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A primer on the synthesis, water-solubilization, and functionalization of quantum dots, their use as biological sensing agents, and present status

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The use of nanomaterials, specifically fluorescent semiconductor quantum dots (QDs), for biological imaging and sensing has become very topical. Here we present a historical synopsis of research in this field to help elucidate the origins of the most recent advances in QD-based technology. We further aim to educate the novice researcher concerning many important aspects of QD synthesis, water-solubilization, functionalization, and usage in biological imaging and sensing that are generally not discussed in the literature. We will also summarize several recent transformative examples of using quantum dots for *in vitro* and *in vivo* studies.

I Introduction

Semiconductor quantum dots (QDs, dots, or nanocrystals) are fluorophores that have unique and tuneable optical and electronic properties.[1–5] This is engendered by their size-dependent bandgaps that result from quantum confinement, which is essentially the change of the kinetic energy of localized electrons or holes *via* the Heisenberg Uncertainty Principle.[1] Small QDs have wider bandgaps compared to large ones; furthermore, the chemical composition of the QD may also be altered for the purpose of bandgap engineering. Shape control is possible as well. Due to the development of advanced synthetic protocols that create highly crystalline and surface-passivated nanomaterials, fluorescent quantum yields (*i.e.* emission efficiencies) can approach 100%. All of these effects are illustrated in Fig. 1. The emission of large water-soluble CdZnS/ZnS (blue),[6] small CdSe/CdZnS (green), and large CdSe/CdZnS QDs (red) are shown in Fig. 1A. The electron micrograph of Fig. 1B demonstrates the nanoscopic size, variety of shapes, and sharp crystallinity of QDs.[7]

Quantum dots have two main utilities; as semiconductors, they may effectively generate hydrogen from sunlight[8] or function as the active elements in solar cells.[9] They are also effective bioimaging agents[10] due to their high quantum yields and resistance to photobleaching as well as their continuous and strong optical absorption profiles. The latter is especially important as the figure of merit for a chromophore is not just the emission quantum yield (QY) nor the absorptivity, but the product of the two. QDs are also good energy transfer donors to sensing fluorophores. The surface areas of dots are very large on a molecular length scale; as such, they can be multifunctionalized with a significant number of chemical or biological vectors.

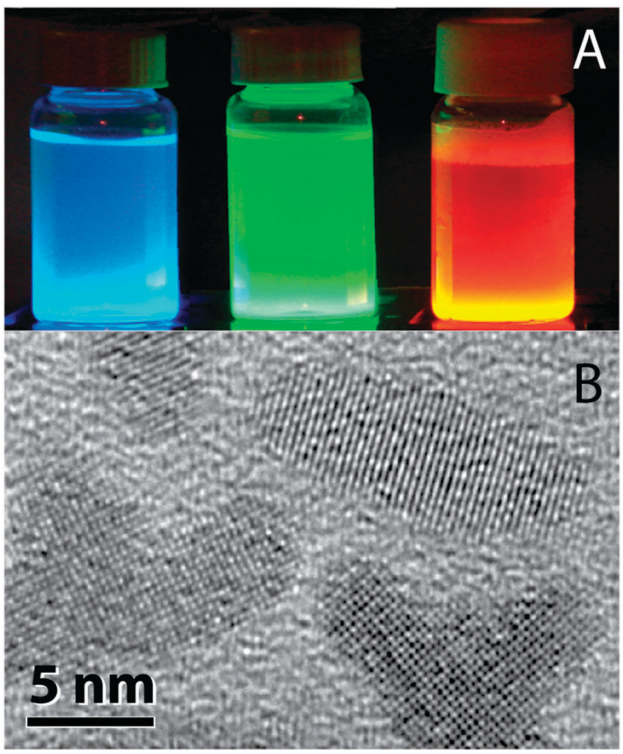

Fig. 1 (A) Emission from large CdZnS/ZnS (blue), small CdSe/CdZnS (green), and large CdSe/CdZnS (red) quantum dots in water. (B) The sharp crystallinity and variety of shapes of nanocrystals, here PbSe, are illustrated in this TEM micrograph.

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This allows for the materials to have enhanced biocompatibility, by which we mean QDs can be programmed to have a biological utility with little-to-no toxicity.

This perspective has been written for the non-expert who is interested in entering the field of biological imaging and sensing with quantum dots. As the use of QDs in biology is a saturated field, and many reviews have been published previously,[10–15] we hope to add value to this perspective by revealing many practical aspects of dot synthesis, water-solubilization, functionalization, and usage that are not often discussed in the general literature. Let us impart the first pivotal lesson here: quantum dots are not organic dyes. One cannot be substituted for the other trivially. Thus, for a research scientist to explore the usage of a QD bioimaging/sensing agent, we believe that all aspects of the dot must be known. We begin at the beginning: the synthesis, water-solubilization, and then functionalization of QDs. Next, the usages of functional QDs for chemical and biological sensing are discussed, and finally we describe exploring cell biology with QD sensors. Every section provides a brief historical synopsis, which is necessary to understand the follow-up discussion on the most recent advances in the field. We will attempt to guide new investigators in terms of every method's strengths and weaknesses and what physical parameters are affected by the choice of synthetic or functionalization methodologies. This is important as almost all processes yield materials with some undesirable attributes, yet simultaneously, other essential properties.

II Colloidal quantum dot synthesis

Cadmium selenide core–shell quantum dots

For those interested in semiconductor quantum dots, becoming cognizant of their origin is a good first step. Brus' group first prepared CdS QDs† using simple cadmium and sulphur salts in an aqueous solution containing an amphiphilic polymer.[16] This surfactant, a diblock copolymer, forms micelles that served as the nanocrystal nucleation sites and simultaneously coated the dot surfaces. Subsequent addition of zinc created a core/shell CdS/ZnS structure that nearly doubled the quantum yield to ~1%. These observations provide two very important lessons: QDs must be stabilized by an outer coating (almost always organic in nature) and the passivation of the surface matters. In our experience, control over these two variables is most important to make a functional biosensor. Due to the modest quantum yields and reported stability issues with aqueous synthesized QDs, most studies are now performed with nanocrystals prepared in hydrophobic solvents. For topicality, we won't discuss aqueous synthesized quantum dots further.

The next significant development occurred in 1993 as reported by Murray *et al.*[17] The group presented a procedure that produced the finest CdS, CdSe, and CdTe QDs at the time by the rapid injection of precursors into a very hot amphiphilic coordinating solvent (essentially, a "soap" that coats the QDs). The elements become supersaturated and precipitate in the form of nuclei.[18] After injection, the temperature of the solvent quickly decreases to allow the nuclei to develop into nanocrystals with a narrow size distribution; this is referred to as "focused-growth."[18] A range of dot sizes is achieved by control of relatively obvious conditions such as precursor concentrations, temperatures, and growth times. The great significance of this work is that semiconductor nanoscience became infinitely more accessible to the scientific community due to the quantum dots' narrower size distributions and increased quantum yields (~10%). It must be noted that dimethylcadmium was used as a precursor. This reagent should be avoided due to its toxicity, pyrophoricity, and the fact that recent reports have shown that cadmium salts,[19] or better, cadmium phosphonates,[20] are preferred precursors. Our group has occasionally observed the synthesis of nearly unit quantum yield CdSe cores *via* the use of cadmium salts.

After this publication, the field remained primarily focused on cadmium chalcogenide systems. Furthermore, as the fluorescent characteristics were significantly improved by the rapid injection process, there was interest in water-solubilizing the materials. However, water-solubilization quenches core CdSe emission significantly. Water-soluble core dots are also known to be fairly cytotoxic. These problems were resolved by coating the CdSe core with an inorganic passivating shell of zinc sulphide[21,22] as in the original report on CdS/ZnS QDs. Core/shell CdSe/ZnS quantum yields were reported to be as high as 50%; furthermore, samples were significantly more stable photochemically and were less prone towards precipitation when dissolved in solvents other than their growth solution. Modification of the shell to include a portion of cadmium resulted in the formation of CdSe/CdZnS dots that may have unit emission efficiencies as discussed below. With these materials as starting points, the community was ready to develop water-solubilizing procedures. We have explored the syntheses of a large number of semiconductor nanocrystals and can state that only core/shell materials display appreciable and long-term emission in water.

Now if the reader is as excited about high quantum yield core/shell QDs as we are, one may be tempted to find a detailed synthetic procedure from the very large number that exist and attempt to repeat it the next day. Unfortunately, making quantum dots is a process of material *vs.* molecular chemistry; this makes synthetic repetition problematic. Material chemistry is extremely sensitive to conditions, and note that one cannot separate the "good" fluorescent quantum dots from the "bad" ones in a flash chromatography column as one can isolate organic products from by-products and starting materials. More importantly, reagent purity is rarely controlled in reports on QD syntheses. Our group has found that using refined chemicals is essential; after all, the use of a ~70% pure solvent doesn't *engender the possibility* of irreproducibility, it *assures* irreproducibility.‡

† The discovery of CdS quantum dots in glass melts was also reported in ref. 2.

