP) to facilitate conversion of carboxylated quantum dots (coated with poly [acrylic acid] octylamine) to hydroxylated and crosslinked dots (Kairdolf et al., 2008). As a result, hydroxylated quantum dots displayed a dramatic 140-fold reduction in nonspecific cellular binding compared with that of carboxylated dots.

To sum up, nonspecific cellular binding of quantum dots is a limiting factor in a range of biological applications, such as live cell imaging, tissue staining, and in vivo studies. Therefore, the would-be quantum dot user must show great care in defeating the issue of nonspecific binding and be skeptical of data that are presented without rigorous control experiments.

Applications of Quantum Dots in Biology

The utility of quantum dots has been demonstrated in a variety of biological and clinical applications, such as immunohistochemical detection, drug delivery and therapeutics, biosensing, small animal imaging, and single-quantum dot tracking of extra- and intracellular targets (Medintz et al., 2008; Delehanty et al., 2009; Medintz and Mattoussi, 2009; Gao et al., 2004; Pinaud et al., 2010). This review focuses on examples of how quantum dots are being used to unravel biological function at the molecular level, especially in the field of neurobiology. Particular emphasis is placed on the use of individual quantum dots to investigate membrane dynamics of cell surface proteins involved in cellular signaling.

Systems aimed at detecting proteins at single-molecule level have been a long-term desire for biologists to understand the molecular mechanisms of protein binding, signaling, and regulation. Using bright and stable quantum dots as a fluorescent tag for single protein tracking is a revolutionary and exciting tool in recent life science research (Giepmans et al., 2005; Michalet et al., 2005; Pons and Mattoussi, 2009; Pinaud et al., 2010). The approach to single protein tracking using quantum dots can be divided into three steps: (1) time-series fluorescent imaging; (2) trajectory construction; and (3) data analysis (Figure 5). In single protein tracking, target proteins of interest are labeled with a quantum dot in a very dilute fashion to avoid interparticle reactions. The movement of individual target proteins is monitored over time from an optical fluorescent microscope system (Figure 5A). Before generating the trajectory of each protein, subpixel location estimation from digital signals is essential for improving the accuracy of 2D position of single quantum dots in each frame. This is generally accomplished by fitting the individual spot intensity values into two-dimensional Gaussian distributions (Figure 5B). According to Pinaud et al., the quantum dot localization accuracy can be as low as 10 nm with 10 ms integration time (2010). When trajectory construction is complete, the dynamic properties of the target proteins can then be derived through various statistical analyses of the trajectories (Figure 5C). Dynamic properties (i.e., displacement, velocity, and diffusion coefficient) provide direct access to the influence of the intracellular microstructure or extracellular stimulus on the movement of the target protein.

In 2003, Dahan and colleagues performed the first single protein tracking experiment based on quantum dot probes (Dahan et al., 2003). In brief, the authors tracked the lateral diffusion of individual glycine receptors with antibody-conjugated quantum dots in living neuronal cells by time-series fluorescent microscopy. Their experiment revealed that diffusion dynamics of glycine receptors varies in synaptic, perisynaptic, and extrasynaptic domain of spinal neurons. Using a similar tracking strategy in a different study, the same group elegantly demonstrated that the molecular mechanisms of $GABA_A$ receptors respond to the chemotactic signaling in neurons (Bouzigues et al., 2007). In the presence of an extracellular GABA gradient, the authors showed that single quantum dot-labeled $GABA_A$ receptors redistribute asymmetrically across the growth cone (GC), located at the axon tip, toward the gradient source in a microtubule- and calcium-dependent manner (Figure S4).

A similar concept was applied by Chu and colleagues to investigate the retrograde axonal transport of nerve growth factor (NGF) signals (Cui et al., 2007). The authors labeled the biotinylated NGF with streptavidin-conjugated quantum dots and used total-internal-reflection fluorescence (TIRF) microscopy to track the movement of NGF in live DRG neurons in real time. Their experiment revealed that quantum dot-labeled NGF exhibited "stop-and-go," unidirectional retrograde motion within the axons with an average speed of 1.31 ± 0.03 μm/s (Figure S5).

