Quantum Dots in Biomedical Applications

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Abstract

Semiconducting nanoparticles, more commonly known as quantum dots, possess unique size and shape dependent optoelectronic properties. In recent years, these unique properties have attracted much attention in the biomedical field to enable real-time tissue imaging (bioimaging), diagnostics, single molecule probes, and drug delivery, among many other areas. The optical properties of quantum dots can be tuned by size and composition, and their high brightness, resistance to photobleaching, multiplexing capacity, and high surface-to-volume ratio make them excellent candidates for intracellular tracking, diagnostics, *in vivo* imaging, and therapeutic delivery. We discuss recent advances and challenges in the molecular design of quantum dots are discussed, along with applications of quantum dots as drug delivery vehicles, theranostic agents,

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single molecule probes, and real-time *in vivo* deep tissue imaging agents. We present a detailed discussion of the biodistribution and toxicity of quantum dots, and highlight recent advances to improve long-term stability in biological buffers, increase quantum yield following bioconjugation, and improve clearance from the body. Last, we present an outlook on future challenges and strategies to further advance translation to clinical application.

Graphical Abstract
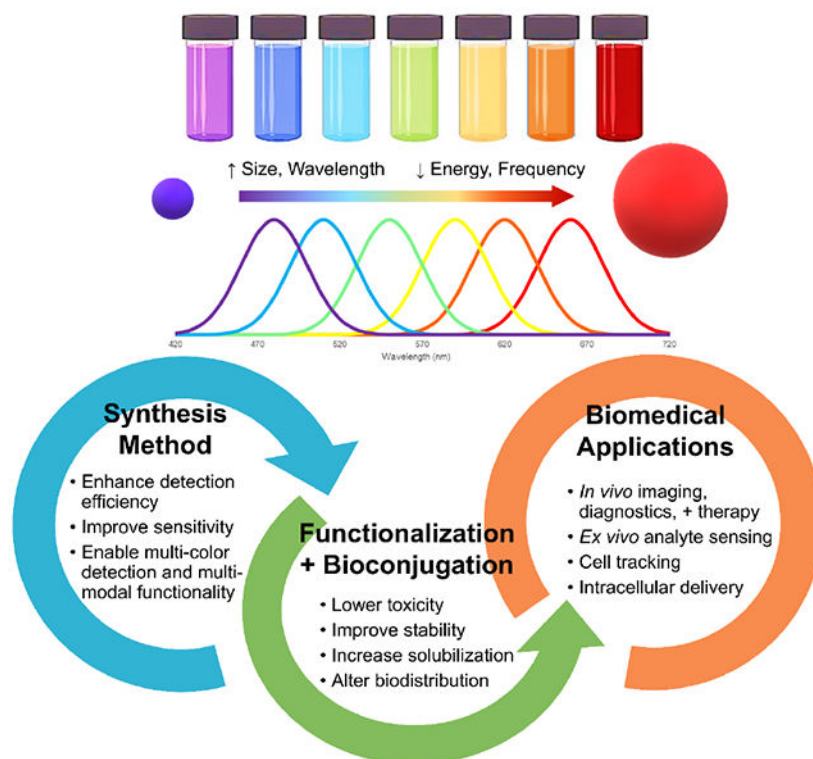
Keywords

bioimaging; nanotechnology; theranostics; drug delivery; molecular probes

1. Introduction

In the field of biomedical imaging, inorganic particles (e.g., iron oxide, gold, quantum dots) have gained significant interest in the last few decades. Specifically, they are being extensively explored for applications such as DNA-functionalized probes for real-time identification of disease-relevant biomolecules, diagnostic agents for tumor detection through tumor-specific membrane antigens, and molecular probes for simultaneous tracking and monitoring of drug delivery and therapeutic efficacy [1]. This interest is due to the unique optical, electrical, and magnetic properties of inorganic particles, which can be altered through changes in their size, composition, geometry, and structure [2]. Further, inorganic materials offer a relative ease of processing and resistance to degradation under

physiological conditions compared to organic materials [2]. To this extent, the tunability and resistance makes inorganic particles a very attractive and enticing modality.

Inorganic particles with nanometer sizes are especially exciting in the biomedical field. Many common proteins and biomolecules exist on a similar size scale, and such small length scales allows the inorganic materials to interact very closely with biological entities on a molecular level. This close interaction can enable a higher detection specificity and sensitivity, as well as the ability for intracellular uptake [3, 4]. In tandem, the nanometer size also increases the particle surface area to volume ratio. The increased ratio, coupled with the wide array of chemistry available, enables easy surface modifications to enhance the biocompatibility, solubility, and reactivity [2, 4, 5].

In bioimaging applications, the nanometer-sized particles also offer a significant advantage towards overcoming the severe toxicity often accompanying inorganic materials. The decreased size can enable increased sensitivity and a lower effective dose, without needing to sacrifice function. Many types of inorganic nanoparticles have been developed in the field, including superparamagnetic nanoparticles, metal-based nanoparticles, and semiconductor nanoparticles. The latter is referred to as quantum nanodots (QDs) in layman terms and is the key focus area of this review.

2. Semiconductor Quantum Dots

The initial discovery of QDs in the early 1980's has been credited to Brus, Efros, and Ekimov by the Optical Society of America [6–8]. Notably, they display unique size and shape dependent optoelectronic properties that in recent years have found useful application in bioimaging, among many other areas including photodetectors, light-emitting diode (LED) fabrication, solar cell technology or computing. Several types of QDs are currently available commercially, and are listed in Table 1.

2.1. Properties of Quantum Dots

A semiconductor nanocrystal, illustrated in Figure 1, is distinguished by its bandgap energy, which is the energy required to excite an electron from one electronic band into another one at a higher energy level. This excitation scheme ultimately creates an electron-hole pair known as an exciton. Upon relaxation back to its ground state the exciton emits energy in the form of a fluorescent photon [9]. When the semiconducting nanocrystal size becomes similar to that of the bulk Bohr exciton radius, a characteristic length in the range of 2-10 nm, the particle gains unique electrical and optical properties [10].

The most well-documented and understood property is the inverse relationship between nanocrystal size and energy band gap; in other words, as the size of the nanocrystal decreases, the energy band gap increases and the corresponding excitation/emission wavelengths decrease. This is known as the quantum size effect [11], and a comparison between excitation and emission profiles of QDs of various sizes [9] is portrayed in Figure 2. In Figure 2A, the fluorescent color emitted by the QDs upon ultraviolet exposure can be tuned simply by varying the size of the particle. In Figures 2B **and** 2C, respectively, tuning

the particle size and distributions of the QDs can enable a wide absorbance range with a highly symmetric and narrow emission spectra.

2.2. Synthesis Methods

There are two important methodologies behind semiconductor nanoparticle synthesis. This first is a top-down synthesis method, including processing techniques such as molecular beam epitaxy (MBE) and electron beam lithography [10]. The other is achieved by a bottom-up method, where precursor materials self-assemble and react in solution to yield colloidal QDs. As the optical properties relevant to bioimaging are unique to colloidal quantum dots, these top-down synthesis methods will not be discussed in detail here, and we will solely focus on the bottom-up method instead.

A breakthrough in the bottom-up process came in 1993, when Bawendi and coworkers reported a more facile synthetic method yielding nearly monodisperse QDs with a relatively high quantum yield [12]. The organometallic reagents dimethyl cadmium (Me_2Cd) and either trioctylphosphine selenide (TOPSe), trioctylphosphine telluride (TOPTe), or bis(trimethylsilyl)selenium [$(TMS)_2Se$] were injected into a solution of coordinating ligand, trioctylphosphine (TOP) or trioctylphosphine oxide (TOPO) heated to ~300°C [12]. The rapid introduction of the cooler organometallic reagents into the reaction vessel induced brief nucleation that ceased as the temperature of the reaction solution dropped abruptly. Nanoparticles were grown by gradual heating and annealing while the coordinating ligand stabilized the crystal solution. Rate of growth and crystal size were dependent upon growth temperature, and once the reaction was quenched by cooling, crystals were separated by size-selective precipitation with 1-butanol and methanol.

This important work by Bawendi et al. resulted in a synthesis method for CdSe QDs with excellent size control and an unprecedented degree of monodispersivity and crystalline order, which enabled researchers to perform detailed investigations of size-dependent optical properties [13]. The method is still largely used today to synthesize QDs, with some changes to the cadmium species and coordinating ligand [14]. However, use of milder reaction conditions to synthesis monodisperse QDs have recently become more common. These methods use lower temperatures (~100°C), green chemicals such as cadmium acetate ($Cd(Ac)_2$), and a thiolated capping agent in aqueous solution to produce CdTe QDs with quantum yields as high as 75% prior to passivation [15].

In these methods, both the size and core composition of the crystal will affect the emission wavelength. For example, CdSe QDs typically emit in the 450-650 nm wavelength range, while CdTe QDs may display an emission wavelength in a slightly higher range (500-750 nm) [9]. Nie and associates have even developed Se:Te alloyed QDs with different morphologies and unique optical properties independent of size by controlling the cadmium feed into the reaction mixture [16]. The ability of CdTe to emit in the near-infrared (NIR) region (~700-1400 nm) is directly applicable to deep tissue imaging, which will be discussed in detail in a later section.

2.3. Passivation

Though there are many advantages attributed to the high surface area to volume ratio of nanoparticles, there is at least one drawback. Coordinating atoms at the surface of the crystal have "dangling" molecular electronic bands that serve to quench the photoluminescence (PL) and decrease the PL quantum yield [9]. Therefore, a passivating layer of a wider bandgap is necessary; this passivated QD is known as a "core-shell'' structure, shown in Figure 3.

The synthesis developed by Bawendi et al. was at the expense of long fluorescence lifetimes and did not yield a well passivated surface [13]. To this extent, Hines and Guyot-Sionnest developed a key method to cap the core nanocrystal with a higher bandgap inorganic shell to improve the luminescence, stability, and electrical properties more could be observed with an organic capping layer [13]. Inorganic passivating materials are preferred over organic, as they are more robust and amenable to processing conditions. Typical passivating compounds include CdS, CdSe, and ZnS [13, 17]. ZnS is the most common passivating compound, as this coating results in less pronounced red shift than materials of a lower bandgap similar to that of the core material, such as CdS or CdSe [10, 13]. A ZnS shell also prevents oxidation and enhances colloidal stability of the particles [9, 13]. Dabbousi et al. showed that a passivating layer of ZnS can increase the quantum yield to 30-50% from 5-15% for unmodified dots, and that the quantum yield reaches a maximum value with about ~1.3 monolayers of ZnS [17].

The ZnS layer is added to the QD core in a manner similar to the synthesis described earlier, the major difference being the use of diethylzinc ($ZnEt_2$) and bis(trimethylsilyl)sulfide (($TMS)_2S$) as reagents. The nanoparticles are then purified and precipitated in a size-selective manner with a nonsolvent such as methanol [13, 17]. ZnS shell growth is improved by the addition of a small amount of Cd to promote even coating across the QD surface, achieving particles with a quantum yield of up to 95% [18].