‡ Common solvents and purities are: oleylamine (70–90%), oleic acid (70–90%), trioctylphosphine (90–97%), and trioctylphosphine oxide (90%). We have found that chemicals with purities <97% to be of insufficient quality for use. Higher purity chemicals are available, but can be prohibitively expensive.

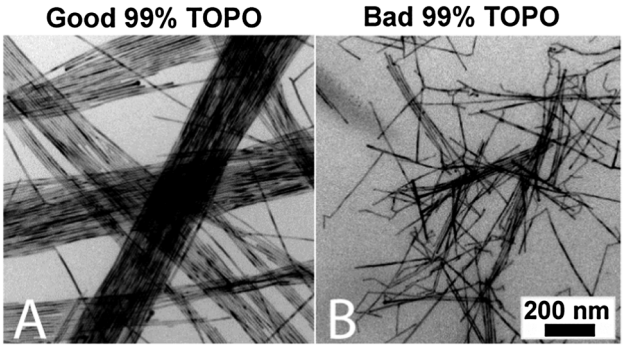


Fig. 2 An example of CdSe rod syntheses where dependence on the identity of a 1% impurity can drastically affect the quality and morphology of products. Reprinted with permission from ref. 23. Copyright 2008 American Chemical Society.

To present a dramatic example of this we must briefly move away from discussing dots for bioimaging and rather focus on the synthesis of CdSe rods. In a series of publications, Wang *et al.*[23,24] noted that the quality of CdSe wires grown by the solution–liquid–solid method displayed significant variations over time. The results are shown in Fig. 2, where clear differences in the morphologies are noted between samples A & B that were made using the same solvent from different production lots; but there is a catch. Both lots had the same nominal purity level of 99%. By careful examination and subsequent identification of the contaminants in the solvents, and then by controlled addition of the right impurities while removing the wrong ones, the group was able to consistently produce high quality nanowires. The significant dependence on solvent or precursor identities is referred to as the ''magic bottle effect''.

State of the art and future directions. QD synthesis

The refinement of CdSe/CdZnS QD synthesis over several years has made it the ''king'' of quantum dots. Their emissions can span over the ~500 to ~680 nm range, making them the most practical for visible-light optical detection. Note that the other cadmium chalcogenides have received similar albeit somewhat less attention. From the perspective of a QD chemist, this is most likely because hydrophobic cadmium sulphide and cadmium telluride dots are significantly more air sensitive than cadmium selenide nanocrystals. This is a trend we have observed over several years: if there exist few publications on the syntheses of QDs out of obviously interesting semiconductor material systems, then there are good reasons for it such as in the above example concerning air sensitivity.

In terms of robust methods to make CdSe/CdZnS QDs, we have found the method of Schrier *et al.*[25] to create 100% quantum yield emitting QDs to be the best.§ We have consistently reproduced the method and provided full synthetic details in ref. 26. The solvents and many precursors must be refined, the process is hazardous and has to be performed under a vigorously pure nitrogen atmosphere, and the proper techniques may take months for a new student to learn. While daunting, the gain is that this method only fails to make the best dots when one of the precursors or solvents has aged too much; the reagent can then be repurified. This has allowed us to develop robust post-processing derivations as the material's nature never changes from batch to batch. For the non-expert, there are a large number of procedures significantly simpler than that of Schrier *et al.*; however, one must be very wary of the magic bottle effect.

If the synthesis of core/shell cadmium chalcogenide nanocrystals is an answered question, albeit with a difficult solution, what then are the next steps in quantum dot syntheses? There are two: the first being to synthesize cadmium-free emissive QDs and the other being to create near-infrared fluorophores. For short-term cell or animal imaging studies, the cadmium content in CdSe dots is not a significant issue; however, note that cadmium may be detected over months in the tissues of exposed animals that none-the-less showed no signs of ill health.[27] Regardless, it is very unlikely that cadmium-containing materials will be used for human health purposes. To this end, the community has investigated the development of several non-cadmium binary semiconductor quantum dots. These include, but are not limited to (from UV to near-infrared), ZnS,[28] ZnSe,[29] ZnSe/ZnS,[30] InP,[31] InP/ZnS,[32] InAs,[33] InAs/ZnSe,[34] PbS,[35] and PbSe.[36] To discuss the merits and foibles of each, the UV to blue emitting zinc chalcogenides are the least toxic; however, such short wavelength chromophores are not desirable for bioimaging due to issues with UV tissue damage and poor tissue penetration resulting from efficient scattering. Indium compounds emit in the visible to near-infrared (NIR),[32] yet the chemistry of these systems is difficult to master[31,33] and the materials are prone towards oxidation.[32] Lead chalcogenides are near-infrared emitters; however, they are very air and water sensitive if prepared hydrophobically. Furthermore, the PbS and PbSe dots are usually passivated with cadmium chalcogenides,[37] defeating the purpose of synthesizing less-toxic materials.

An exciting direction towards synthesizing less toxic QDs is the doping of non-toxic QD hosts with phosphorescent guest ions. In this regard, the community has synthesized doped QDs,[38–40] termed ''d-dots'',[41,42] mostly using a ZnSe host often doped with Ag, Cu, or Mn ions.[43] Two recent examples are shown in Fig. 3A. The synthesis of d-dots is very challenging due to the ability of dopants to anneal out of the material and a strong dependence on reaction conditions;[40,44] regardless, processes have been developed to produce high quantum yield (>50%) doped materials.[45] The resultant d-dots emit over a significant portion of the visible spectrum, have large Stokes' shifts, and emit on micro- to millisecond timescales. They can also be good energy transfer donors to organic dyes.[46] The only drawback is the fact that doped ZnSe must have UV or blue light excitation, which is undesirable for biological imaging. Counter to this is the very recent demonstration of copper-doped InP/ZnSe QDs that can absorb over the entire visible wavelength and emit at near-infrared wavelengths.[47]

§ Additionally, see ref. 246 and 247 for recent examples of the synthesis of bright CdSe/CdS QDs.

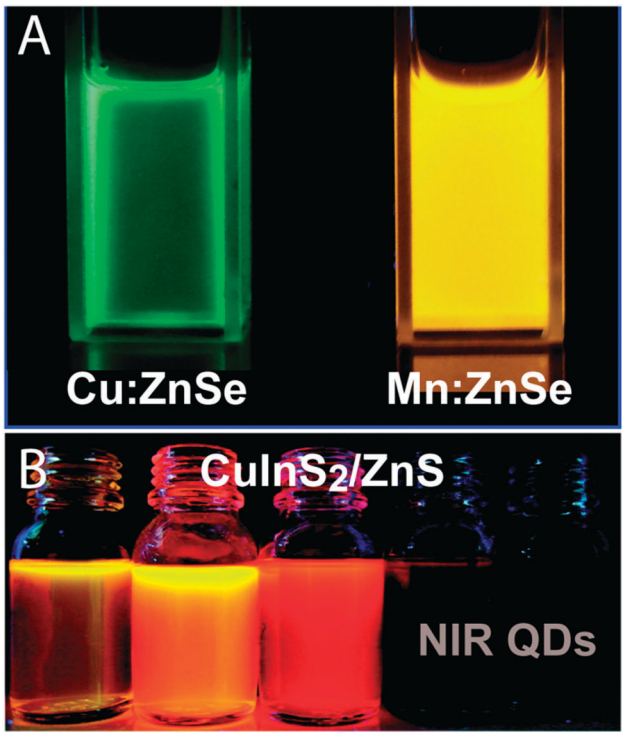


Fig. 3 (A) Copper and manganese doped ZnSe d-dots. (B) Visible to near-infrared emitting $CuInS_2$/ZnS quantum dots. Reprinted with permission from ref. 43 and 54. Copyright 2009, 2013 American Chemical Society.

The newest development in cadmium-free quantum dot synthesis is the exploration of ternary I–III–VI_2 QD systems. Recent examples include $AgInS_2$,[48,49] $AgInS_2$/ZnS,[48,50] $CuInS_2$,[51] and $CuInS_2$/ZnS, see Fig. 3B.[52–54] The syntheses and photophysics of these multi-cation dots are very unusual compared to CdSe and CdSe/ZnS nanocrystals. In terms of syntheses, it is very important to balance the reactivity of cations as copper and silver are more reactive than indium; this is controlled by the judicious choice of metal-coordinating solvents. Overcoating with zinc sulphide blue-shifts the emission, which is indicative of alloying the outer surface of the core. Photophysically, ternary QDs are similar to d-dots in that they have very large Stokes' shifts coupled with sub-bandgap emission. Furthermore, the fluorescent lifetime is in the hundreds of nanoseconds. In our own explorations, we have found the published procedures to be fairly facile and reproducible; as such, we consider the exploration of these systems one of the most exciting of recent developments. This is especially true as the emission of ternary dots can be tuned to the near-infrared for *in vivo* imaging as discussed in the last section.