Another example of how quantum dots may provide valuable insights into the molecular details of complex biological processes is the visualization of the initial stages of receptor tyrosine kinase-dependent signaling (Lidke et al., 2004). In his extensive review in 2000, Schlessinger discussed the significance of transmembrane receptor tyrosine kinases (RTKs) in cellular signal transduction and the sheer complexity of the signaling networks associated with RTKs (Schlessinger, 2000). Since RTKs play a critical role in the development and proliferation of many types of cancer, Schlessinger emphasized the need

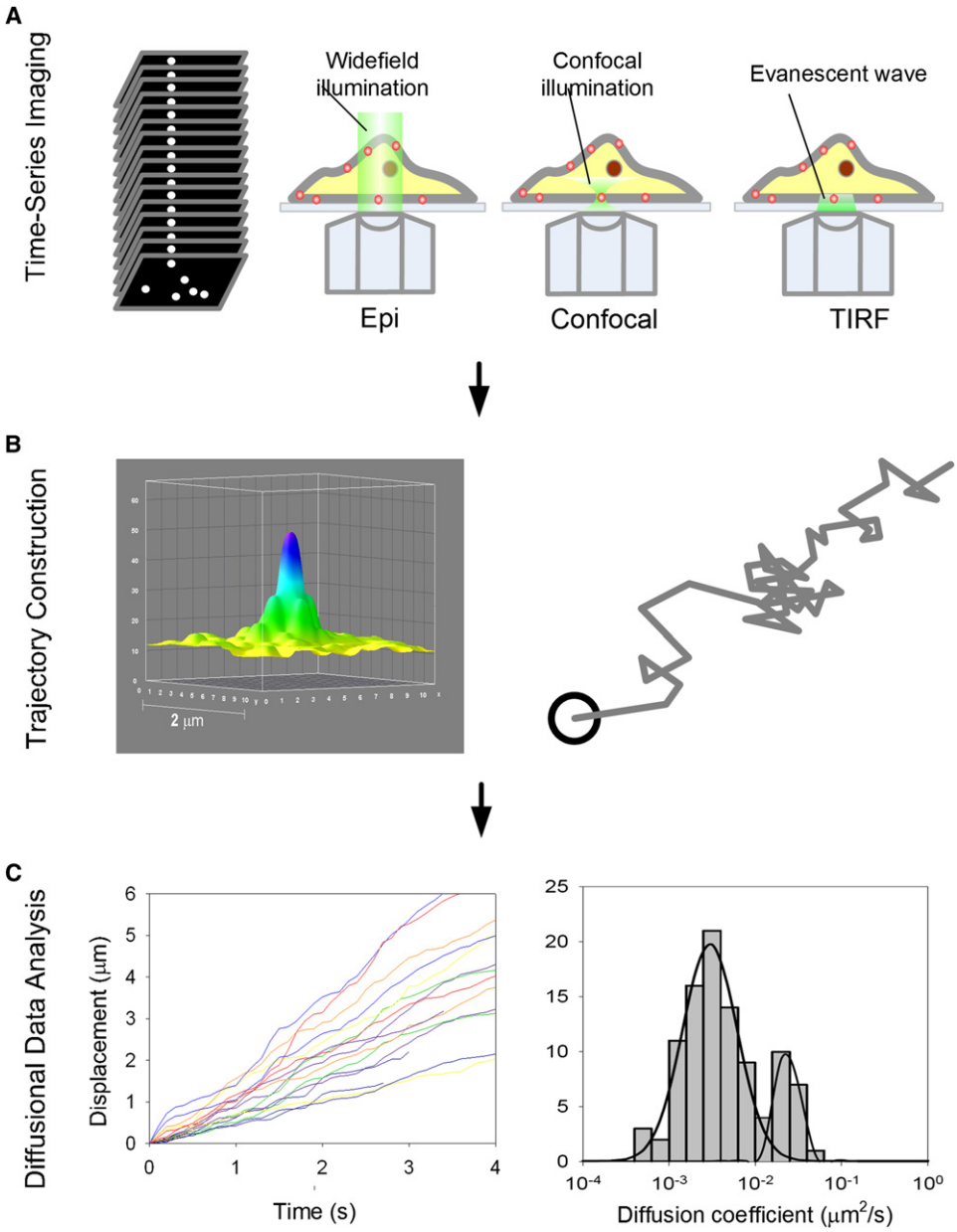


Figure 5. Approach to Single Protein Tracking Using Quantum Dots

(A) Time-lapse images of single quantum dot tagged proteins in living cells are acquired from an optical fluorescent microscope system (e.g., epifluorescence, confocal, or total internal reflection fluorescence [TIRF] microscope).