3. Quantum Dots in Biomedical Applications

Historically, QDs have been widely employed in non-biological applications due to their appealing size and composition tunable optical and electrical properties. In the biomedical field, QDs offer many advantages over traditional organic dyes and fluorescent compounds [19–22]. However, there are certain aspects that need to be considered when the materials are intended for contact with biological environments, such as aqueous solubility, stability, and toxicity.

In this work, we discuss several comparative advantages of QDs over conventional organic dyes in biological applications. Further, we discuss current challenges and limitations in the application of QDs, to which we provide possible solutions, advanced techniques, and future perspective in later sections. We concentrate on relatively recent, successful or promising methods. A comparative analysis of quantum dot properties versus conventional organic fluorophores is summarized in Table 2.

3.1. Comparative Advantages

There are a number of competitive advantages that makes QDs desirable in numerous biomedical applications. In comparison to common organic dyes and luminescent proteins, QDs have broad excitation spectra, narrow emission spectra and large Stokes shifts. QDs also possess superior optical properties including increased intensity (up to nearly 100 times brighter) and significantly improved stability against photo-bleaching [9, 22]. Traditional organic dyes are highly prone to photo-bleaching, and the quantum yield can often be less than 15% in a biological environment [20, 23]. Further, as opposed to most conventional organic label dyes, QDs offer broad optical range of fluorescence, from near ultraviolet to near infrared (NIR). In particular, QDs emitting in the NIR have greatly expanded the potential of fluoresce in biomedicine due to its lower tissue absorption and relatively low autofluorescence [22, 24].

The variety of excitation and emission properties available through the quantum size effect is highly attractive in biomedical applications. For instance, QDs of various sizes can be injected into a patient, excited with a single wavelength, and yield a reproducible and narrow emission with minimal cross-talk between channels and spectral overlap [4, 25]. The reduction in overlap is critical as it enhances detection efficiency, improves sensitivity, and can enable multi-color detection and multi-modal functionality. In contrast, it is challenging to control and modulate the spectral wavelengths of traditional organic dyes as these properties are dependent upon the chemical structure, conformation, and reactivity. They cannot be readily or easily modified to emit different colors, which limits their applicability in multiplex imaging applications [20, 22].

3.2. Current Challenges and Limitations

In spite of these advantages, there still remain several significant limitations in the widespread use of QDs as bioimaging agents. Compared to conventional organic fluorophores, QDs behave as nanocolloids. This complicates their long-term use in biological environments and raises additional safety concerns.[21] Specific challenges include low aqueous solubility, complicated surface chemistry, low biological specificity, poorly controlled biodistribution to target tissues, and the potential for severe long-term toxicity [19, 21, 22].

QDs synthesized using more traditional organic methods typically result in hydrophobic surface properties, leading to a poor aqueous solubility and stability for *in vivo* applications [26–29]. The surface needs to be modified post-synthesis through encapsulation, ligand exchange, bioconjugation, or other means (detailed discussion in the following section). The type and density of the surface coating strongly affects the achievable fluorescently intensity, target recognition, particle size, solution stability, and biocompatibility [22, 30]. However, many published strategies lack reliable methods to produce the surface coating or neglect to fully characterize the surface modifications.

Additional key limitations include the ability to control the QD biodistribution only to target organs, reduce long-term accumulation in the body, and limit the long-term toxicity [31, 32]. The type of QD chemistry and resulting physicochemical properties will ultimately

determine the fate of the nanocolloids *in vivo.* The particles encounter multiple lines of defense upon administration to the circulatory system and must overcome several significant biological barriers from organ to cellular level (detailed discussion in later section). The absorption, distribution, metabolism, excretion, and toxicity depends on a multitude of properties, and must be studied in careful detail before clinical application [31, 32].

QD composition and properties such as particle size, surface charge, and hydrophilic coating can be rationally designed to slow the body's natural clearance mechanisms and improve interaction with target cells [21, 22, 27–30, 33]. In particular, surface modification with biological entities targeted to specific receptors (e.g., aptamers, peptides, antibodies, antigens) has shown remarkable success in improving both the aqueous solubility and cell / tissue specificity of imaging and diagnostic modalities. However, despite many published studies, there have been no resulting widespread conclusions to understand all the factors that influence particle biodistribution and target cell interactions [26–30, 33–40].

Ultimately, the field needs more systematic studies to understand the influence QD properties and demonstrate control of biodistribution and long-term safety. Successful strategies to address these limitations will enable the potential of QDs to be fully realized in clinical applications. To the extent, recent progress and potential solutions to major challenges will be discussed in the following sections. We also provide detailed discussion on techniques to improve the long-term stability in biological buffers, increase the quantum yield following bioconjugation, and improve clearance from the body.

4. Surface Modification Strategies to Improve Solubilization, Stability, and Specificity

4.1. Encapsulation

One of the greatest challenges with using QDs in medical and theranostic applications is related to their water-insolubility, as QDs must be water-soluble for proper use in bioimaging applications. In overcoming this limitation, many researchers have focused on encapsulating the QDs in a hydrophilic, soluble material. For instance, QD particles are often encapsulated in polymers or hydrogels. In another strategy, ligand exchange or surface capping can be used to substitute hydrophobic moieties with more water-soluble counterparts. To this extent, Mattoussi et al. have collected a broad list of surface functionalization strategies studied in literature [10]. Ultimately, the choice of coating material and method is primarily driven by the desired application and environmental requirements.

One of the more common general encapsulation methods is the utilization of amphiphilic polymer chains to coat the QD. To achieve the highest quantum yield the original ligands are left intact, and the hydrophobic interactions between the ligands and the hydrophobic regions of the polymer chain result in coating of the QD [41]. The polymer is often crosslinked to create a stable particle with a fully encapsulated core. Quantum yields following solubilization with this method are very high, even nearly identical to the quantum yields prior to encapsulation [42].

Design of the amphiphilic polymer coating has gained much interest, since a facile process that will not alter the colloidal stability of the particles is desirable. Functional groups may even be incorporated into the amphiphilic polymer coating to eliminate a functionalization step and impart the particles with greater utility. An encapsulation protocol employing click chemistry has recently been reported using a functionalized, amphiphilic anhydride-backbone polymer with acetylene groups to coat CdSe QDs (Figure 4). The method is advantageous because it includes common polymeric precursors, simple functionalization of the polymer incorporated into the encapsulation coating, and yields robust, stable, water-soluble particles that require no additional crosslinking or functionalization [43].

More recently, multi-dentate polymer coatings have been widely adopted in the field over the last decade to minimize the QD size and increase aqueous stability [44–49]. Multi-dentate polymers are capable of interacting with the QD surface via multiple sites, where the multivalent interactions serve to improve the overall stability of the interface of the QD and ligands [49]. Common example ligands may include bidentate thiol polymers, oligomeric alkyl phosphine polymers, general amine-containing polymers, and thiol-containing polymers [47–54]. These multi-dentate polymer coating strategies typically yield enhanced colloidal stability in solution as well as provide a robust platform for further modification [49]. However, structure analysis, quantification, and control over the spatial distribution of functional groups is often not addressed literature studies [49, 51, 53, 54]. This becomes more important to consider and address with increasing biological complexity of the intended application and with progress towards the clinical application of QDs.

4.2.   Ligand Exchange

The other predominant solubilization method is ligand exchange. This technique entails replacement of the organic capping ligand with a bifunctional hydrophilic ligand or polymer that can be anchored to the nanoparticle surface with one end and induce water solubility with the free end. The technique does not greatly increase the hydrodynamic diameter, but it does decrease the PL quantum yield due to direct binding to the QD surface [55]. Examples of hydrophilic ligands include mercaptoundecanoic acid (MUA), dihydrolipoic acid (DHLA), and thiolated agents. Though a simple process, known problems with ligand exchange are the large amounts of ligand required to drive the reaction and the lack of long-term stability of the particles, especially in extreme pH buffers [10].

The ultimate success of this method hinges upon the affinity of the anchoring group to the nanoparticle surface and the degree of colloidal stability promoted by the hydrophilic ligand. It has been shown that hydrophilic ligands incorporating a polyethylene glycol (PEG) chain have increased colloidal stability, high water compatibility, and resistance to immunogenicity and nonspecific interactions. The incorporation of multiple coordinating groups per ligand further enhances colloidal stability over a wide range of pH, from pH 1.1 to pH 13.9, and over various ionic strengths [56].

One modular approach to ligand exchange first uses the unique ability of the amino acid L-histidine to completely exchange the initial TOPO ligand, followed by further ligand exchange with the histidine intermediate in aqueous solution to functionalize the surface [55]. This method resulted in a high degree of phase transfer and a modest preservation of

quantum yield, from 53% to 30%, though this is still lower than that of polymer encapsulation techniques. The histidine can be further exchanged with a range of self-assembled monolayers, including DHLA and PEG, in a homogeneous solution [43].

In the specific application of simultaneous imaging and therapeutic delivery, cationic QDs are of interest as they can electrostatically bind with negatively charged proteins and nucleic acids [57]. These particles, shown in Figure 5, resist deprotonation and consequent aggregation at high pH due to quaternary ammonium end groups. Figure 5B shows an overview of the ligand exchange process: hydrophobic QDs are first converted to amphiphilic QDs by exchange with DHLA capped tetra(ethylene glycol) (TEG) chains, then further modified with the quaternary ammonium terminus. The ultimate surface functionality of the particle can be tuned by altering the ammonium head group. The cationic QDs showed high stability under biological conditions, but similar to other ligand exchange methods, preserved only 40-60% of the original quantum yield [57].

4.3. Bioconjugation

Quantum dots were first used as biological labels in 1998, when it was reported that biotin functionalization and protein conjugation of QDs could enable cellular uptake, specific binding, and intracellular imaging [58, 59]. QDs can be conjugated to a number of biomolecules, including proteins, oligonucleotides, antibodies, and small molecule ligands, to direct their *in vitro* or *in vivo* pathway to a specific target or to impart therapeutic capability [9, 60].

Conjugation of the QD surface with biomolecules depends heavily upon the ligand chemistries available to provide suitable functional groups for covalent attachment or noncovalent binding [61, 62]. In this respect, conventional organic dyes offer a wider variety of bioconjugation strategies. Controlling the QD surface via passivation and phase transfer of QDs has improved tremendously over the last couple decades. Particularly important are mild processing conditions, long-term stability in a biological buffer, biocompatibility, and maintenance of optimal quantum yield following passivation and solubilization [63–69].