To conclude, for the non-expert interested in the use of quantum dots for bioimaging and sensing, one has to decide whether to buy the QDs or synthesize them. As in all things, this is a balancing act. On one hand, in-house dot syntheses afford the research scientist unlimited and ultimately inexpensive access to any known or unknown material system. Also note that many of the QDs discussed above are not commercially available. Unfortunately, the price paid is the time and expense of mastering a new discipline and developing the corresponding infrastructure. On the other hand, purchasing QDs from commercially available sources means that a product can arrive *via* next-day delivery, which is of great convenience. Presently, visible to NIR emitting dots can be bought with several reactive functionalities appended to their surfaces. However, the cost per sample is very high, and the exact chemical nature and quality of the material is not under the research scientist's control.

III Water-solubilization methods

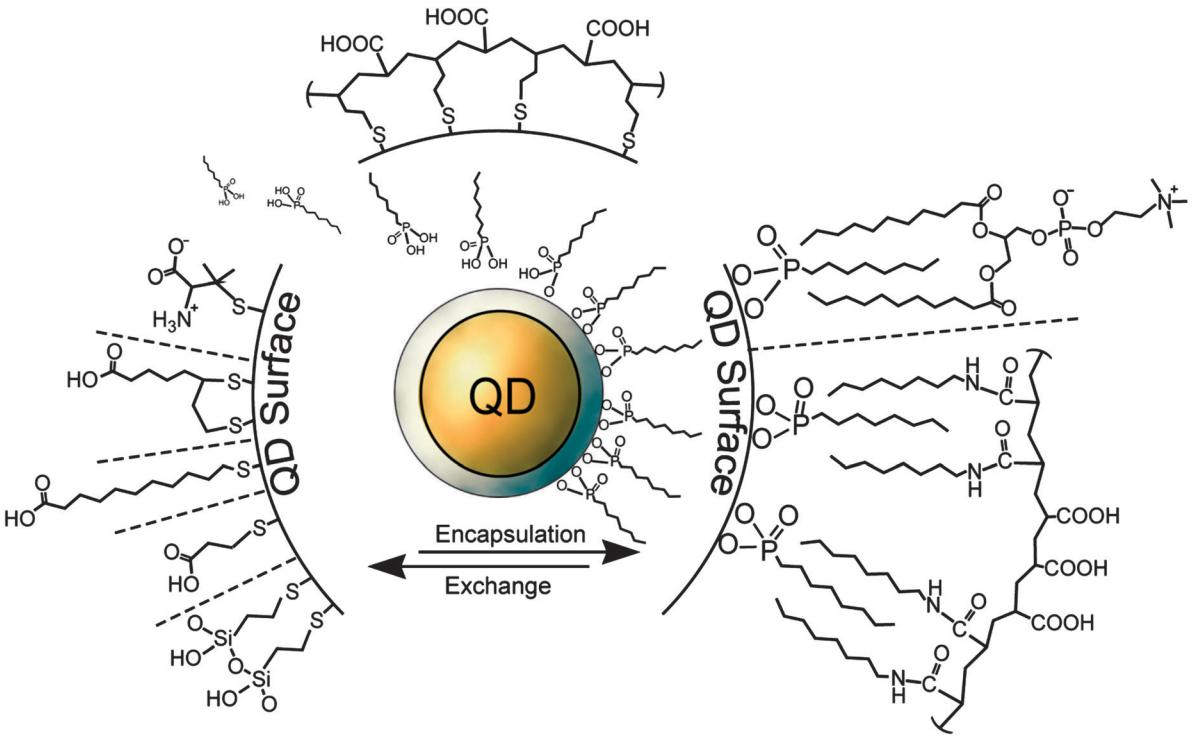
Regardless of how one obtains high-quality samples of hydrophobic QDs, the next task is to water-solubilize them. Many methods to create aqueous QD dispersions have appeared since 1998.[56,57] The water-solubilization process begins by purifying the QDs by precipitation, in which hydrophobic QDs flocculate by polarizing their growth solution with a non-solvent such as methanol.[17] This process may need to be repeated one or more times as removal of the growth solution is essential. However, this can be overdone as a significant loss of surface caps ruins nanocrystal optical properties. Precipitation of dots is a non-trivial procedure that may be sample-to-sample dependent. It takes practice, especially to avoid forming an oil-like precipitate, which should be avoided. It is also not desirable to store dots outside of their growth solution beyond a few days. After purification, there are several processes of water-solubilization to choose from; a schematic representation of many of these is provided in Scheme 1.

Cap exchange

In this method, the purified QDs are dispersed in a solution containing hydrophilic organics such as mercapto-acids that replace the original hydrophobic caps after sufficient exposure time.¶[56,58] After precipitation with a hydrophobic non-solvent, the now-hydrophilic QDs can be dispersed into water of the appropriate pH. The significant advantage of the use of the cap exchange method is that the QDs have the smallest hydrodynamic diameter possible, usually on the order of ~1 nm greater than that of the QD itself. However, cap exchange causes a reduction in quantum yield,[59] and some samples may have short, one day shelf lives. This has spurred the development of chemical processes that manipulate the mechanism of cap exchange;[60–62] the use of these reported procedures created aqueous cap-exchanged QDs that retained a significant portion of their original quantum yields and were stable on month-long timescales. For new investigators, we recommend employing these procedures during the cap exchange process, regardless of the nature of the nanocrystal or cap.

The most important aspect concerning cap exchange is the chemical nature of the new ligand.[63] Almost all research in this area has examined thiol-functional organics as the re-capping agents; especially prevalent are short alkyl chain monodentate

¶ Other methods for cap exchange include the biphasic exchange method, see ref. 65 and 248.



Scheme 1 Various methods of water-solubilizing hydrophobic quantum dots with cap exchange (left) or encapsulation (right). Adapted from ref. 55.

mercapto-acids such as mercaptoacetic acid (aka thioglycolic acid).[56] However, it is well known that the use of these species results in the formation of unstable water-soluble QDs, generally with significantly reduced quantum yields.[64] While these problems are somewhat mitigated by lengthening the alkyl chain using 11-mercaptoundecanoic acid, we recommend the use of one of the many alternatives discussed below.

Given that minimizing the hydrodynamic diameter of a QD sample is perhaps the best reason for using the cap exchange method, several recent studies have shown that zwitterionic ligands are exceptional in this regard. A significant demonstration used cysteine to coat CdSe/CdZnS QDs, resulting in an aqueous dispersion of dots with a 5.9 nm hydrodynamic diameter.[65] These small QDs were shown to undergo renal clearance; this should significantly reduce issues with toxicity. A drawback was the reported instability of the cysteine-coated QD dispersion; the samples precipitated after a single day. Bulkening the ligand using zwitterionic penicillamine was reported to mitigate the effect significantly, as the caps were resistant to oxidation.[66] Overall, the use of zwitterionic ligands for cap-exchanging semiconductor QDs is a major thrust of research,[67–69] as this creates the smallest, most compact water-soluble QDs.

Stabilizing cap-exchanged QDs is important; in this regard, several groups have explored the use of ligands with an increasing number of coordinating functionalities. Multidentate thiol ligands, starting with bifunctional dihydrolipoic acid (DHLA)[58] and derivatives of the same,[70,71] have sparked the most intense research. For the new investigator, DHLA is the usual capping ligand of choice among chemists who synthesize their own QDs and prefer the cap exchange method. Its usage is also a good starting point when learning how to synthesize and functionalize cap-exchanged aqueous dots. Most recently, tridentate ligands have been introduced,[72–75] with one report demonstrating significantly enhanced stability of QDs capped with tridentate ligands over DHLA.[73] Dots have even been cap-exchanged with recombinant proteins containing a polycysteine binding unit.[76] Obviously, the trend in the field of cap exchange is to increase the number of ligand anchoring points, which is discussed in the next state of the art and future directions section.

To transition to the next section on encapsulation, we note that one of the first methods of water-solubilizing QDs was to cap exchange them with silane precursors such as mercaptopropyl trialkylsilane and further encapsulate the dots with additional SiO_2 precursors.[57,77] These processes resulted in water-soluble QDs that could be functionalized with standard silica and/or thiol chemistry;[78] however, the initial water-solubilization processes involved a large number of steps that are known to be temperamental. Since these early reports, Ying's group developed a reverse-emulsion process to cap-exchange and encapsulate nanocrystals in silica in a one-step method.[79] The resultant water-soluble dots were found to be non-toxic and could be coated with PEG to increase biocompatibility. However, the method has not been as utilized as other encapsulation methods outlined below.