(B) Estimation of the positions of single quantum dots with subpixel accuracy is accomplished by fitting the individual spot intensity values into a two-dimensional Gaussian distribution. After the positions of single quantum dots are identified, trajectory of target protein (gray line) can be subsequently derived from the time-series imaging data.

(C) The final aspect of single protein tracking is to analyze the single-quantum dot trajectories. Motion properties (i.e., displacement, velocity, and diffusion coefficient) of the target proteins can then be characterized to understand how the motion dynamics of the target protein is associated with intracellular microstructure or extracellular stimulus.

for the development of more sophisticated tools to facilitate biological inquiry into signaling networks associated with RTKs. It turned out that quantum dots might be just the tool needed for understanding the intricacies of cellular signaling.

In 2004, Lidke and co-workers used epidermal growth factor-conjugated quantum dots to target erbB1 receptor, a member of the RTK family of erbB proteins often dysregulated in cancer (Yarden and Sliwkowski, 2001; Lidke et al., 2004). As a result, they discovered a previously unreported mechanism of retrograde transport of the receptor-quantum dot complexes from the filopodia to the cell body in human epidermoid carcinoma cells. Furthermore, Lidke and co-workers revealed the protein-protein interactions (heterodimerization) between quantum dot-bound erbB1 and erbB2 receptors and, consequently, the active

Table 2. Calculated Quantum Dot Molecular Weight

Inorganic Core/Shell	Band Edge Absorption (nm)	Core Diameter (nm)	Core Molecular Weight (kDa)	Shell Molecular Weight (kDa)	DDPA (kDa)	MUA (kDa)	AMP* (kDa)	Cysteine (kDa)	AMP + Core/shell (kDa)	Cysteine + Core/Shell (kDa)
CdSe/ZnS	640	7	615	535	147	128	380	71	1530	1220
	585	4	133	191	63	55	163	30	487	354
	460	2	15	74	26	23	68	13	157	101
CdTe/CdS	635	4	120	235	67	58	172	32	527	388
InAs/ZnSe	900	4	114	244	63	55	163	31	521	388

Molecular weights were determined assuming spherical structures with 100 % ligand coverage and 2 nm of shell coverage. The ligands chosen were dodecylphosphonic acid (DDPA), mercaptoundecanoic acid (MUA), an amphiphilic polymer-poly(maleic anhydride alt-1-tetradecene) (AMP), and cysteine. For reference, streptavidin has a molecular weight of 52.8 kDa and GFP has a molecular weight of 26.9 kDa.
[a]The AMP molecular weight was calculated as the sum of the DDPA and AMP ligands, assuming a one to one ratio of AMP and DDPA.

role erbB2 receptor plays in conferring higher aggressiveness to breast cancer. This was the first successful attempt of visualizing the erbB signaling network at the molecular level in living cells in real time.

While quantum dots have been utilized to investigate the diffusion dynamics of individual membrane-associated proteins, their use in small, crowded cellular locations such as neuronal synaptic clefts (10–50 nm) has been limited by the relatively large size and suboptimal biocompatibility (De Koninck et al., 2007). However, with the introduction of amphiphilic polymers as surface-passivating agents, it became possible to decrease the size of quantum dots while simultaneously improving their colloidal stability and mitigating nonspecific binding (Wu et al., 2002). In 2008, Heine and co-workers used streptavidin-conjugated quantum dots in conjunction with a biotinylated antibody to visualize intrasynaptic lateral mobility of AMPA glutamate receptors (Heine et al., 2008). This report illustrated the bright future of quantum dots as probes of first choice to study synaptic neurotransmission at the molecular level.