To this extent, conjugation of biomolecules using conventional methods has been fairly successful and various detection and targeting applications have been documented [29, 61–63, 65–72]. However, significant work remains to be done to further improve suitability of QDs to enable more site-specific and a wider variety of bioconjugation strategies [28, 73]. Some additional concerns remaining to be addressed include maintaining high quantum yields after bioconjugation and improved efficiency of bioconjugation, to which research has recently shifted focus [28, 64, 65, 68, 69, 73].

As attachment of biomolecules to QDs relies on available surface ligands, there are limited chemistries commonly used to attach the biomolecule to the QD [61–64, 68, 69]. The first is via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemistry, which combines a carboxylic acid-terminated QD to an amine-terminated biomolecule. A second, yet similar method uses Traut's reagent or N-hydroxysuccinimide (NHS) to fix an amine-terminated QD to amine-terminated biomolecules. Additionally, sulfosuccinimidyl 6-(3′-[2-

pyridyldithio]-propionamido)hexanoate has been used to link amine-terminated QDs to thiol-terminated biomolecules.

For example, EDC/NHS chemistry was used to conjugate carboxy-terminated CdSe/ZnS core-shell QDs with an amine-terminated IgG antibody, shown in Figure 6A. A QD to antibody ratio of 50:1 was selected as the optimal formulation based upon fluorescence intensity as well as biological activity, and the size of the particles were in the range of 25-31 nm [74]. The IgG-conjugated QD were then shown to rapidly and specifically detect *Salmonella typhi* captured on a modified polycarbonate membrane at bacterial concentrations of up to 100 cells/mL. Additionally, the QDs were found to be 100 times more sensitive than a comparable common organic dye labeled with IgG [74].

However, coupling via carbodiimide chemistry is often very inefficient as NHS esters can be readily hydrolyzed in the required conjugation conditions. In an alternative strategy, ligands present during the QD synthesis may be exchanged with biomolecules (e.g., DNA, RNA) to enable electrostatic attachment [61–64, 68, 69]. However, this method may release very toxic or heavy-metal ligands, and must be followed by stringent purification and characterization methods. Most often, this requirement leads to a labor intensive and low yield process. Similarly, ligand exchange with thiol-labeled biomolecules can be used. However, this method is often limited as surface-bound thiols can be readily oxidized or degraded by light. In another common method, the native avidin-biotin interaction can be utilized to attach avidin-functionalized QDs to biotinylated biomolecules [41]. However, this method significantly increases the overall QD size and often limits the applicability to *ex vivo* studies or imaging of superficial tissues.

As discussed above, these traditional coupling methods have some limitations, including inefficient binding and cross-reactivity [10]. Bioorthogonal reactions and click chemistry (1,3-dipolar cycloaddition between azides and terminal alkynes) are powerful techniques, and advantageous in functionalizing QD with targeted biomolecules as they enable specific reaction sites without undesired side products [75–84]. Many of these methods have been widely adopted and can be found as part of commonly used commercial antibody or protein conjugation kits. Additionally, the stability of terminal alkynes and azides used in click chemistry mean that these methods are compatible with a wide variety of biomolecules (e.g., antibodies, peptides, proteins, enzymes, polysaccharides) and susceptible to hydrolysis or crosslinking as standard coupling reactions often are (e.g., esterification, thiol-maleimide addition) [77, 85]. Importantly, these methods can also be utilized in a high-throughput manner to rapidly produce a library of QDs for subsequent characterization [77].

In click chemistry, reactions can typically occur at room temperature with high yield to form a thermodynamically stable carbon-heteroatom bond [85]. Copper catalyzed azide-alkyne Hüisgen cycloaddition (CuAAC) is commonly used to perform the reaction. However, the successful use of copper in QD functionalization has been not been frequently demonstrated due to the surface trapping of Cu ions and subsequent inhibition of QD photoluminescence, especially in the case of CdSe QDs [85–88].

To address these limitations, methods to enable copper free click chemistry with QDs have progressed rapidly within the last decade [77–79, 89–95]. For example, strain-promoted azide-alkyne cycloaddition (SPAAC) is a very promising and interesting copper free click reaction, with an example reaction scheme shown in Figure 6B. In this reaction, azido-biomolecules can be directly conjugated to QDs surface-functionalized with strained cyclooctyne. Schieber et al. reported the successful use of the copper free cyclooctyne click approach to attach transferrin protein onto the QD surface [78]. The high stability, biocompatibility, and versatility of SPAAC reactions also enables its direct use for dynamic cellular imaging. Using polymeric imidazole ligand-capped QDs prepared through SPAAC reaction, Zhao et al. recently demonstrated the ability to surface label enveloped viruses via the metabolic incorporation of a choline analogue into host cells as a method to facilitate the development of new targeted, therapeutic agents [95].

Additionally, Bawendi et al. developed a tetrazine derivative [3-(4-benzylamino)-1,2,4,5-tetrazine (BAT)] that undergoes click chemistry with strained olefins such as norbornene [96]. To this end, QDs were functionalized with norbornene using polymeric imidazole ligands in a simplistic synthesis. The functionalized QDs were then reacted with BAT-modified biomolecules to conjugate QDs in what has become known as a bio-orthogonal method. After conjugation, the QDs displayed a quantum yield of approximately 60%, maintained approximately the same size as prior to conjugation, and were shown to target endogenously expressed cell receptors [96].

More recently, a novel ligand, 5-norbornene-2-nonanoic acid, has been explored for the flexible functionalization of QDs via click chemistry at nM scale. Interestingly, this new approach avoids the conventional ligand-exchange step by providing a carboxyl at one end of the ligand which binds to the QD surface and providing a norbornene group at the other end of the ligand to enable phase transfer into aqueous solutions and efficient norbornene/tetrazine click reaction [97]. This method is capable of producing water-soluble QDs with a high quantum yield and a small hydrodynamic diameter of around 12 nm at an order of magnitude higher scale than previous methods [97].

QDs can also be derivatized with a modified haloalkane dehalogenase (HaloTag ligand) and subsequently reacted through HaloTag protein mediated site-specific conjugation [80, 98–104]. The haloalkane to HaloTag protein interaction is highly specific and sensitive, with a fast on-rate of $\sim 10^6$ $M^{-1}s^{-1}$, and can be used to study cellular processes and signaling with minimal toxic effects on regulatory proteins [105]. For example, QDs functionalized with HaloTag ligand conjugates can covalently bind biomolecules tagged with HaloTag protein or to cellular receptors genetically fused to HaloTag protein (i.e. to enable specific labeling of living cells or chemically fixed cells). The Halo-Tag ligand can also react with ligated bromodecanoic acid to form covalent adducts. In another example of dynamic cellular imaging, Liu et al. developed a platform for single-molecule imaging on living cells through QD targeting to cellular proteins using *Escherichia coli* lipoic acid ligase and HaloTag [106].

5. Approaches to Alter Biodistribution and Toxicity

As discussed in the prior section, substantial progress has been made to improve the surface properties of QDs and improve the solubility and stability in biological environments. However, several challenges still exist in realizing the clinical potential of QDs. Key limitations include the ability to control the QD biodistribution only to target organs, reduce long-term accumulation in the body, and limit the long-term toxicity [22, 28, 30, 32, 73, 107]. Highlighted here is a discussion of potential solutions and recent progress to these ongoing challenges.

5.1. Biodistribution

Investigation of the biodistribution of biological compounds, biomarkers and related systems is of utmost importance in our understanding of the behavior of such systems. For the majority of *in vivo* applications, QDs are administered directly into the bloodstream via systemic intravenous (*i.v.*) injection. Since QDs are nanocolloids, their behavior is significantly more complicated than conventional organic dyes in this biological environment. To this extent, the particles encounter multiple lines of defense upon administration to the circulatory system and must overcome several significant biological barriers from organ to cellular level [26–30, 33–40]. These barriers inhibit accumulation and reduce the efficiency of targeted interactions.

First, the bloodstream is comprised of a variety of serum proteins, and those proteins can adsorb to the particle surface through an immune process known as opsonization [36, 38, 40, 107–109]. Consequently, nanocolloids are marked through the formation of a protein corona, and recognized as foreign invading objects by phagocytic cells (predominantly resident macrophages). The particles are then sequestered by the reticuloendothelial system and mononuclear phagocytic system (RES/MPS), and eventually cleared by intracellular lysosome degradation. If not recognized by macrophages, particles can also undergo non-specific uptake by lymphocytes. Additionally, first-pass renal filtering and extraction is another barrier in which foreign particles are quickly removed from circulation. All of these processes combined result in high non-specific and uncontrolled QD accumulation in the liver, spleen, lymph nodes, and kidney [36, 38, 40, 107–109].

QD composition and properties can be rationally designed to slow these clearance mechanisms and increase circulation time in the bloodstream. Gambhir et al. were the first to show the biodistribution of commercially available CdSe QDs in living mice [110]. The results indicated that there was rapid uptake by the liver and spleen, with the most accumulation occurring in the liver on the order of a couple minutes. Once particles entered the RES organs, there was no observation of clearance or metabolism.

Since these studies, there has been significant additional research in controlling and altering the biodistribution of nanocolloids [111–118]. Composition, particle size, particle shape, and surface characteristics are all critical properties that affect the ability to avoid removal by the immune system and extend circulation time in the blood stream [36, 38, 40, 107–109]. In terms of particle size, it has been shown that QDs with sub-10 nm particle sizes are prone to rapid clearance from the body by first-past renal filtration [39, 115, 119, 120]. Additionally,

a diameter less than 200 nm is desired to help evade immune recognition and clearance by macrophages [39, 115, 119, 120]. In general, larger particles (in the range of 50–200 nm) have slower removal from the circulation compared with those having smaller size. In terms of particle shape, a rod-like particle shape is advantageous for avoiding protein accumulation, but can lead to a less effective cell internalization compared to spheres [39, 115, 119, 120].

Further, surface charge, hydrophilicity, and material stiffness are very influential for determining the level of protein absorption and play a critical role in determining cell interactions. For example, a net neutral to slightly negative surface charge is desired for circulation as a positive charge enhances uptake by the cells of the phagocytic system and results in a short circulation half-life [39, 115, 119, 120]. In terms of material stiffness, a low elastic modulus is desired to lower the rate of protein accumulation and internalization by macrophages as compared to their stiff counterparts [39, 115, 119, 120].

Coating particles with hydrophilic polymers reduce the clearance rate of particles by the MPS/RES system [39, 121–123]. In particular, polymers can prevent nanoparticles from being recognized as foreign substance, opsonized, and removed from the body. A common strategy is to employ a surface coating of a neutral, soft, hydrophilic polymer, such as polyethylene glycol (PEG).

Depending on the coating density, the PEG can take on a mushroom or extended brush conformation and reduces the interaction with serum proteins [39, 121, 122]. Gambhir et al. found that PEGylation of CdSe QDs did serve to increase particle blood half-life from 20 seconds to 3-4 minutes as well as retard organ uptake to a small extent, from 2 to 6 minutes in the liver. However, it also increased low-level bone uptake. Additionally, all QD formulations tested were still cleared from circulation within 10-20 minutes of injection [110].