Encapsulation

The encapsulation of QDs with their native ligands intact occurs by mixing the purified QDs with molecular or polymeric amphiphilic encapsulants in a co-solvent such as chloroform

polarized with a minimum of methanol.[80] Upon drying, it is believed that the hydrophobic portions of the amphiphiles interdigitate with the original ligands, leaving an outer corona of water-solubilizing functionalities that allows the QDs to form micellular dispersions in water. Gallic acid derivatives,[81–83] lipids,[84] and much more commonly phospholipids[85] have been used as molecular encapsulants; the resultant water-soluble QD micellular dispersions were reported to be stable for months even within a biological milieu. The advantages of using phospholipids are their commercial availability and ease of preparation; furthermore, phospholipids can have a number of biocompatible and reactive functionalities. However, the downside is the cost of functionalized phospholipids.

In the polymer encapsulation method,[86–88] the purified QDs are coated with an amphiphilic polymer such as 40% octylamine-modified poly(acrylic acid)[86] and derivatives of the same.[89] The QDs can then be dispersed into water of the appropriate pH, which can be neutralized with dialysis that removes excess polymers at the same time.[80] The latter process must be performed thoroughly as we found that exposing a culture to a polymer-encapsulated QD dispersion containing excess amphiphilic polymers results in significant cell death. In our lab, we work with ~3000 MW amphiphilic polymers, yet it is necessary to use 100 000 MW dialysis filters to properly remove the excess encapsulants.‖

The polymer encapsulation method is extremely robust, producing aqueous QD dispersions with minimal loss of starting material. Since the original ligands are intact, the dots are only partially quenched (~25% loss of QY)[26] in the water-solubilizing process. As such, when purchasing aqueous QDs, most likely the research scientist is receiving some variant of encapsulated dots. This is also our lab's preferred method of water-solubilization as we can make a sample and store it indefinitely for future research. There are negatives; the amphiphilic polymers must be synthesized, although note that the wet chemistry is rather minimal. The resultant encapsulated dots have large hydrodynamic diameters (>25 nm for a typical CdSe/CdZnS QD),[90] and this reduces biocompatibility. Most importantly, polymer-encapsulated QDs can be notoriously difficult to functionalize.[61] As such, many groups have resorted to pre-functionalizing the polymers, under non-aqueous conditions, before overcoating hydrophobic QDs.[88,89,91,92] Examples include coupling PEG and biotin to the polymer backbone to enhance biocompatibility, as well as energy transfer accepting dyes for sensing applications. Without access to reagents designed to functionalize such aqueous quantum dots,[93,94] we suggest this approach.

State of the art and future directions. Cap-exchanging polymers

One of the most interesting developments in QD water-solubilizing techniques is the blending of the cap exchange and encapsulation methods.[95–99] Such ligands can be created by grafting short alkyl-thiol functionalities onto an existing hydrophilic polymer backbone,[95,98] which is synthetically facile. Other reports have synthesized polymer precursor monomers containing QD-binding motifs such as thiol, or imidazole,[96,99–101] functionalities as well as monomers with water-solubilizing and reactive moieties. The advantage here is that the research scientist has better control over the size of the polymer cap or complete control in the case of living free radical polymerization. Obviously, this comes at the expense of greater synthetic effort. The use of an imidazole-functional anchor point[96] is especially interesting as the moiety is un-oxidizable, unlike thiols. This motif has also appeared in other areas, such as functionalizing QDs with peptides and recombinant proteins containing a large number of dot-binding cysteine residues.[102] This topic is discussed further in Section IV.

Typical processes for the use of polymer caps invoke procedures more akin to cap exchange *vs.* encapsulation, although, in some cases, replacing the original QD ligands required two steps. All reports have demonstrated that the hydrodynamic diameter of QDs coated with polymer caps is comparable to or just slightly larger than that expected with pure cap-exchanged QDs (and obviously less than that observed with amphiphilic polymers). Significantly enhanced stability over cap-exchanged QDs was also universally reported, and, in the case of water-soluble CdTe dots, enhanced brightness was observed.

To conclude, while this recent blending of water-solubilizing methods is interesting, overall, it is difficult to say where the field will move. The large number of reports on the subject of QD water-solubilization has made a wide range of claims concerning stability and optical properties, regardless of the method employed. In our experience, QD water-solubilization is a matter of matching the material with the method with the chemist, a process also known as trial and error. We believe the idea of using redox-inactive ligands will likely be a part of future studies given that thiol oxidation is the bane of cap-exchanged dots.

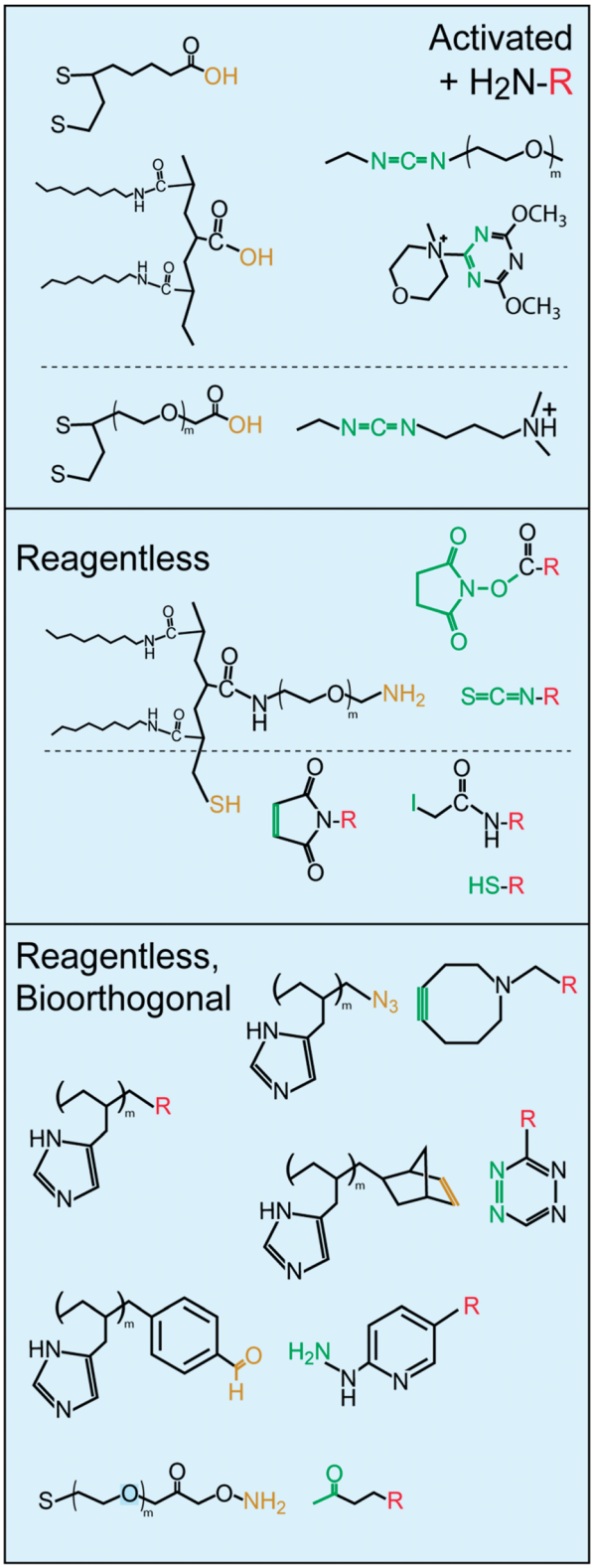
IV QD functionalization methods

Recently, an extensive review on methods to synthesize functional metallic, semiconductor, and organic nanoparticles was published.[103] For the purpose of brevity, we will summarize here just a few of these methods that are pertinent to the water-soluble semiconductor QDs that we have more intimate knowledge of. A schematic synopsis of this section is provided in Scheme 2.

Water-soluble QD have very little utility unless they are functionalized with some chemical or biological vector. It is well-known that QDs and many chemical and biological species have strong non-specific interactions,[104–106] most likely due to electrostatics,[107,108] and simply mixing a dot and protein can result in the formation of a functional adsorbate.** However, it is difficult to know how such constructs may behave *in vitro* or *in vivo*. Nanocrystals may also be cap-exchanged with singular-thiol

‖ We believe that the amphiphilic polymers form aggregates that have almost no transient instability. Thus, they form strongly bound <100 000 MW species.