Why is so much attention focused on neuronal synapses? A synapse is the junction between two adjacent neurons that permits the propagation of electrical or chemical signals, and proper synaptic transmission is critical for all neuronal *functions*, including movement, sensation, learning, and memory (Eccles, 1964). Signal transmission in chemical synapses is facilitated by vesicular secretion of neurotransmitters (Eccles, 1964). Synaptic vesicles may release their contents into the synapse via either full-collapse fusion (FCF) or transient fusion and retrieval, also known as Kiss-and-Run (K&R) (Smith et al., 2008b; An and Zenisek, 2004). The preferred vesicular fusion mode has been the subject of ongoing controversy since the 1970s (Smith et al., 2008b; An and Zenisek, 2004). In the most remarkable display of the utility of quantum dots to understanding neurotransmission to date, Zhang and co-workers loaded amphiphilic polymer-coated quantum dots into synaptic vesicular lumen (~24 nm) and successfully visualized and identified FCF and K&R fusion modes of single quantum dot-loaded synaptic vesicles (Zhang et al., 2009). Individual vesicle imaging was facilitated by the small size of quantum dots (15.0 ± 0.5 nm), pH dependency of quantum dot photoluminescence (reversible 15% difference between intravesicular (pH 5.48) and extracellular environment (pH 7.34)), and excellent photostability. Zhang and co-workers discovered that K&R and FCF can each be the preferred fusion mode dependent on the type of stimulus applied. K&R was found to be the predominant fusion mode for readily releasable pool of synaptic vesicles upon rapid stimulation. In addition, the duration of fusion pore opening, which allows complete release of neurotransmitters, was determined to be ~0.5–1 s long.

In addition to specific labeling of individual cell surface biomolecules, quantum dots have also been used to target intracellular single biomolecules, such as mRNA and molecular motors (Courty et al., 2006; Peirobon et al., 2009; Ishihama and Funatsu, 2009). However, several challenges must be overcome before single-quantum dot tracking can be employed to probe the dynamics of endogenous molecules inside live cells. First, it is difficult for quantum dots to cross the plasma membrane due to the critical effects of size, charge, and surface coating (for intracellular delivery strategies, see Delehanty et al., 2009). Second, once internalized, quantum dots are prone to endosomal entrapment and aggregation in the acidic lysosomal environment, resulting in nonspecifically bound quantum dot probes that cannot be easily washed away. Third, specific targeting of endogenous intracellular molecules without in vitro preconjugation and genetic manipulation remains an important technical challenge (Pinaud et al., 2010).

Limitations of Quantum Dots as Biological Probes and Possible Solutions

Probe Size

While quantum dots are intrinsically small (large, red-emitting, as-synthesized CdSe/CdZnS core/shell nanocrystals are less than 14 nm in diameter with their native organic coating), their size can grow substantially after being made buffer-compatible with an AMP strategy, attaching streptavidin and an antibody, or by using a long polyethelene glycol linker arm to attach small molecules. Quantum dots containing heavy metals also have substantial mass. The diameter and approximate molecular weight of various quantum dot constructs is tabulated in Table 2.

There are several reasons to push for a smaller probe size. Perhaps the most pressing is the desire to use quantum dots in vivo in humans in the future. Many investigators have performed studies that point to the utility of quantum dots for in vivo use as diagnostics and therapeutics (for reviews, see

Nie et al. [2007], Jin and Ye [2007], and Medintz et al. [2008]). In 2004, Nie and coworkers demonstrated both passive and active targeting of quantum dots to image prostate cancer tumors in mice (Gao et al., 2004). Waggoner, Bruchez, and co-workers also demonstrated the capability to image quantum dots in vivo in mice and found that the surface coating on the quantum dot dictated where the quantum dots localized (Ballou et al., 2003). In that same year, Frangioni and co-workers demonstrated using large animals that NIR-emitting quantum dots enabled a type of cancer surgery, sentinel lymph node mapping, using image guidance provided by the quantum dots (Kim et al., 2004). This group has also found that the biodistribution and elimination routes of quantum dots depend on their size (Choi et al., 2009). Recently, Kim and coworkers showed that hyaluronic acid-functionalized quantum dots could be used to visualize lymphatic vessels in vivo in small animals, demonstrating the potential toward imaging the real-time development of lymphatic vessels around tumor mass such as in lymphangiogenesis (Bhang et al., 2009).