The same group expanded their study to investigate the effect of particle size, surface functionalization, and PEGylation on biodistribution of QDs in living mice [124]. Six QD conjugations were studied: two Qdot800 conjugations from Invitrogen (diameter 19-21 nm), one with PEG (2000 MW linear PEG in all instances) and one without; two peptide coated QDs (diameter 11-12 nm), one with PEG and one without; and two indium arsenide (InAs) QDs (diameter 5 nm), one with PEG and one without. Uptake of Qdot800 without PEG by the liver and spleen was rapid, becoming a significant amount in less than 1 minute compared to 6 minutes for the PEGylated Qdot800. Liver activity for both stayed nearly constant for the remainder of the study. Accumulation of peptide coated QDs was slower, taking 19 minutes to reach one standard deviation of the peak liver activity for the PEGylated formulation, and levels in the liver had statistically decreased after 12 hours. The InAs QDs experience rapid accumulation in the liver and spleen, about 2 minutes for both with and without PEG, as well as some activity in the bladder. As with the peptide coated QD, the InAs were cleared from the liver to a significant extent within 6 hours. Different from the other PEGylated QDs, the InAs QDs did not show significant accumulation in the bone. The study concluded that PEGylation and peptide coating both slow RES uptake of QDs, and that the small InAs QDs are able to be renally excreted to some extent [124].

There are additional challenges and biological barriers if the intended QD use is for tumor imaging, diagnostics, or therapy [38, 40, 120, 125]. In this case, a thorough understand and characterization of the tumor microenvironment is important to understand the ability of particles to penetrate and accumulate in the tissue. Abnormal vessels in tumors create a hostile microenvironment: characterized by extreme hypoxia, low pH, and high interstitial fluid pressure. The tumor vessel tortuosity and higher interstitial pressure can lead to an outward convective fluid flow.

Additionally, tumor vasculature grows so rapidly that they develop ~200 nm gaps in the blood vessels. Fortunately, nano-colloid properties can be designed to enable to penetration into the tissue through the gaps and be retained due to poor lymphatic drainage. This passive targeting is commonly referred to as the enhanced permeation and retention (EPR) effect, and is of particular importance in cancer diagnostics and imaging. However, to be able to diffuse through the tumor interstitium and accumulate within organs and tissues, particles should be less than 150 nm in diameter [38, 40, 120].

Ultimately, a key challenge is that many of the strategies used to enhance the biodistribution to target tissues or organs also significantly reduce target cellular interactions and subsequent uptake [36, 108, 113, 120, 126]. For example, a rod-like particle shape is advantageous for avoiding prolonging circulation and passive accumulation in tissue, but it can lead to a significantly lower rate of cellular internalization when compared to spheres or ellipsoids [39, 115, 119, 120]. Additionally, a dense coating of hydrophilic polymer reduces protein accumulation and recognition by the immune system. However, that same coating significantly hinders cell interactions and uptake [39, 121–123]. Surface modification with biological entities targeted to specific receptors (e.g., aptamers, peptides, antibodies, antigens) has shown remarkable success in improving the cell specificity of imaging and diagnostic modalities. Despite many published studies, there have been no resulting widespread conclusions to understand universal factors that influence the biodistribution and target cell interactions [26–30, 33–40].

5.2. Toxicity

The long-term toxicity of QDs is another major concern in biomedical applications, and represents one of the most challenging obstacles to overcome in clinical translation. As discussed above, QD particles have been shown to accumulate and reside in the liver and spleen of living mice [110]. The toxicity stems primarily from four areas: i) heavy metal materials commonly used in the particle core; ii) chemistries with the potential to release toxic components from the surface (i.e. especially for coatings composed of Cd, Se, and Hg); iii) any free radicals or reactive species generated from excitation; and iv) the tissue and nano-colloid interaction in biological environments (e.g., small particle size, positive surface charge, chemical composition) [26–30, 72, 127–132].

Heavy metal toxicity has been well documented in the literature, especially as the metals have shown an extremely long biological half-life (greater than 10 years) and been shown to readily cross the blood-brain barrier [30–32, 107, 133]. Although surface coatings can be used to limit the release of toxic agents from the QDs surface, it has been recently reported that in some occasions the coating itself might be even more toxic [26–29]. The greatest risk

of toxic effect is due to the release of Cd^{2+} ions from the nanoparticles, which can be somewhat controlled by encapsulation of the QDs in a passivating ZnS shell or crosslinked polymer coating [134, 135]. However, adequate long-term *in vivo* studies have not yet been performed to determine if these measures offer protection against ion release into the body. Consequently, there has been movement toward Cd-free NIR QDs composed of III-V or I-III-VI$_2$ semiconducting metals such as CuInSe [136] and $CuInS_2$ [135].

Pons et al. developed a novel synthesis of $CuInS_2$ using a primary amine precursor [135]. The result was brighter QDs of about 3 nm in diameter with an emission peak around 800 nm and a quantum yield of 10-20%. After addition of a ZnS shell, the diameter increased to about 5.5 nm and quantum yield increased to 30%. After transfer into water by phospholipid micelle encapsulation, the QDs maintained relatively high quantum yields and were stable for multiple weeks. These $CuInS_2$/ZnS QDs, as well as traditional CdTeSe/CdZnS QDs, were then injected subcutaneously into the paws of live mice, and the effect of the QDs on the regional lymph nodes was observed. Both compositions showed accumulation in the lymph nodes, but only the CdTeSe/CdZnS QDs were accompanied by a significant amount of inflammation, which presumably reflects the acute toxicity of the Cd^{2+} ions [135].

Another report described $CuIn_xSe_y$/ZnS QDs with a much higher quantum yield of 40-60% following passivation [136]. However, the stability of these QDs was limited to 1-2 days, mainly due to aggregation. Modification of the coordinating ligand improved the stability to 10-14 days, but this is still not optimal for commercialization. These QDs were coated in a lipid layer and injected into mice; after 90 minutes, fluorescence intensity started to decay. At 48 hours, the fluorescence level had returned to the baseline, indicating destabilization of the particles or clearance from the body. There were no observed bright spots, suggesting that no aggregation or accumulation in organs occurred, but this needs to be confirmed by further *in vivo* studies [136]. Though there is need for improvement in the synthesis, quantum yields, and stability of these cadmium-free QDs, preliminary studies indicate favorable toxicity over traditional cadmium QDs and further investigation is worth pursuit.

To further advance in reducing toxicity of QDs, it is recommended to fabricate the semiconductor nanoparticles with optimized colloidal stability under physiological conditions, to control QD size and to improve surface properties for better cellular uptake. Moreover, QDs can be passivated or bioconjugated with antibodies, polymers such as polyethylene glycol (PEG) or nanoparticles like micelles. The latter reduce QDs toxicity without altering their surface and optoelectronic properties. Last but not least, it should be reminded that toxicity of QDs could also be helpful for cancer treatment. For instance, photo-induced toxicity of QDs coupled to molecular photosensitizers has been explored for causing apoptosis of cancer cells [137].

6. Recent Progress and Trends in Biomedical Applications

From the time quantum dots were first conjugated to a biological molecule in 1998, they have evolved into a potent tool for fluorescent bioimaging, including intracellular and single molecule imaging. Highlighted here is a selection of recent and progressive reports of QD

development and application to biological imaging, diagnostic tools, and therapeutic application.

6.1. *Ex vivo* Analyte Detection and Molecular Diagnostics

Recent studies in literature have also focused on the development of functionalized QDs to create diagnostic tools to enable more sensitive *ex vivo* identification of disease-relevant biomolecules [138–150].

In a recent study, Zhang et al. reported the development of dopamine-functionalized QDs for use in a redox-mediated immunoassay in order to detect the cancer biomarker alpha-fetoprotein (AFP) in human serum samples [70]. The authors developed ~12 nm CdSe/ZnS QDs functionalized with dopamine through self-assembly of surface-capped ligand dopamine onto the QD surface through the sulfhydryl group. In the sandwich redox-mediated indirect fluorescence immunoassay (RMFIA), the authors used tyrosinase in order to catalyze enzymatic oxidation of dopamine on the surface of the functionalized QDs, leading to fluorescence quenching in the presence of the analyte AFP. The authors successfully demonstrated the ability to detect AFP concentrations as low as 10 pM, concluding that this assay has great potential as a rapid, sensitive detection method [70]. Further, the authors subsequently demonstrated clinical application through the detection with serum samples collected from hepatocellular carcinoma patients versus normal subjects, confirming the method applicability in clinical *ex vivo* biological testing.

Avidin-biotin binding has also been used in a similar application of QDs in competitive fluoroimmunoassay development for the detection of human serum albumin (HSA) in human urine [151]. Biotinylated QDs were prepared via the bioconjugation of CdTe QDs with denatured bovine serum albumin (dBSA) at the QD surface. The conjugation strategy employed also allowed for flexibility in further surface modification, as the albumin can be used as a bridge for additional ligand functionalization [151]. It was observed that for this application, an excess of 6.7 dBSA to QD was required for complete derivatization of QDs, and the highest quantum yield of 15% was achieved by the resulting 400 nm diameter bioconjugated QDs. These were much larger particles with a lower quantum yield compared to unconjugated QD, but they were shown to be stable for up to two months and able to detect increased levels of HAS in urine using a fluorescence assay [151]. The authors successfully demonstrated the ability to detect HSA concentrations in urine as low as 1.0 μg/mL, with a relatively broad working range from 1.7 to 10 μg/mL.

Qiu et al. developed a bioresponsive QD-paper-based analytical device as a high-performance molecular diagnostic method to monitor disease-relevant levels of carcinoembryonic antigen (CEA) analyte in human serum samples [71]. In this research, the authors used CdTe/CdSe QDs and glucose oxidase immobilized on paper to develop a visual fluorescence assay based on CEA aptamer/complementary DNA (cDNA)-gated release of glucose from mesoporous silica nanocontainers. The glucose is released from the nanocontainers, which are oxidized by the glucose oxidase and produce hydrogen peroxide to quench the fluorescence of the CdTe/CdSe QDs. The authors demonstrated that the fluorescence detection could be visualized by the naked eye and on a commercial

spectrometer. Sensitive and accurate discrimination of CEA was reported with a linear range of 0.05-20 ng mL^{-1} and high reproducibility [71].