** Recently, the same method was applied to form QD–dye FRET pairs using highly cationic[249] or hydrophobic[26] dyes.



Scheme 2 Representative chemistries to functionalize water-soluble quantum dots. Top: reagents used to activate carboxylic acid-coated QDs for cross-linking to amine-functional vectors. Middle: reagentless crosslinking strategies that may indiscriminately tag biological molecules. Bottom: bioorthogonal coupling schemes. Orange and green: QD and reactive functionalities. Red: chemical or biological vectors. Some chemical moieties have been simplified for presentation purposes.

functional DNA, peptides, and proteins, but these linkages are unstable.[109] Thus, the development of more robust conjugation methods for cellular studies is necessary, which leads to two problems. The first is that, despite the breadth of organic bond-making reactions, not that many are functional in water. Also working against the research chemist is the QDs' colloidal nature; this makes dots prone towards precipitation at the slightest provocation. For example, as water-soluble QDs are often coated with carboxylic acids, an obvious way to functionalize them is to chemically crosslink the organic coating with a primary amine-containing chemical or biological species using the commercially available carbodiimide activator 1-ethyl-3-(3-dimethylaminpropyl)carbodiimide (EDC).[110] While generally reported to be highly efficient in water, EDC causes quenching and precipitation of carboxylic acid-coated QDs.[58,80,93,111,112] The QD aggregation can cause the loss of an entire sample if the material is exposed to enough EDC.[93] The lesson here is that textbook methods of chemical conjugation usually fail when applied to quantum dots.

There are three general strategies to resolve this issue; we will discuss each of them, provide examples and references, and then examine the merits of each. They are: (a) protein modification, (b) electrostatic manipulation for carbodiimide chemistry, and (c) reagent-free and bioorthogonal chemistries, which represent the most recent and effective methods to date.

Protein modification

One of the first reported methods to create biologically functional nanocrystals without EDC was to engineer an electrostatic linking between dots and proteins.[58] Specifically, QD–protein conjugates were prepared by combining a highly cationic recombinant maltose-binding protein containing a terminal leucine zipper region to an anionic DHLA cap-exchanged QD. This motif was further used in several follow-up studies[113–117] and was also used to make QD–RNA conjugates;[118] however, the field has been moving away from the usage of electrostatic interactions. Given the effort to create recombinant proteins, why not engineer one to attach directly to the surface of QDs? To accomplish this goal, researchers recognized that the imidazole-functional amino acid histidine has a strong affinity for metal sulphides.[119,120] Hypothesizing that the interaction would transfer to zinc sulphide coated quantum dots, proteins and peptides were modified by the addition of a polyhistidine tail and were found to adhere to either unpassivated portions of cap-exchanged or lipid-encapsulated dot surfaces or outer carboxylate (or nitrilotriacetic acid) ligands in the presence of nickel ions.[75,119–139] Protein binding *via* adherence to the QD surface is evident through the observation of increased QD quantum yield, and as many as $\sim 10 \rightarrow 30$ proteins could bind per dot.[119,120,140] Smaller peptides can be loaded at higher ratios, with valencies reported as high as 50 ± 10.[140] QD-based sensors were created by binding dye-functional proteins to the surfaces of energy-donating QDs,[119,120,125] or by modulating luminescent protein–QD energy transfer *via* ligation with a metalloprotease.[124] Other examples are provided in the next section.

Overall, the benefits of this method are the ability of the QD–protein to self-assemble and the enhancement of QD emission upon protein binding, although the latter has not been observed in every study. Polyhistidine coordination is also stable within a biological milieu[133] and bioorthogonal as discussed in the state of the art and future directions section below. To the best of our knowledge, it is the most utilized method of forming QD–peptide or QD–protein couples at present. Polyhistidine–peptide conjugates are readily accessible *via* synthesis; however, larger recombinant proteins must be grown and isolated, which requires a broader range of skill and knowledge.

Electrostatic manipulation for carbodiimide chemistry

Another successful approach has been to ''fix'' carbodiimide chemistry to conjugate carboxylic acid-functional QDs to species with free amine groups. The reasons are three-fold: (a) many commercially available water-soluble QDs are coated with carboxylic acids, (b) most proteins contain residues with free amine groups, and (c) amide bonds are very strong. While the reason for the failure of carbodiimide chemistry was initially not clear, it is due to the cationic nature of the EDC reagent that neutralizes the negative charges of a carboxy-functional QD.[94] Such sensitivity is the result of the colloidal nature of aqueous dot dispersions; either a high degree of charge density or long-chain PEG stabilization[70] is necessary to keep the materials suspended. This led to the synthesis of cap-exchanged QDs coated with poly(ethylene glycol) (PEG), where a percentage of the PEG ligands are terminated with carboxylic acid groups. With dot buoyancy a result of neutral PEG groups, carbodiimides were able to activate the materials without precipitating them.[71,126,141,142] This allowed for the synthesis of dye- and protein-functional quantum dots for sensing applications. Our group rearranged the same idea by synthesizing neutral PEG carbodiimides. PEG carbodiimides functionalized 40% octylamine-modified poly(acrylic acid) polymer-encapsulated QDs with proteins and dyes with a record of 95% efficiency;[93] the reagents were also shown to activate cap-exchanged dots. The merit of both methods is that they can be used to functionalize QDs with simple chemical procedures to make amide bond-conjugated species; however, implementation requires somewhat complex syntheses of either the QD caps or the dot activators. Furthermore, carbodiimide coupling does not allow for control of the orientation nor the number of biologicals coupled to the dots.[103]

In our further investigations, a single commercially available reagent (DMTMM)[143] was shown to functionalize 40% octylamine-modified poly(acrylic acid) polymer-encapsulated QDs with $\sim$75% efficiency.[94] The benefit of this reagent is that some of the least expensive commercially available QDs are coated with acrylic acid; on the down side, DMTMM is not as effective as PEG carbodiimide activators and overuse causes dot precipitation.

Between both strategies lie commercially available polymer-encapsulated QDs where the coating is coupled to amine-terminated PEG ligands. These materials may be functionalized with chemical and biological vectors that have been activated with amine-reactive *N*-hydroxysuccinimide functionalities. No external reagent is needed, and the reaction byproduct can be removed in a desalting column. Unfortunately, convenience comes with a higher price tag; other reagentless conjugation strategies are discussed next.

State of the art and future directions. Reagent-free and bioorthogonal chemistries

Thus far, we have detailed how dot synthesis and water-solubilization necessitates having significant inorganic and organic chemical transformation experience and skill. Functionalization may require knowledge of genetic manipulation and protein isolation, and subsequent usage of QDs in *in vitro* or *in vivo* assays must be performed by experts in biology. As such, we contend that there is significant value in removing at least one layer of complexity. Fortunately, the most recent trend in nanocrystal research is the development of conjugation methods that are ''spring-loaded'' to react without the use of external coupling reagents and that produce no byproducts.

Early on, reagent-free protocols created functional nanocrystals by coupling biotinylated chemical and biological vectors to avidin- or streptavidin-coated dots (and *vice versa*).[144] This methodology was spurred by the commercial availability of starting materials;[145,146] however, by today's standards, this is a very expensive method that creates overly-large and sometimes unstable QDs. Recently developed methods for conjugating dots are based on chemistries employed by biologists to tag proteins, DNA, RNA, *etc.* These include thiol–maleimide,[80,147–153] thiol–iodoacetate,[154,155] and amine–isothiocyanate[71,156] coupling, as well as disulphide formation.[151,152] In our experience, these chemistries are the easiest to implement and result in the least problems with QD precipitation. Yields may be very high, and purification can be performed with dialysis. The only issue with these methods concerns the fact that they cannot be performed to label biologicals *in vitro* or *in vivo*, and some of the bonding motifs are not entirely stable in biological environments.

The future of quantum dot functionalization lies in the development of bioorthogonal strategies, which are chemical coupling schemes that can occur in a biological milieu without disruption of non-targeted biologicals. Recent examples to create biologically-functional QDs include the aforementioned polyhistidine coupling method, the SNAP and HALO tag techniques,[157,158] the reaction of tetrazine derivatives with norborene and *trans*-cyclooctene,[99] hydrazone ligation[159] by the reaction of hydrazinonicotinoyl and formylbenzoyl groups,[127,136,147,160] oxime formation by aldehyde and aminooxy coupling,[161] and strain-promoted azide to alkyne cycloaddition.[100,162–164] While these approaches require chemical or genetic modification of biologicals, the advantage is that a research scientist is assured that only the entity of interest is labelled by the quantum dot.

To highlight the importance of such coupling, we must reiterate that bioorthogonal ''spring-loaded'' chemistries are designed to avoid tagging all known naturally occurring biological organic and inorganic functionalities. Using such methodologies allowed Boeneman *et al.* to demonstrate the labelling of fluorescent proteins in the cytosol of live cells with quantum dots. In this

study, COS-1 cells expressing mCherry fluorophores bearing a polyhistidine tag were microinjected with polymer-encapsulated QDs pre-loaded with polyhistidine-coordinating nickel ions.[165] Interaction of the two was quantified by FRET energy donation from the QDs to the fluorescent proteins. Proteins localized on the surfaces of live cells have also been interrogated with QDs using bioorthogonal ligation methods.[75,82,166] These demonstrations are some of the most important to date as they prove that protein–protein interactions in live cells may be probed with quantum dots. This also enables single particle imaging to develop a microscopic description of cellular processes, as well as possible uses for dots in flow cytometry assays for personalized medicine (theranostics).