If quantum dots, especially those containing heavy metals, are going to be used in in vivo applications such as diagnostic imaging, they must clear the body. Frangioni, Bawendi, and co-workers determined the conditions for renal filtration and urinary excretion of quantum dots by systematically varying their size and surface charge and have arrived at a set of design parameters for targeted nanocrystals that can be eliminated through the kidneys (Soo Choi et al., 2007; Choi et al., 2009). A hydrodynamic diameter of less than 5.5 nm is the benchmark for efficient elimination of quantum dots from the body. The surface chemistry of the quantum dots is important to prevent the adsorption of serum proteins, which increase the hydrodynamic diameter of the quantum dot. Zwitterionic and neutral organic coatings have been shown to prevent the adsorption of serum proteins (Soo Choi et al., 2007).

A second reason to reduce the hydrodynamic radius of the nanocrystal and its mass pertains to their use for in vitro experiments. For example, the synaptic gap can be as narrow as 10 nm across. If one would like to label both presynaptic neurotransmitter transporters and post synaptic receptors at the synapse in a multiplexed experiment, the quantum dots will have to be compact. Also, for single protein tracking experiments in which one is trying to elucidate the velocities of the protein, one would not like the mass of the quantum dot label to alter the intrinsic dynamics. Finally, quantum dots will have to be small to study intracellular compartmentalization.

Several groups are developing synthetic strategies for compact, fluorescent quantum dots (Choi et al., 2009; Law et al., 2009; Perrault et al., 2009; Tromsdorf et al., 2009). The challenge in designing quantum dots with small hydrodynamic diameter is to maintain high quantum yields and photostability while keeping the shell and organic coatings at a minimum. One solution to minimize the core/shell diameter is to integrate the shell into the core nanocrystal via alloying. Photostability with water solubility will likely require short bidentate ligands with carboxylic head groups, such as dihydrolipoic acid (Zimmer et al., 2006). Liu et al. synthesized quantum dots with a hydrodynamic diameter less than 5.5 nm which clear the renal system, utilizing ultrasmall CdSe/$Zn_xCd_{1-x}S$ nanocrystals capped with cysteine, a small zwitterionic molecule (Liu et al., 2007). The quality of the alloy shell helps minimize the reduction in fluorescence quantum yield when the quantum dots are transferred to water. An alternative way to prepare smaller quantum dots is to use semiconductor materials other than CdSe-ZnS, such as CdTe, InP, and InAs (Smith and Nie, 2008; Xie et al., 2007; Zimmer et al., 2006).

Multivalency

The large surface area to volume ratio of a water-soluble, biocompatible quantum dot enables conjugation of multiple copies of various biomolecules, such as proteins, peptides, DNA, and small molecules. For instance, commercial streptavidin-quantum dot conjugates have five to ten streptavidin molecules per quantum dot (Jaiswal and Simon, 2004). Such multivalency effect is considered to be another distinct limitation of quantum dots as biological probes and can be a concern in single-quantum dot tracking experiments, where multivalent interactions of a quantum dot with a target cell surface protein can lead to protein crosslinking and consequently activate signaling pathways as well as significantly impair surface protein mobility (Saxton and Jacobson, 2003). Another undesirable outcome of quantum dot multivalency may be increased cytotoxicity of the multivalent quantum dot conjugates. Recently, Wang et al. (2010) demonstrated that cytotoxicity of PRINT nanoparticles conjugated to nontoxic transferrin and transferrin receptor (TfR) antibody was a function of surface ligand density. Trf-targeted PRINT nanoparticles activated apoptopic pathways upon receptor-mediated internalization in Ramos lymphoma cells in a ligand density-dependent manner (Wang et al., 2010).