To enable a more rapid and accurate approach for colorectal cancer diagnosis, Park et al. developed a multiplexed detection approach using the combination of an enzyme-sensitive fluorescence dye and antibody-QD conjugates [152]. In this study, a cresyl violet-glutamic acid (CV–Glu) derivative was used as the fluorescent molecular probe, as it is capable of rapidly switching between two fluorescent colors in response to enzymatic activity of λ-glutamyltranspeptidase (GGT). A complementary QD probe was developed through bioconjugation of matrix metalloproteinase-14 antibody (MMP14) to biocompatible, 690 nm emitting AgInS2/ZnS (core/shell) QD. The probes were tested in both *in vitro* and *ex vivo* applications, using human colorectal cancer cell lines, *ex vivo* murine model tissues, and *ex vivo* patient tumor colon specimens. The authors used two-photon microscopy to demonstrate that co-application of both probes were able to rapidly permeate from the tissue surface at 10-20 μm depths. Ultimately, the study showed that co-application of both probes enabled rapid (within 5 minutes) and accurate visualization of tumor lesions difficult to detect via conventional colonoscopy techniques [152]. Additionally, the dual-probe technique was able to identify regions with hyperplastic polyps and adenomas possibly in the early stages of colorectal cancer [152].

Molecular changes in tissue are extremely complex and difficult to study the progression of disease. This represents a significant and major challenge still remaining in the biomedical field, particularly in cancer [153]. Additionally, heterogeneity in tumors leads to significant challenges in the development of targeted therapeutics. To this extent, the use of QDs has shown great promise to enable noninvasive *ex vivo* cell labeling and diagnostic imaging in tumor specimens, and *ex vivo* investigation of the molecular markers and major dynamic processes of cancer – migration, invasion, and metastasis [19, 142, 154–160].

In particular, one study explored the effect of breast cancer invasion on the extracellular matrix (ECM) using QD-based multicolor imaging of human epidermal growth factor (HER2) and type IV collagen [161]. It is difficult to achieve multicolor imaging with traditional stains and organic molecules due to unreliable specificity and photo-bleaching caused by excitation over many different wavelengths, but different color QDs can be excited by a single wavelength and have more consistent biomarker quantification relationships, alleviating these issues to some extent [161]. Clinical human HER2 positive breast cancer tissue was incubated with HER2 and type IV collagen primary antibodies, followed by incubation with QDs conjugated with the secondary antibody, and imaged using a fluorescent microscope. The researchers were able to visually show that visually that high levels of HER2 expression is in fact correlated with increased ECM destruction, breast cancer cell invasion, and possibly even angiogenesis. The researchers concluded that this imaging modality could provide a beneficial method of diagnosis and aid in determination of treatment strategy [161].

In another recent study, multiplexed QDs-antibody conjugates and wavelength-resolved spectral imaging were used to enable high-throughput molecular mapping of tumor heterogeneity using clinical prostate cancer specimens [153]. Here, a sequential staining

method of primary antibodies with QD-secondary antibody conjugates was used to build the degrees of multiplexing. The study evaluated four protein biomarkers (E-cadherin, high-molecular-weight cytokeratin, p63, α-methylacyl CoA racemase), and demonstrated accurate detection and identification of structurally distinct prostate glands and single malignant tumor cells from human specimens [153]. Further, the authors used high-throughput molecular mapping to create profiles of the tumor tissue, revealing the extensive heterogeneity at both the molecular, cellular, and architectural levels. Fundamentally, the study demonstrated the ability to visualize gland morphological and structural transitions during prostate cancer progression, from a double layer of basal and luminal cells to a single layer of malignant cells [153].

6.2.   Real-Time *in vivo* Imaging

In order to be more applicable in real-time biomedical imaging, diagnosis, and image-guided surgical applications, imaging probes need to be capable of deep tissue imaging [20, 22, 26–28, 30]. Light in the visible range (below 700 nm) is not capable of penetrating into deep tissue due to high levels of scattering from natural absorbers (e.g., lipids, deoxyhemoglobin, water) [23, 26]. QDs have a large two-photon cross section, meaning it is possible to use near-infrared (NIR) irradiation with nanoparticles that emit around 650-900 nm wavelengths [135]. This is known as the tissue transparency window, as absorption by water and tissue is very weak in this range, making deep tissue imaging possible. Further, QDs make excellent probes for real-time *in vivo* imaging as they are 100 to 1,000 times more intense than conventional organic fluorophores and are capable of multiphoton imaging with excitation by a single wavelength [22, 135].

Recently, there has been significant success reported using QDs for real-time imaging application and image-guided surgery [155–160, 162–165]. In particular, near-infrared (NIR) QDs have emerged as a remarkable *in vivo* imaging modality [157, 166–180]. Compared to the visible spectral window commonly employed for conventional fluorescence imaging, the ideal spectrum to enable deep *in vivo* imaging needs to have reduced scattering, negligible tissue or biological autofluorescence, minimal absorption, and yield a higher signal-to-noise ratio [166, 172, 176, 179]. To this extent, both the first NIR (NIR-I, 700 to 900 nm) and the second NIR (NIR-II, 1000 to 1700 nm) spectral windows offer significant advantages to enable ultrahigh spatial resolution with increased penetration depths [166, 176, 177].

Comparably, the NIR-II window is more advantageous than NIR-I as it can yield higher imaging clarity than achievable in NIR-I, owed in part to the dramatically reduced photon scattering, absorbance, and tissue autofluorescence at the higher wavelengths [166, 176, 177]. Though the NIR-II range is defined broadly, imaging in the NIR-IIa (1300 to 1400 nm) and NIR-IIb (1500 to 1700 nm) windows can result in additional improvements [166, 181, 182]. However, imaging in the NIR-II window is a relatively newer research area as it has only been readily achievable within the last decade [166, 168, 172, 176, 177, 179, 180]. Recent advances in both chemistry and optical imaging capabilities have enabled the development of NIR fluorophores and QDs with longer wavelengths as well as the

development of more advanced photodetectors with sufficient sensitivity and efficiency for NIR-II imaging [157, 166–171, 173–175, 178, 183–185].

Recently, Bruns et al. have reported the development of next-generation NIR-II QDs capable of deep tissue penetration, high spatial imaging resolution, multicolor imaging, and rapid acquisition for a variety of *in vivo* imaging applications [185]. In this work, a class of heavy metal-free indium/arsenide (core/shell) QDs was developed and demonstrated to exhibit a narrow, size-tunable emission and significantly higher quantum yield (up to 30%) than other literature reports. Further, the authors demonstrated composition-tunability of the QDs and showed that the surface is easily modified with a variety of functional ligands for broad use in applications such as bioimaging, molecular tracking, and structural mapping. Applicability of the QDs was demonstrated using an *in vivo* murine model to enable the real-time imaging and quantification of lipoprotein metabolic turnover rates, heartbeat and breathing rates, and three-dimensional quantitative flow maps of the mouse brain vasculature [185].

In a similar application, Li et al. reported the ability to visualize tissue blood flow and angiogenesis real-time *in vivo* through the development of NIR-II QDs [186]. Hydrophilic, PEGylated silver sulfide (Ag_2S) QDs were synthesized through ligand exchange and carbodiimide coupling, resulting in peak emission at 1200 nm and a quantum yield of up to 16%. Mouse studies confirmed the high stability and long circulation time of the QDs *in vivo*, and the QDs were capable of deep tissue penetration with ultrahigh spatial and temporal resolution (~40 μm) [186]. Ultimately, the study demonstrated the ability to perform real-time, noninvasive and dynamic visualization of lymphatic drainage and vascular networks, which may aid in image-guided surgery as well in assessment of blood supply in tissues and organs and the screening of anti-angiogenic therapeutic treatment efficiency [186].

Another of the remaining challenges in the *in vivo* application of QDs has been in the successful engineering of water-soluble, biocompatible QDs that emit in the 650-900 nm range [20, 23, 30]. In one approach, CdTe/CdSe QDs were loaded into poly(lactic-co-glycolic acid) (PLGA) nanospheres for the ultimate purpose of tumor imaging [134]. Unmodified QDs were encapsulated so that the highest possible quantum efficiency could be preserved, while the PLGA coating created a stable, highly water-soluble shell. PLGA also has a high degree of biocompatibility and can biodegrade. The particles had an emission peak of 760 nm, and exhibited a quantum yield of approximately 50% [134]. These are ideal properties for NIR bioimaging. It was also shown that pH range had minimal effect on the stability PLGA encapsulated QDs, and that exposure to proteins in biological serum did not greatly change the optical properties of the QDs. The results indicate that there is very little escape of QDs from the polymer nanosphere, causing insignificant cytotoxicity on the order of two days, but further studies are needed to assess the effect of PLGA biodegradation over an extended period of time [134]. Confocal laser scanning microscopy revealed high cellular uptake of the particles by two different cell lines [134]. These are promising results for transforming QDs into a real-time *in vivo* tissue imaging tool, with the acknowledgement that a deeper investigation into long-term, repeated toxicity and biodistribution still remains.

Another major application of quantum dots is targeted tumor imaging, as the small size of QDs facilitates tumor accumulation by leaky vasculature around tumor sites due to the enhanced permeability and retention (EPR) effect [2]. With only passive accumulation, applications are limited by poor tumor specificity. To this extent, the use of the previously discussed bioconjugation strategies with cancer-specific targeting moieties has significantly enhanced the targeting efficiency.

For example, Liu et al. recently reported the development of biocompatible NIR QDs capable of *in vivo* cation exchange to enable tumor-specific imaging [187]. The system was designed to achieve tumor-specific imaging first through passive accumulation of the QDs in tumor tissue, followed by induction of cation exchange in excess QDs remaining in the circulation to quench any remaining background signals [187]. The QD core was comprised from Zn^{2+}, Hg^{2+}, Se^{2-}, and S^{2-}, and PEGylated to reduce rapid clearance by the MPS system. A membrane-impermeable etchant was developed from Ag^+ ions stabilized with the metal chelator thiosulfate (TS) to serve as a cation donor to the QDs, where the etchant can provide silver ions to rapidly quench the QD photoluminescence when exchanged with the QD cations (Zn^{2+}, Hg^{2+}) [187]. Using orthotopic breast and pancreatic tumors in mice, the study demonstrated a highly tumor-specific signal with minimal toxicity, demonstrating *in vivo* cation exchange as a promising strategy to enhance the specificity of QDs in tumor imaging [187].

6.3. Intracellular Imaging, Delivery, and Therapeutic Modalities

In the therapeutic application of QDs, photodynamic therapy (PDT) has been extensively studied [188–208]. PDT utilizes the application of light to activate a photosensitizing agent, which in turn transfers its triplet state energy to form reactive singlet oxygen species (ROS) [189]. These reactive species can then initiate apoptosis of local cancer cells. PDT has been widely studied due to its potential for more selective and specific treatment than conventional chemotherapy [189]. However, application of conventional PDT agents is often limited by low singlet oxygen quantum yields, photo-bleaching, and *in vivo* toxicity [209].

The use of QDs as photosensitizing agents has demonstrated the potential to overcome some of these limitations. QDs comprised from CdSe, CdTe, carbon-based materials, and others have been studied in the literature for PDT applications as either QDs alone, QDs in the presence of other photosensitizing agents (e.g., phthalocyanine, phthalocyanine derivatives, porphyrin, chlorine e6), or QDs conjugated to another photosensitizing agent [189, 194, 196, 198, 200, 203–208]. QDs have been shown to display superior photostability and aqueous solubility, but the clinical translation has been hindered by significant toxicity to health tissue and low ROS-generation efficiency [196, 198, 200, 204, 209].