To conclude, the field of functionalizing quantum dots is clearly moving towards the development of reagent-free bioorthogonal coupling protocols. However, the syntheses are not simple. For example, it is true that azide-functional dots will react with cyclooctyne-bearing chemical and biological vectors with no external reagents and byproducts; however, the ''spring-loaded'' reactive functionalities still have to be conjugated to the QDs and other species beforehand. Regardless, there is still great benefit to these methods as the functionalization of bioorthogonal reactive moieties can be done on the friendliest of terms. Dot ligands can be conjugated to azides in non-aqueous solvents before they are used for cap exchange; furthermore, cyclooctyne-functional chemical and biological substrates can be synthesized and purified using well-established high-yielding protocols. Commercial sources for such reactive substrates are also increasingly available, leaving research scientists with a question: what do we want functionalized QDs to do?

V Sensing with quantum dots

QD–dye FRET

In the history of nanocrystal-based sensing, initial studies used poorly passivated QDs synthesized in water because the most robust methods for dot syntheses had yet to be developed. This led to a rather large number of publications where as-prepared dots were exposed to chemical or biological agent X resulting in Y change of the QD's fluorescent quantum yield. The issue is that, if one catalogues the number of X analytes, it appears that dots are sensors for just about anything. Furthermore, QD emission intensity can also be a time-dependent function of light exposure.[167] Our own experience with highly robust core/shell dots is that quantum yields are insensitive towards non-targeted analytes in all but a few cases, such as exposure to metals. Also note that even this is non-specific as several metal ions, notably mercury[168] and copper,[169] can irreversibly quench core/shell QD emission.

The development of very high quantum yield water-soluble dots actually presents an interesting problem for sensing applications. Namely, how can one engender a response to a supermolecular inorganic chromophore buried under a thick layer of another solid material? Several groups have found that the control of energy transfer is a novel way to impart sensing capability to semiconductor nanocrystals,[170,171] see ref. 171 for a more thorough review of these seminal works. Briefly, in 2001, van Orden's group first demonstrated efficient Förster Resonant Energy Transfer (FRET)[172] from a QD donor to an organic dye acceptor.[173] Other groups quickly confirmed that QDs are highly desirable as FRET donors in biological applications as long as the donor emission properly overlaps the acceptor dye absorption.[174] This work became the basis for a general strategy to create QD chemical and biological sensors; while it is true that highly passivated QDs do not directly sense their environment, the observation of efficient energy transfer led to the concept of manipulating energy transfer as a sensing strategy. Mattoussi's group was the first to show that QDs may sense chemical agents by designing a dot/fluorescence quencher conjugate where the quencher was permanently displaced by trinitrotoluene (TNT).[175] Thus, FRET energy transfer from the QD to the quencher was removed in the presence of TNT, resulting in increased QD emission. Later, energy transfer sensing was extended to detect biological species such as maltose.[119] Biological processes such as DNA replication were also studied using QD energy transfer manipulation.[176] Presently, there are a very large number of examples of manipulating QD emission *via* analyte-specific energy transfer; as such, we cannot discuss them all and will instead focus on sensors for monitoring cellular processes.

There is an issue with using a singular turn-on or turn-off response to an analyte within a complex biological environment. As an extreme example, imagine that a turn-off sensor for mercury ions is applied to live cells; after incubation, cells are washed and examined with fluorescence microscopy. As discussed in the next section, nanocrystals are very difficult to deliver to the cytosol of a live cell, and even when possible, the cytosol may not be evenly stained throughout. Thus, how can one know if non-fluorescent regions in a cell are observed due to high levels of mercury ions or due to a failure to deliver the dot sensors to begin with? Furthermore, how does one calibrate the emission observed from within such a highly scattering medium? Fortunately, a simple solution is to use a dual-emissive (but non-overlapping) system composed of a recognize-relay-reporting probe[177] tethered to another fluorophore. This is especially true if an energy transfer relationship (FRET) has been engineered between the two emitters that can be perturbed by a targeted analyte. In this situation, probe concentration is irrelevant as long as the ratio between the emissive intensities of the coupled chromophores can be measured. The fluorescence spectrum, rather than the emission quantum yield, then becomes the quantitative and calibratable metric for analyte concentration.

Quantum dots are invaluable elements in dual-emissive, or ratiometric, fluorescent sensors. When used as energy transfer donors, the continuous and strong absorption manifold of dots largely results in an excitation-wavelength independent response. If the sensing chromophore is an organic dye, then photobleaching can be mitigated through the attachment of multiple copies of the dye to a single QD. It is also easy to tune

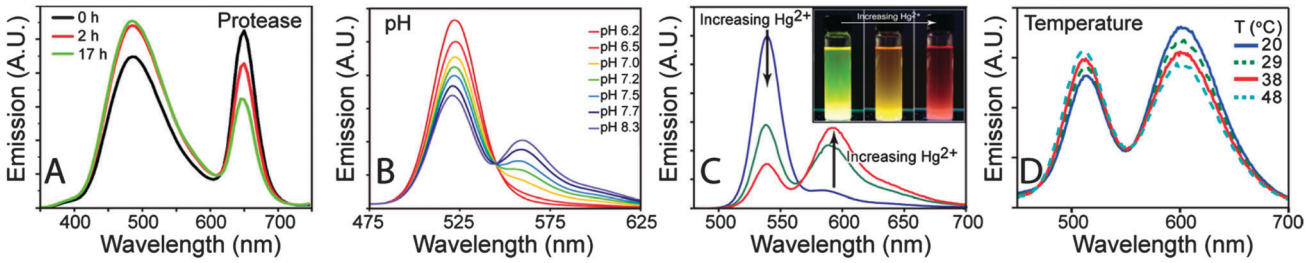


Fig. 4 Ratiometric fluorescent responses of QD sensors for (A) the time-dependent activity of protease, (B) pH, (C) mercuric ions in water, (D) temperature in cells. Reprinted with permission from ref. 187, 194, 200 and 211, respectively. Copyright 2008, 2012, 2011, and 2011, American Chemical Society.

nanocrystal emission to manipulate energy transfer efficiencies to dyes. The high dot valency allows for further sensor functionalization to target specific biological entities or to reduce toxicity and non-specific interactions. Given these properties, it is no wonder that so many reports on ratiometric sensors using quantum dot energy transfer donors to organic dye acceptors have been published.

The problem of altering the FRET efficiency from a dot donor to a dye acceptor can be solved by manipulating the QD–dye spatial distance or by altering the dye's optical properties. The choice of method is also somewhat dependent on the nature of the analyte; we will start with donor–acceptor spatial modulation. In this regard, there is a very large number of reports on sensing proteases,[124,135,139,178–185] especially metalloproteases,[124,186–188] which are overactive in a number of pathological diseases.†† These sensors function by establishing an energy transfer relationship between a QD and another chromophore through a peptidal linkage that is a substrate for a targeted protease. Motifs for energy transfer include dot to dye, dot to quencher, dot to dot, and lumophore to dot; action of the protease reduces or completely negates energy transfer in an optically detectible way as shown in Fig. 4A. Cells have also been interrogated with these probes.

One of the best examples of this work is the dual detection of urokinase-type plasminogen activator and human epidermal growth factor receptor 2 by Steven's group.[139] Overexpression of these proteins together is associated with breast cancer metastasis,[189] making this study of high clinical significance. Furthermore, the report demonstrated multiplexed detection with quantum dots, which is an often cited reason to develop nanotechnological sensors. The group also developed an assay where substrates do not need to be pre-loaded onto the surfaces of QDs before the action of the analyte is detected, similar to a previous report from Medintz's group.[135,139]

The manipulation of FRET *via* distance has largely been used for sensing biologicals, although a recent report demonstrated ratiometric pH sensing *via* spatial modulation of a dot donor and a dye acceptor that are connected *via* a cytosine-rich oligonucleotide.[190] Below pH 7, the DNA forms a folded structure that increases dot to dye FRET efficiency; the probe was successfully used to monitor the time-dependent acidification of endocytotic vessels. A similar design was reported where dot–dye distance was manipulated by coating the QDs with a dye-labelled polymer that swells when acidified.[191] A dye displacement strategy was also recently reported for detecting heavy metals.[192]

The second design for ratiometric quantum dot sensing involves the use of a FRET accepting dye or fluorescent protein that has an optical response to the presence of an analyte. Generally, these systems are for the fluorescent detection of local chemical elements such as calcium, H^+, *etc.* One of the first examples was created by coupling a squarane dye that has a pH sensitive absorption spectrum to an energy-donating QD.[193] FRET efficiency was modulated by the dye absorption moving into or out of resonance with the pH-insensitive QD emission; several reports have also demonstrated pH sensing, as well as other ions, with similar motifs as shown in Fig. 4B and C.[126,194–197] Oxygen sensors were recently developed where increasing O_2 levels lower a chromophore's fluorescent quantum yield while the QD's QY is invariant to the same;[198,199] this is relevant to cancer detection as tumours are hypoxic. A sensor for mercuric ions has also been reported; while mercury normally quenches QD emission, Page *et al.* coupled a QD to a mercury-reactive turn-on dye that simultaneously sequesters the ions into insoluble HgS.[200] Ratiometric sensing was achieved, and the group demonstrated dot "protection" *via* mercuric ion sequestration.