To date, several approaches to address quantum dot multivalency have been reported; however, it has been problematic to obtain quantum dots with exactly one surface targeting group until Howarth et al. generated monovalent quantum dots in 2008 (Sung et al., 2004; Lévy et al., 2006; Fu et al., 2004; Howarth et al., 2006, 2008; Howarth and Ting, 2008). In their report, CdSe-ZnCdS core-shell quantum dots were conjugated to a single copy of monovalent streptavidin or antibody to carcinoembryonic antigen. The quantum dot conjugates were analyzed by electrophoresis in agarose gel, and a striking electrophoretic mobility of quantum dots was used to purify monovalent conjugates. As a result, monovalency resulted in a reduced hydrodynamic radius of the quantum dot conjugates and consequently improved access to synaptic cleft as well potential prevention of cell surface receptor complex activation.

While quantum dot multivalency is a disadvantage for imaging protein dynamics at the single-molecule level, it can be exploited to produce quantum dot drug carriers and imaging agents with higher avidity for the target cells of interest as well as highly sensitive quantum dot-based diagnostics and biosensors. In parallel, many effective antiinflammatory and antiviral agents are based on the multivalent drug design to amplify their potency (Mammen et al., 1998). However, current lack of control over the number of surface ligands and their exact orientation and conformation is a significant obstacle that must be overcome to fully harness the advantages associated with quantum dot multivalency.

Blinking

Another characteristic property of quantum dots that poses significant challenge to monitoring protein dynamics at the single-molecule level is the photoluminescence intermittency

exhibited by single nanocrystals, known as blinking (Nirmal et al., 1996; Kuno, et al., 2001). The photoluminescence spectra of single quantum dots are characterized by large intensity fluctuations, leading to the appearance of ''on'' and ''off'' states. A single nanocrystal spends a significant fraction of time in the ''off'' or ''dark'' state even under continuous illumination, with values as high as 100 s for commercially available avidin-conjugated quantum dots (Kagan et al., 1996). As a result, it can become difficult to establish correspondence between consecutive frames of the time-lapse image sequence for blinking quantum dots in tracking applications (Bannai et al., 2007). On the other hand, the advantage of blinking is that it can be used as a criterion for distinguishing single quantum dots from aggregates ((Nirmal et al., 1996; Kuno, et al., 2001).

Despite extensive interrogation over the last decade, fundamental processes responsible for photoluminescence intermittency of quantum dots have not been fully understood. Currently, blinking is believed to be primarily caused by long-lived extra charges residing in deep-trap energy states at the nanocrystal surface, thereby enhancing the rate of nonradiative recombination processes (Efros and Rosen, 1997; Frantsuzov et al., 2008; Kuno et al., 2001, Shimizu et al., 2001). It has been demonstrated that surface-bound ligands (Hohng and Ha, 2004; Fomenko and Nesbitt, 2007) and thicker shells (Chen et al., 2008; Mahler et al., 2008) significantly reduce blinking, albeit not completely, by eliminating deep-trap surface states. In a recent effort to achieve complete blinking suppression, Wang et al. (2009) synthesized continuously emitting alloyed CdZnSe-ZnSe core-shell nanocrystals. Although the use of such nonblinking quantum dots makes for easier fluorescence detection and identification, the broad multipeaked photoluminescence spectra of CdZnSe-ZnSe quantum dots make them unsuitable for a multicolor experiment that involves simultaneous monitoring of several fluorophores.

Sensors

Although the utility of quantum dots in unraveling biological function at the molecular level has been successfully demonstrated, their potential for biosensing applications has largely been untapped. Owing to their unique spectral properties and physicochemical stability, it is apparent that quantum dots effectively address some of the limitations encountered by conventional fluorophores currently forming the basis for biosensors and bioanalytical assays, yet quantum dot use in biosensing applications has thus far been limited to proof-of-concept experiments. Recent advances in adapting quantum dots for predominantly in vitro biosensing applications, such as immunoassays, nucleic acid detection, and fluorescence resonance energy transfer (FRET)-based sensing have been extensively described (Sapsford et al., 2006; Medintz and Mattoussi, 2009). Here, we focus on the limitations that have precluded quantum dots from finding widespread use in biosensing applications.