To this extent, Ge et al. have reported the development of a novel PDT agent with high singlet oxygen generation based on highly water-dispersible graphene QDs [209]. The graphene QDs were synthesized through a hydrothermal method using polythiophene derivatives as a carbon source [209]. The QDs were able to produce ROS through a multi-state sensitization process, exhibited a broad absorption band with a strong emission peaking at 680 nm, displayed superior photostability to the more conventional CdTe QDs, and resulted in a ROS quantum yield (~1.3) significantly higher than similar studies reported in

literature. An *in vivo* murine xenograft model was used to evaluate the graphene QDs for dual PDT and bioimaging in breast cancer. In terms of real-time tumor imaging, the study demonstrated a high signal-to-noise ratio of 229.5, and no apparent fluorescence intensity decay or diffusion observed at the injection sites, even 1 week after injection [209]. In terms of efficacy, tumors began to decompose 9 days after light exposure and QD activation, and were fully destroyed after 17 days with no tumor regrowth observed for up to 50 days [209]. In a second study, the authors further demonstrated the applicability of the graphene QDs as multifunctional fluorescent, photoacoustic, and thermal theranostics for simultaneous diagnosis and therapy of cancer [210].

Using nitrogen-doped graphene-based QDs, Kuo et al. reported dual photodynamic antimicrobial therapy and bioimaging [211]. The developed nitrogen-doped QDs were capable of generating a higher amount of reactive oxygen species than a nitrogen-free QDs after only 3 minutes of photoexcitation (670 nm laser, 0.1 W cm^{-2}), and demonstrated an increase in PDT antimicrobial efficiency with higher nitrogen-binding compositions [211]. Additionally, the study demonstrated that the NIR and high photostability properties of the graphene QDs enabled better contrast and imaging of the bacteria.

QDs can also be utilized to deliver and monitor the efficacy of a therapeutic payload, as attached cargo quenches the fluorescence of the particles by fluorescence resonance energy transfer (FRET) [60]. These platforms hold great promise to enable the study of the pharmacokinetic and pharmacodynamic behavior of chemotherapeutic agents, and monitoring of disease progression and the therapeutic effect.

To this extent, Minko and colleagues recently reported a QD carrier with doxorubicin (DOX) conjugated via a pH sensitive hydrazone bond, coupled with a DNA aptamer targeted to MUC1 [60]. This system was engineered to advantageously increase delivery into ovarian cancer cells, avoid systemic effects of chemotherapy by employing targeted delivery, and provide an effective imaging modality to monitor drug release. Increased uptake of the QD-MUC1-DOX particles over unmodified QD by DOX-resistant A2780-AD human ovarian cancer cells was confirmed, as was release of the anti-cancer agent DOX within the cells. *In vivo* distribution of the targeted and non-targeted QD, evaluated in a nude mouse model, indicated that the targeted QD conjugate showed higher accumulation in the tumor and less accumulation in other organs, compared to the non-targeted QD, shown in Figure 7 [60].

QDs represent an exciting candidate to enable tumor theranostic platforms, and developing nanocarriers co-loaded with chemotherapeutic agents and QDs has been a prominent goal in recent literature. For example, paclitaxel along with CdTe/CdS/ZnS QDs loaded in nanostructured lipid carriers showed an encapsulation efficiency of around 80%, drug loading of 4.7% and a tumor growth inhibition rate of more than 77% [154]. Other additional recent approaches include quercetin loaded CdSe/ZnS QDs as efficient antibacterial and anticancer nanoplatforms [212] or 5-Fluorouracil loaded onto Mn-ZnS QDs encapsulated with chitosan biopolymer and conjugated with folic acid to enhance targeting efficacy towards folate receptors expressing malignant tissues [213].

QDs have also been utilized to facilitate gene therapy through intracellular delivery and imaging of treatment with small interfering RNA (siRNA). siRNA are short chain s of double-stranded RNA that have the ability to specifically silence genes via a endogenous pathway known as RNA interference (RNAi) [214]. RNAi is defined as the process of messenger RNA cleavage by sequence-specific complementary siRNA, and may be applied as a therapeutic for diseases that result from aberrant gene expression. For RNA interference to occur, siRNA must be delivered into the cell cytoplasm.

Typical intracellular vehicles for siRNA include cationic polymer nanoparticles and micelles, as well as liposomes. One of the earliest reports appeared in 2005, in which QDs were used to observe siRNA transfection *in vitro* [215]. In this study, CdSe-core QDs, ZnS-shell QDs, and/or siRNA (Lamin A/C gene target) were encapsulated in the commercially-available cationic-lipid reagent, Lipofectamine (Invitrogen). Subsequently, fibroblasts were transfected, and siRNA uptake and silencing were analyzed via flow cytometry, western blotting, and immunofluorescence staining. As shown in Figure 8, the study showed a strong correlation between gene knockdown and fluorescent intensity in cells transfected with both the QDs and siRNA, demonstrating that QDs are a suitable probe to track siRNA delivery. Further, the study also demonstrated that the QD-mediated siRNA delivery was able to increase the *in vitro* gene silencing efficiency [215]. The formulation delivering both the QDs and siRNA together resulted in a 90% silencing efficiency. This was significantly higher than the control formulation delivering only siRNA, which resulted in 20-30% silencing efficiency.

QDs with a positive surface charge have also been used as carriers of siRNA themselves, eliminating the need for an additional transfection agent such as Lipofectamine. In one reported study, L-arginine (L-arg)-modified CdSe/ZnSe QDs, both with and without β-cyclodextrin (β-CD), were used as siRNA delivery vehicles to target gene silencing of HPV18 E6 [216]. The purpose of modifying the QD surface with L-arg was to provide a large, positively charged surface area onto which negatively charged siRNA was complexed via electrostatic attraction. Subsequently, the QD and siRNA complexes were transfected into HeLa cells. Cell uptake and trafficking was monitored via flow cytometry and confocal microcopy, respectively, and efficacy was analyzed via RT-PCR and western blotting. The study demonstrated that the complexes were cytocompatible (defined in the study as greater than 70% viability) at QD concentrations less than 70 μg/mL after 24 hours of exposure. Further, the QD and siRNA complexes were able to achieve nearly 80% gene knockdown and approximately 80% target protein suppression [216].

More recently, cadmium sulphoselenide/zinc sulfide quantum dots (CdSSe/ZnS QDs)-based nanocarriers loaded with siRNA have been evaluated for targeting human telomerase reverse transcriptase (TERT) of central nervous system tumor cells [127]. Results showed high gene transfection efficiency (>80%) on two glioblastoma cell lines. Furthermore, silencing of TERT gene expression significantly inhibited the proliferation of glioblastoma cells.

Another intriguing approach involves using QDs and siRNA for exploring the role of genes such as SOX9 in the chondrogenic differentiation of adult mesenchymal stem cells (MSCs). Authors fabricated functional quantum dot (QD) nanoplexes by sulfosuccinimidyl-4-(N-

maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) activation of PEG-coated CdSe/ZnS QDs as the gene carrier of siRNA to explore the effect of SOX9 RNA interference on MSCs [217]. Interestingly, SOX9 knockdown inhibited the expression of cartilage-specific markers in MSCs, delaying cartilage repair.

7. Conclusions and Future Outlook

Quantum dots (QDs) present a versatile tool to enable more accurate diagnostic tools and fluoroimmunoassays, multiplexed imaging, dual imaging and therapeutic platforms, real-time *in vivo* and cellular process imaging, and tracking of single cells and biological molecules. With the widely increased interest in inorganic particles over the last decade, the design and development of QDs has advanced significantly.

Recent advances in synthesis and bioconjugation strategies have rapidly progressed the ability to ensure accurate control over QD size, polydispersity, quantum yield, and surface properties. The developed strategies are versatile and flexible, enabling QDs to also be used in more complex hybrid nanomaterials. Additionally, these strategies can yield enhanced colloidal stability as well as provide a robust platform for further bioconjugation. However, investigation, quantification, and control over the spatial distribution of functional groups is often not addressed literature reports. This will become more important to consider and address with increasing biological complexity of the intended application.

While QDs as biological probes have demonstrated their potential across a variety of fields, the most promising reports to date are limited to *in vitro* and *ex vivo* applications. In these applications, QDs can be readily substituted for and are significantly more advantageous than traditional fluorophores, outperforming in nearly every aspect. For example, the development of novel and advanced QD conjugation strategies with a wide variety of biologically-active molecules has opened up several avenues towards creating advanced bioimaging techniques. As discussed previously, conjugation with molecules such as proteins, oligonucleotides, and cell-targeted ligands can enable profound discoveries in the study of cellular mechanisms and molecular structures. Additionally, QD applications with *ex vivo* patient tissues and fluids have the potential to significantly improve our understanding of disease progression, enable more rapid and accurate diagnosis, and create improved tools to aid in the identification and design of new drugs for a variety of diseases.

As acute and long term toxicity are not major concerns, *in vitro* and *ex vivo* applications of QD-based approaches are likely to continue progressing rapidly in the next decade. With further validation of the technologies, these QD-based approaches could also make significant strides towards enabling the pre-treatment analysis and prediction of therapeutic efficacy in specific patients – a significant goal towards the realization of personalized medicine.

In spite of these advances, there still remain several significant limitations and regulatory issues that need to be addressed before the application of QDs in medicine and *in vivo* imaging in human subjects [218]. A key challenge remaining is the inefficient delivery from low biological specificity and poorly controlled biodistribution to target tissues. The

biodistribution of current QD compositions show inadequate clearance from real-time *in vivo* studies, and the long-term accumulation of QDs is still largely unknown.

However, the greatest challenge to the clinical translation of QDs is the risk of severe acute and long-term toxicity. More specifically, there is limited understanding of the biological interaction of QDs reported in literature, and there is a critical need to develop a detailed understanding of the relationship between then QD physicochemical properties and their pharmacokinetic and pharmacodynamic behavior. In an effort to reduce safety concerns, research should also focus towards a better replacement of the heavy metal-based QDs, as these materials have dominated current bioimaging studies especially with cadmium being used a prominent core material [219, 220].

The ultimate clinical success of QDs will depend on the ability of future research overcome these limitations. If research does not continue to work towards addressing these limitations, QDs will remain only for use in animal and laboratory research. The advent of advanced synthesis routes, improved heavy-metal-free QDs, and a deeper understanding of the structure-function relationship of QDs at biological interfaces are critical. Ultimately, we believe that researchers will be able to address these challenges and continue to push QDs forward towards their clinical potential.