QD charge transfer sensing

Aside from using FRET as a means of sensing, other reports have detailed the use of charge or electron transfer to create analytical platforms.[201,202] These systems work because a QD's absorption and photoluminescence properties are affected by charge transfer. This method has been used to sense many biologically relevant analytes such as pH,[203,204] enzymes,[205] anions,[206] zinc ions,[207] chlorides,[208] and glutathione.[209]

A recent example by the Mattoussi group described a pH sensor where QDs were coupled to dopamine.[203] At high pH, the dopamine is oxidized, making it a good electron acceptor that causes quenching of the QD emission. At low pH, the dopamine is reduced and does not allow for charge transfer with the QD. Thus, QD emission is much more visible at low pH. The group used this system to successfully measure pH in cells using steady state and time resolved data.

†† The subject of using quantum dots to detect proteases has recently been reviewed, see ref. 250.

Another recent example by Ruedas-Rama *et al.* also described a pH sensor using mercaptopropionic acid capped QDs.[204] The protonation or deprotonation of the cap caused changes in the optical properties of the QDs. Using fluorescence lifetime imaging microscopy, the group showed that the photoluminescence decay curves of these QDs are quantitatively pH sensitive.

While we have not gone into great detail about fluorescence lifetime imaging microscopy, this technique is very useful in avoiding the issues associated with measuring a single enhancement or quenching of emission in complex environments as discussed in the previous section. We would like to note that fluorescence lifetime imaging microscopy is a popular imaging technique that has shown much success in sensing and imaging methods with QDs.[203–205,208]

State of the art and future directions. QD sensing

In terms of developing new platforms for QD sensing, there have been some recent, intriguing demonstrations of all inorganic temperature sensing with d-dots.[210–212] Here, core nanocrystal exciton and dopant emissions are simultaneously observed from a singular nanostructure, where the calibratable emission ratio is modulated by temperature-dependent forward and backward energy transfer as shown in Fig. 4D. This represents the ultimate motif in stability as organics are completely eliminated. As for future directions, we believe that the next critical steps are to target unlabelled proteins, other than proteases, and to monitor the dynamics of cellular protein–protein interactions. This may be impossible with an all inorganic platform such as that discussed above; however, as in the case of water-solubilization, it is difficult to know what paradigms will dominate research in the future.

To conclude, quantum dot-based sensing platforms are actively being developed for the simplest analyte (H^+) to large matrix metalloproteases. Engineering an energy transfer motif, or another method that can be used in complex environments, that is a function of the presence of a targeted analyte is essential. Recently, such analytical platforms have been applied to study cell biology, including measuring the acidification of maturing endosomes, quantifying the presence of membrane proteins, and measuring temperature changes inside a cell due to calcium shock. Most importantly, the field needs to demonstrate biological discovery, rather than compatibility with known outcomes. Using QDs to monitor chemical and biological events in cells would have a significant impact, as singular fragile organic dyes and fluorescent proteins could largely be replaced. Unfortunately, this reveals one of the greatest problems in the use of quantum dot nanotechnology as discussed below.

VI Cytosolic internalization by live cells

The first papers on aqueous solubilization of high quality QDs demonstrated staining the organelles of fixed cells, *i.e.*, dead cells that are encased in poly(formaldehyde).[56,57] Cell fixation is well-known to cause increased permeability, which clearly facilitates QD internalization. However, live cells are quite different. While cell membranes may be permeable to many simple dyes, any cell biologist will tell you that internalization of larger, more interesting cargo like DNA or proteins can be challenging. Although there are many delivery mechanisms, cells generally internalize larger cargo by enveloping it within endosomes that later mature to lysosomes. Thus, cytocellular delivery of QDs must either break through the endocytotic pathway or side-step it to begin with.

To summarize a large quantity of the literature with a sprinkle of our own unpublished data, a simple first step to explore live cell staining with QDs is to incubate them together, wash and fix the cells, and then examine them with fluorescence microscopy. Generally, the results can be summarized as follows: (a) no emission is observed due to the lack of QD internalization or membrane adherence,[94] (b) fluorescence is seen from the outer cell surface due to non-specific interactions,[94] or, most often, (c) specular emission is seen from QDs stuck within endosomes or lysosomes.[67] Case b can be diagnosed with *Z*-stacked confocal microscopy, which can determine whether the dots are located inside the cells, or with the use of cell-impermeable quenchers. The last case is revealed by the observation of a punctated QD emission that is colocalized with endosomal markers, rather than a diffuse glow throughout the cytosol.[213] Obviously, a method for cytocellular delivery must be actively engineered.

The cell biology community has similar issues with delivering large cargo and has developed several methods to do so, such as microinjection, electroportation, the use of cationic polymer vehicles, attachment to the HIV TAT peptide and its analogues, and osmotic shock. Many of these methods have been applied to QDs with mixed and/or irreproducible results as detailed in ref. 214, so we will not re-review them here. Rather, we will focus on four recent results that were based on somewhat unconventional delivery designs.

As stated above, delivery of dots to the cytosol of a living cell is evident by a diffuse emission throughout the cell body as observed in microinjection studies.[70,85,215,216] Thus, one might ask if microinjection or the recently developed nanoblade method[217] are solutions to the cytocellular delivery dilemma; yes, but unfortunately, these processes have low throughputs and are invasive to cell viability in the case of microinjection. A recent report by Lee *et al.* has demonstrated a resolution to these issues by delivering dots into Hela cells *via* a mechanical method.[218] Cells were essentially squeezed through a small gap in a microfluidic device to create a transient membrane disruption in the presence of QDs as shown in Fig. 5A. Cells were stained with a reported 40% efficiency while retaining a cell viability of >80%. The process is disruptive to the mechanical strength of the cell membrane as evident by their initial round shape; regardless, cells became adherent after subsequent incubation. The authors reported the ability to stain 10 000 cells per second; this makes the process amenable to flow cytometry which can be used to determine statistical data on cell health and the presence of biomarkers.

Non-mechanical delivery of cargo into cells often utilizes cationic cell-penetrating peptides (CPP), such as polyarginine

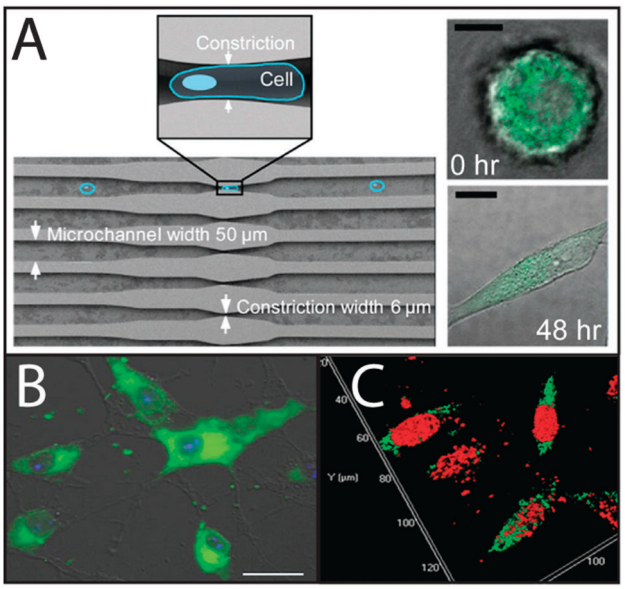


Fig. 5 (A) Mechanical cytocellular delivery of water-soluble QDs *via* disruption of the cell membrane inside a microfluidic device. The cells become adherent after 48 hours of incubation; diffuse QD emissions observed throughout the cell. (B) Cytocellular delivery of QDs coated with a hydrophobically-modified cationic cell-penetrating peptide (CPP). (C) Nuclear staining of cells with QDs (red) coated with a highly anionic CPP conjoined with a nuclear-localizing peptide. Emission co-localizes with DAPI but not endosomes (green). Reprinted with permissions from ref. 218, 128 and 223, respectively. Copyright 2012, 2013, and 2013 American Chemical Society and American Society for Biochemistry and Molecular Biology.

or the transduction domain of the HIV TAT protein. While reports vary, for the most part the literature has shown that conjugating dots to such cell-penetrating peptides affords only endosomal capture of QDs. The endosomes rarely maturate into lysosomes; regardless, cytocellular staining is not observed.[67] For the novice, note that manuscripts often discuss cellular uptake of nanocrystals, but this generally refers to endosomal capture and not cytocellular delivery. Recently, two reports have shown that CPPs can be altered for use as cytocellular delivery vehicles. First, Medintz's group developed a small peptide with a hexahistidine dot-binding segment attached to a polyproline spacer with a glycine hinge. Next, a lysine-rich segment that is a K-Ras4A signalling protein motif is conjoined, followed by a palmitic acid-functionalized diaminopropionic acid residue.[128,214] Essentially, the group partially "oiled" up a cationic delivery vehicle that can be conjugated to DHLA cap-exchanged QDs. After COS-1 and HEK 293T/17 cells were exposed to such functionalized dots, and subsequent 48 hours of incubation and fixation, it was found that 90% of the cells showed uptake with 77% displaying obvious endosomal escape of QDs. Diffuse emission was observed as shown in Fig. 5B. A follow-up study demonstrated that the cationic nature of the peptide is essential for QD cytocellular delivery, most likely to allow the dots to associate with cell surface proteoglycans.[219] Oddly, the delivery was also extremely sensitive to the nature of the palmitic acid conjugation, although palmitic acid itself could be substituted with a variety of other hydrophobic moieties.