Multivalency, as discussed above, renders quantum dots an attractive nanoscaffold that can accommodate multiple copies of different functional elements. Not only can such architecture result in higher sensitivity and lower limits of detection, but it may also serve as a basis for multifunctional biosensors. However, as previously mentioned, it is current lack of control over conjugation stoichiometry as well as the orientation and conformation of a surface sensing element that is a major obstacle to fully capturing the advantages of multivalency. Failure to determine the exact number and orientation in space of a surface sensing element in a reproducible manner can lead to either under- or overestimation of an analyte-specific signal. This is particularly relevant in the case of ligand-binding assays, where the number and conformation of ligand-specific proteins present at the surface of a quantum dot are critical to accurate determination of ligand potency.

Compared with conventional fluorophores, quantum dots are characterized by larger size, which enables multivalency and may be useful in ex vivo sensing applications. However, their relatively large size becomes a significant concern for in vivo sensing applications and FRET-based biosensing. Since the issue of quantum dot renal clearance in in vivo applications has been discussed above, importance of quantum dot size in FRET-based biosensing is considered here. In a FRET-based sensor, Förster or fluorescence resonance energy transfer efficiency between a donor-acceptor (D-A) pair of fluorophores is measured to monitor such biological events as ligand-receptor binding, protein-protein interactions, and conformational changes (Selvin, 2000; Sekar and Periasamy, 2003). Highly efficient FRET requires short D-A separation distances (1–10 nm). The final size of a water-soluble, functionalized quantum dot poses a serious limitation, often exceeding the donor-acceptor distance required for efficient FRET. Nevertheless, as a result of their high quantum yield, superior physicochemical stability, and size-tunable, narrow photoluminescence spectra, quantum dots have been used in a number of FRET-based sensors, predominantly as a donor molecule. For instance, Medintz et al. used a quantum dot-Cy3 FRET pair to detect the maltose sugar in solution in a dose-dependent manner (Medintz et al., 2006). In their design, positively charged, Cy3-labeled maltose-binding proteins (MBP) were prebound with a maltose analog-Cy3.5 dark quencher and then self-assembled on the surface of dihydrolipoic acid (DHLA)-capped quantum dots. Upon quantum dot excitation, FRET photoluminescence of Cy3 was fully quenched by the Cy3.5 dark quencher in a control solution, whereas addition of maltose resulted in the displacement of the maltose analog-dark quencher complex and consequent Cy3 photoluminescence recovery. In another example, Zhang et al. constructed a single quantum dot-FRET biosensor for accurate and sensitive DNA detection in solution (Zhang et al., 2005). The Cy-5-labeled target DNA sequence was detected by hybridization with the biotinylated complementary DNA strand, and the resulting Cy5-labeled DNA complex was captured with streptavidin-conjugated quantum dots in a highly sensitive manner.

To date, quantum dots have been predominantly utilized as donor molecules in FRET-based sensing assays. Their use as FRET acceptors has been limited by two key properties. First, although their broad absorption profile presents a distinct advantage in multiplexed experiments, it results in unavoidable direct excitation. Second, it has been reported that the lifetime of the acceptor relative to that of the donor is critical to efficient FRET. Quantum dots have a longer excited-state lifetime compared to that of organic dyes, and it has been shown that FRET is virtually absent in dye-quantum dot D-A pairs even at favorable separation distances (Clapp et al., 2005; Clapp et al., 2006).

Finally, despite substantial progress in nanocrystal surface chemistry and conjugation strategies, surface modification and functionalization remains the most important issue when utilizing quantum dots as a basis for biosensing applications. A single surface modification step may require several purification steps, often resulting in unwanted aggregation and crosslinking (Jaiswal and Simon, 2004). Nevertheless, quantum dots have come a long way since their introduction to the field and have a large potential in biosensing applications that still remains untapped.

Future Outlook

Quantum dots will become, and one can argue they already are, and indispensable tool for biological research. Quantum dots will see increasing use to elucidate the molecular mechanisms of protein binding, signaling, and regulation. They will be used to establish molecular mechanisms of disease and at the same time become a tool for drug discovery to treat disease. In the future we may also see further incorporation of quantum dots as cell reporters of biological function, implementation as tools for interrogating tissue, and forming the basis of novel biochemical assays. The varieties of commercially available quantum dots for the research community will continue to grow and dual-functional nanoparticles will become available, such as particles that can be used for dual imaging modalites. We believe that the future of quantum dots in biology is as bright as the quantum dots themselves.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.chembiol.2010.11.013.

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