Acknowledgements

Abbreviations

QD	quantum dot
MBE	molecular beam epitaxy
Me$_2$Cd	dimethyl cadmium
TOPSe	trioctylphosphine selenide
TOPTe	trioctylphosphine telluride
(TMS)$_2$Se	bis(trimethylsilyl)selenium
TOP	trioctylphosphine
TOPO	trioctylphosphine oxide
CdS	cadmium sulfide
Cd(Ac)$_2$	cadmium acetate
CdSe	cadmium selenide

CdTe	cadmium telluride
NIR	near-infrared
PL	photoluminescence
ZnS	zinc sulfide
ZnEt$_2$	diethylzinc
(TMS)$_2$S	bis(trimethylsilyl)sulfide
MUA	mercaptoundecanoic acid
DHLA	dihydrolipoic acid
PEG	polyethylene glycol
TEG	tetra(ethylene) glycol
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)
NHS	N-hydroxysuccinimide
dBSA	denatured bovine serum albumin
BAT	3-(4-benzylamino)-1,2,4,5-tetrazine
siRNA	small interfering RNA
RNAi	RNA interference
L-Arg	L-arginine
β-CD	β-cyclodextrin
EPR	enhanced permeability and retention
FRET	fluorescence resonance energy transfer
DOX	doxorubicin
ECM	extracellular matrix
HER2	human epidermal growth factor
PLGA	poly(lactic-co-glycolic acid)
RES	reticuloendothelial system
InAs	indium arsenide