Another recent report by Koshman *et al.* described a mixed protein delivery strategy where a cellular-localizing peptide (cardiac troponin C) was conjugated to a TAT-influenza hemagglutinin protein (HA2) fusion *via* a disulphide bond.[220] The TAT-HA2 combination has been found to be significantly more effective for delivering cargo to live cells in several studies.[221] By using a disulphide linkage, the delivery vehicle broke in the reducing environment of the cytosol, resulting in myofibril staining. The group also conjugated a nuclear-localizing sequence to the QDs that resulted in nuclear staining, as evident by dot colocalization with DAPI emission. A very positive aspect of this work is the fact that the delivery peptides are commercially available.

A recent paradigm shift for cytocellular cargo delivery recently occurred in the gold nanoparticle community. Specifically, coating Au dots with DNA allowed the materials to enter the cytosol of live cells;[222] this work has been followed up by several demonstrations of the same. The importance concerns the fact that conventional wisdom states that delivery vehicles should be cationic, whereas DNA is overall negatively charged. Unfortunately, as metal and semiconductor surface chemistries are very different, it is not easy to adapt these methods for CdSe/ZnS QDs. Despite this, a recent report by Ravindran *et al.* showed that a protein derived from dentin phosphophoryn (DPP) allows for efficient QD delivery into the cytosol of live cells.[223] The protein has a repeated aspartic acid–serine$_2$ sequence where 85–90% of the serine groups are phosphorylated, resulting in a very low isoelectronic point of pH = 1.1. Incubation of MC3T3 mouse osteoblast cells over 24 hours resulted in diffuse staining by QDs. Nuclear staining by QDs was also accomplished by fusing nuclear-localizing protein Runx2 to the DPP derivative; dot emission was clearly localized with DAPI as shown in Fig. 5C.

Overall, it appears that the research community has finally successfully demonstrated that QDs can enter the cytosol of living cells and target various organelles. As for the future, examining protein–protein interactions would be a powerful use for dots in cell biology; this could be quantified statistically with plate readers or flow cytometry.[224] More importantly, the *in vivo* application of QD sensors and/or stains to diagnose pathological diseases such as cancer is and will remain of significant interest as discussed below.

State of the art and future directions. QD imaging agents for cancer detection

Near-infrared emitting water-soluble quantum dots will probably have the greatest impact on human health compared to any other nanocrystalline material. This is a result of their use in through-body imaging,[225] which is relevant for cancer detection.[226] To be useful for this purpose, a NIR emitter must fluoresce in one of four widows of tissue and fluid transparency centred at 800 nm, 1090 nm, 1300 nm, and 1660 nm, as largely dictated by the absorption spectrum of water.[227] NIR emitting dyes have been developed for this purpose;[228] however, they are

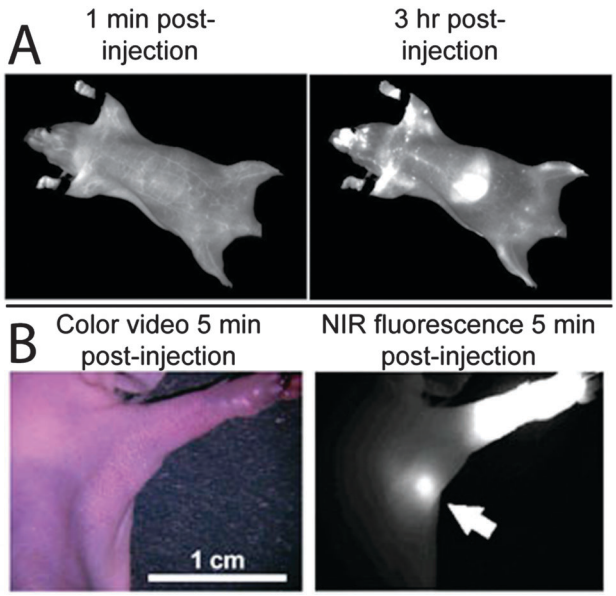


Fig. 6 (A) Through body emission demonstrates that circulation time is greatly enhanced by coating QDs with PEG_{5000}. Dots with a smaller PEG_{750} outer layer are cleared from the circulatory system in <1 h. (B) Quantum dots collect in the nearest (sentinel) lymph node after intradermal injection in a mouse paw. Reproduced from ref. 235 and 226. Copyright 2004, 2004 American Chemical Society and Nature Publishing Group.

generally very photochemically unstable which is why quantum dots' robustness is of supreme importance. There are also several semiconductor materials that may be tuned to emit over a wide range of NIR wavelengths, such as the recently developed cadmium arsenide dots that span all four optical windows of tissue transparency.[229] QD emission through the body of a mouse is readily achievable using several commercially available imaging devices as shown in Fig. 6A.

NIR QDs have been used for the detection of cancer in murine models *via* several mechanisms. First, they can be attached to a targeting vector such as an antibody for an upregulated membrane-bound protein on tumour cells.[12,149,150,230–232] In all cases, targeting the QDs to solid tumours resulted in quantifiably greater dot staining compared to non-functionalized QDs of the same composition. Another very simple way to detect cancer is to rely on angiogenesis, which is the stimulated generation of new blood vessels that provide nutrients to solid tumour tissues.[233] In tumours, blood vessel growth is usually defective and the vasculature is leaky to an extent that quantum dots may escape these vessels.[12,234] Thus, coating QDs with PEG to increase their circulation lifetime[153,235] will afford the opportunity for the dots to extravasate at solid tumour sites;[236] imaging the dots then allows a surgeon to locate the solid tumour. Sentinel lymph node mapping is also important in diagnosing certain types of cancers. Specifically, if a primary tumour is spreading, cancer cells have likely entered the lymphatic system and have become trapped, growing inside the nearest (sentinel) lymph node. Presently, a radioactive tracer is added to the interstitial area around a tumour and is tracked to the nearest lymph node for biopsy.[237] Obviously, cancer patients likely want alternatives to radioactive markers; at present, several studies have shown that NIR emitting quantum dots may be used for this purpose.[226,238–245] One recent example is highlighted in Fig. 6B.

To conclude, traditional methods of cellular cargo delivery have proven to be highly problematic when applied to QDs. As such, only somewhat unconventional approaches have been successful for delivering dots into the cytosol of living cells. While these *in vitro* demonstrations are transformative, we believe that the non-invasive optical detection of cancer will have a prominent role in semiconductor nanoscience. This will likely be realized with NIR emitting nanocrystals, especially those made from ternary I–III–VI_2, cadmium-free semiconductors. This is a very practical use of quantum dot nanotechnology, and will likely be the greatest success story ever told for these materials.

VII Conclusions

Our best advice to a new research scientist in quantum dot nanotechnology is that the Occam's razor principal (the simplest answer is the right one) does not apply to nanocrystals, rather, Murphy's Law applies. One must be keenly sensitive to possible magic bottle effects when developing synthetic protocols; furthermore, one must be wary of apparently simple solutions to intractable problems. As a community, we need to embrace more rigorous chemical standards towards syntheses to mitigate the known irreproducibility problems that occur even within the same research group. Expertise in several disciplines must combine to solve the big issues of the new millennium; after all, research in QD-based technology may have a huge impact on human health, especially concerning cancer.

We hope that this perspective has imparted some ''insider's knowledge'' to the novice scientist concerning significant issues with quantum dot synthesis, water-solubilization, functionalization, and usage. Some critics of quantum dot technology have stated that too much effort has been spent on demonstrating biological compatibility with QDs and not on biological discovery. However, the development of the chemistry of nanocrystals, both in synthesis and functionalization, was not yet mature to the level necessary to realize this goal. At this point in time, we believe a paradigm shift will occur now that many of the most difficult issues with QD chemistry have been resolved. The technology we have reviewed will give biological experts unprecedented ability to make discoveries relevant to human health by studying cellular processes with QD sensors. *In vivo* studies with QD sensors will also make significant contributions to cancer research.

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