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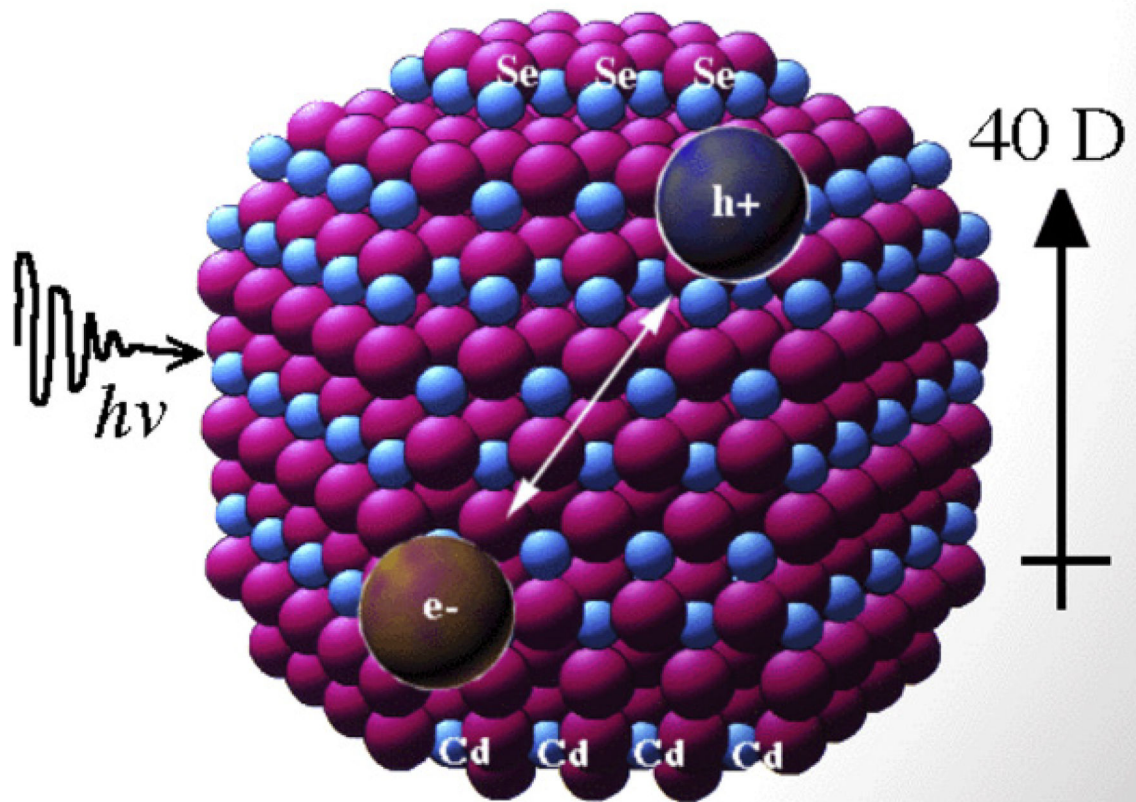
Figure 1.
Illustrative structure of a CdSe quantum dot. Photon excitation causes electrons to move to a higher energy level, creating an electron-hole pair known as an exciton. If the electron and hole recombine as the electron returns to its ground state, fluorescence is emitted. Adapted from [41] with permission from Elsevier.
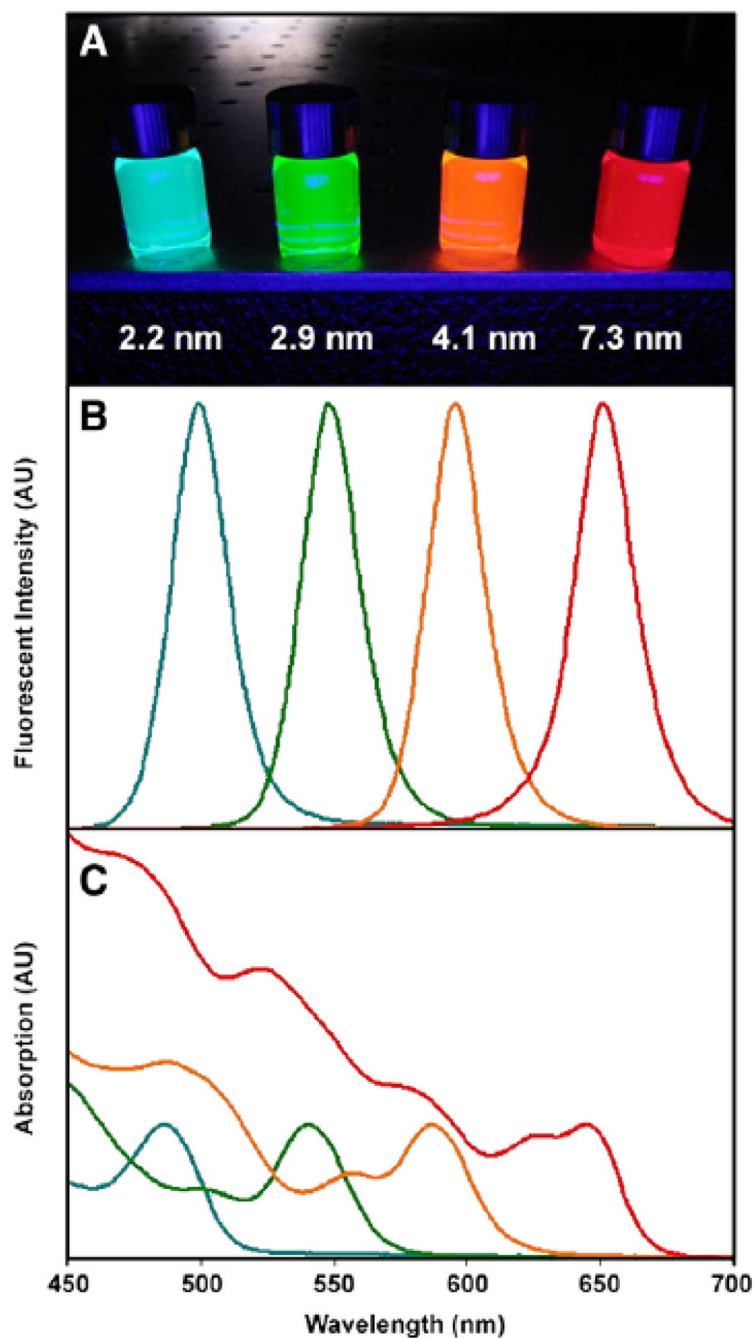
Figure 2.
Optical properties of CdSe QDs (dispersed in chloroform) can be tuned by the size of the particle, known as the quantum size effect. (A) Fluorescence image of monodisperse QDs after ultraviolet illumination, ranging in size from 2.2 –7.3 nm. (B) Fluorescence spectra of the same four QD samples. Narrow and symmetric emission bands are observed, indicating low variance in particle size. (C) Absorption spectra of the same four QD samples. Broad absorption spectra are observed, enabling a wide range for excitation of the QDs. Adapted from [9] with permission from Elsevier.
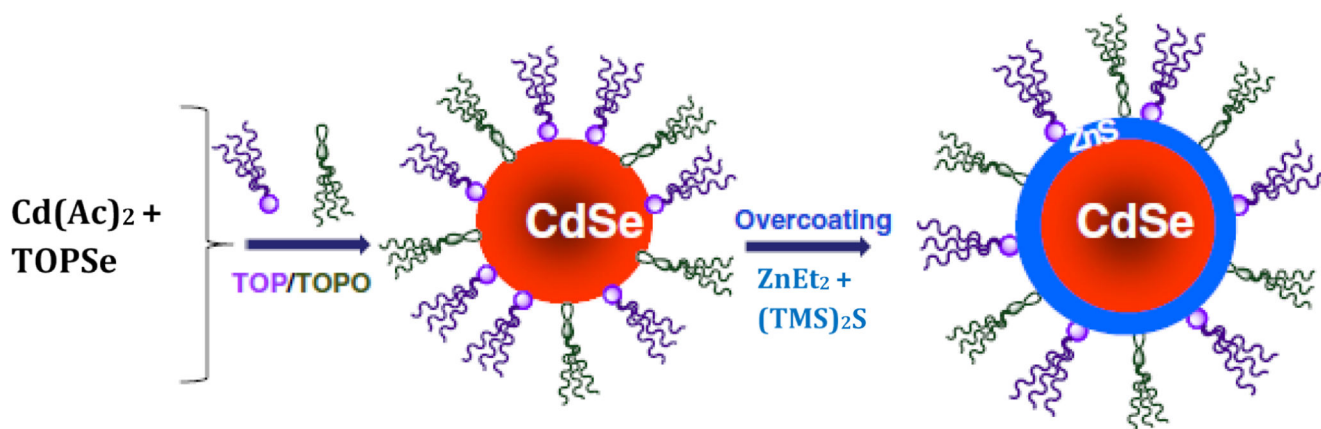
Figure 3.
Schematic depicting the synthesis of a QD and the addition of a passivating layer, resulting in a "core-shell" structure. Typical reagents reported in current literature are shown. Adapted from [10] with permission from Elsevier.
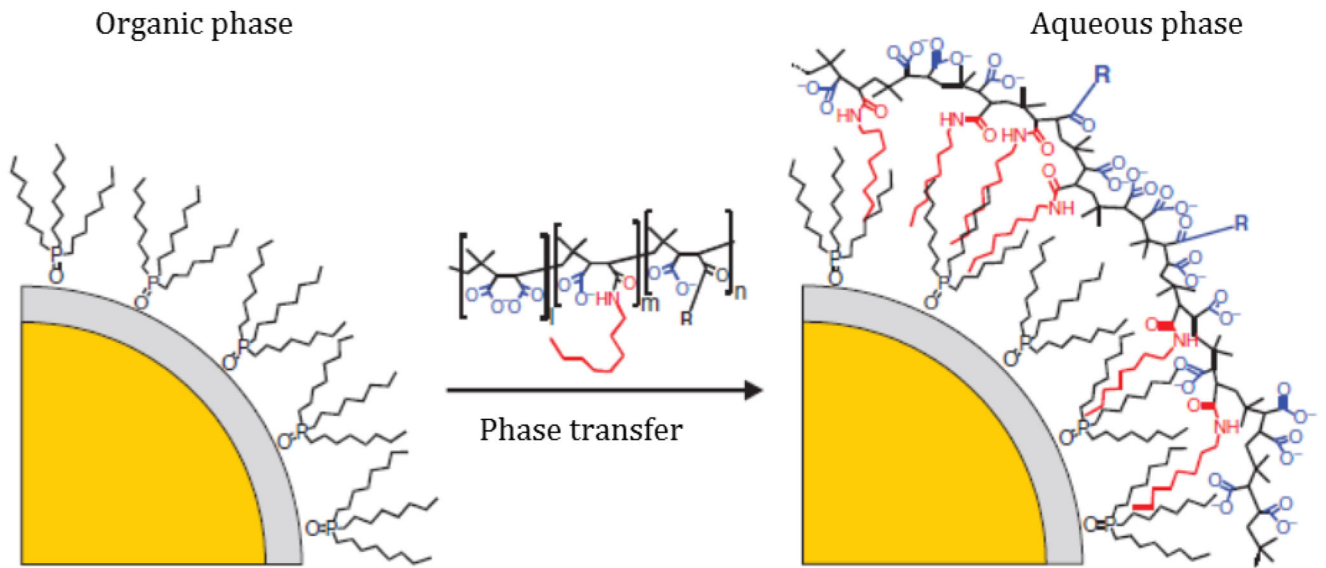
Figure 4.
Polymer encapsulation of a CdSe QD. The polymer is a functionalized amphiphilic anhydride, where R represents a functional group (R= -NH$_2$, -OH, etc.). The hydrophobic ligands interact with the hydrophobic regions of the amphiphile, creating a polymer micelle with colloidal stability and solubility in water. Adapted from [43] with permission from Springer Nature.
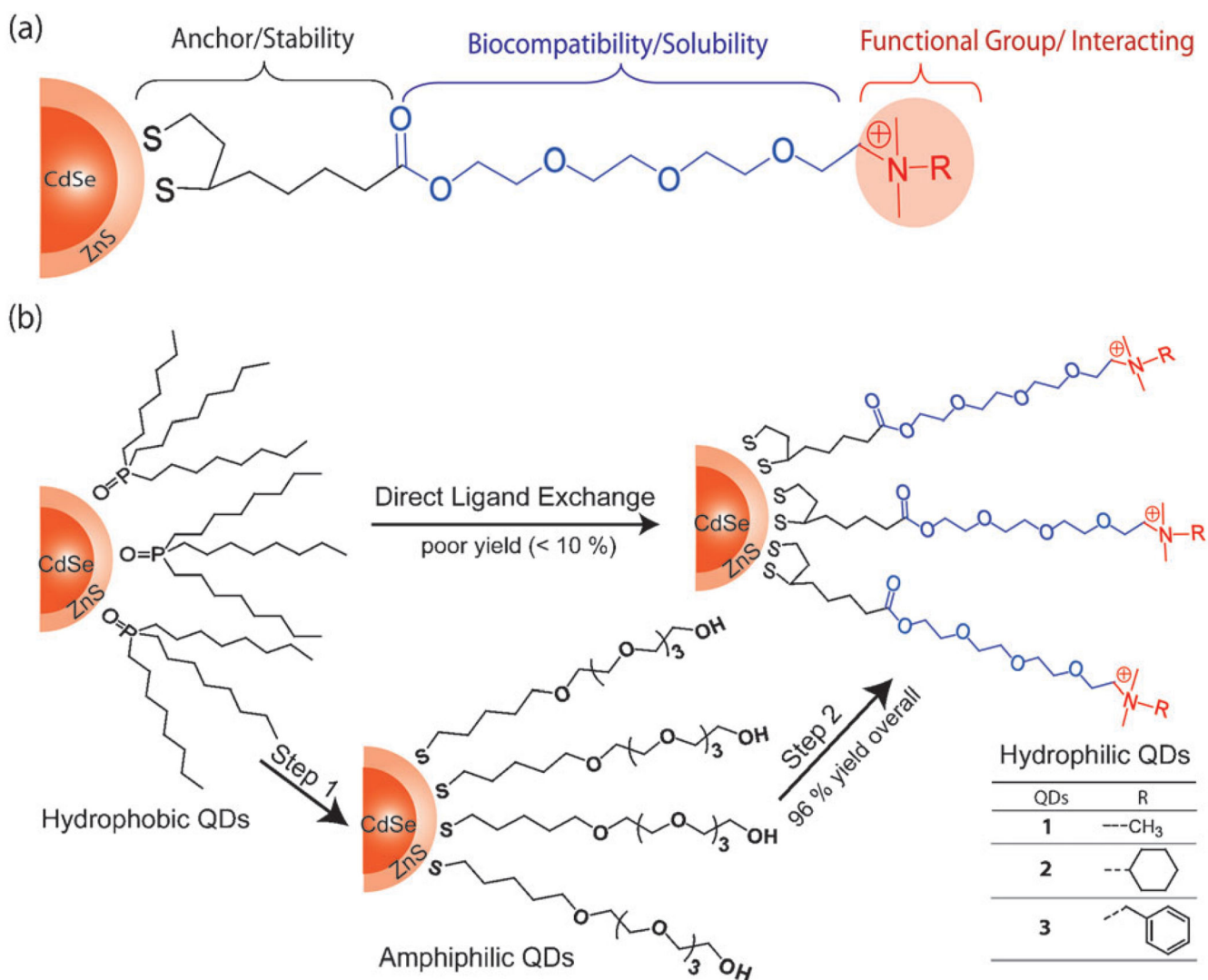
Figure 5.
(A) General structure of DHLA-TEG-quaternary ammonium cationic ligand, where R represents a functional group. (B) Two-step ligand exchange scheme. Step 1: Hydrophobic QDs are converted to amphiphilic QDs by exchange with DHLA capped tetra(ethylene glycol) (TEG) chains. Step 2: Addition of the quaternary ammonium terminus, resulting in hydrophilic, cationic QDs. Adapted from [57] with permission from the Royal Society of Chemistry.
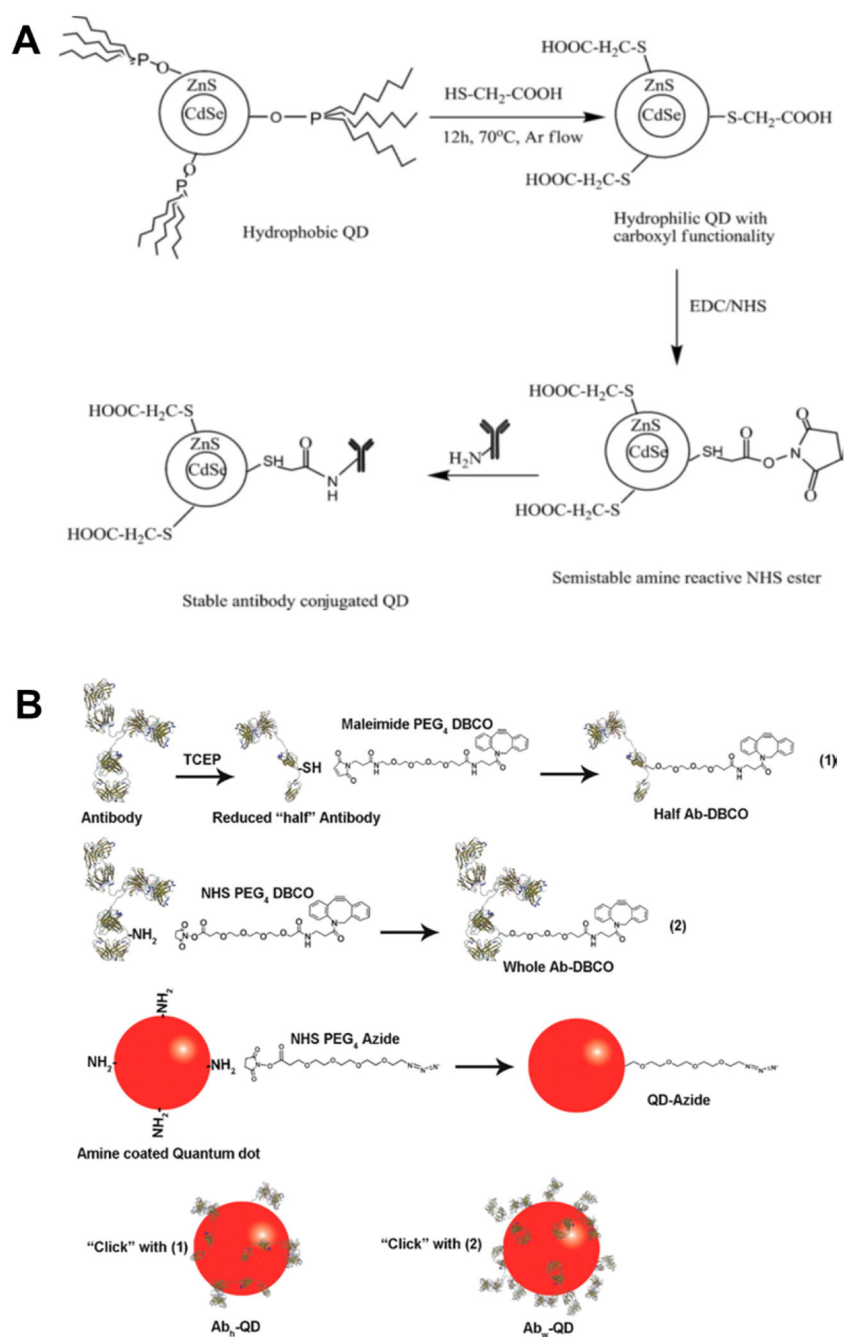
Figure 6.

Conjugation of the QD surface with biomolecules depends heavily upon the ligand chemistries available to provide suitable functional groups for covalent attachment or noncovalent binding. (A) Illustration of phase transfer followed by EDC/NHS carbodiimide chemistry to conjugate a CdSe/ZnS quantum dot with antibody [74]. (B) Schematic of antibody conjugated QD synthesis using copper free cycloaddition 'click' chemistry [221]. Figures adapted from [74] with permission from Elsevier, and [221] with permission from the American Chemical Society.
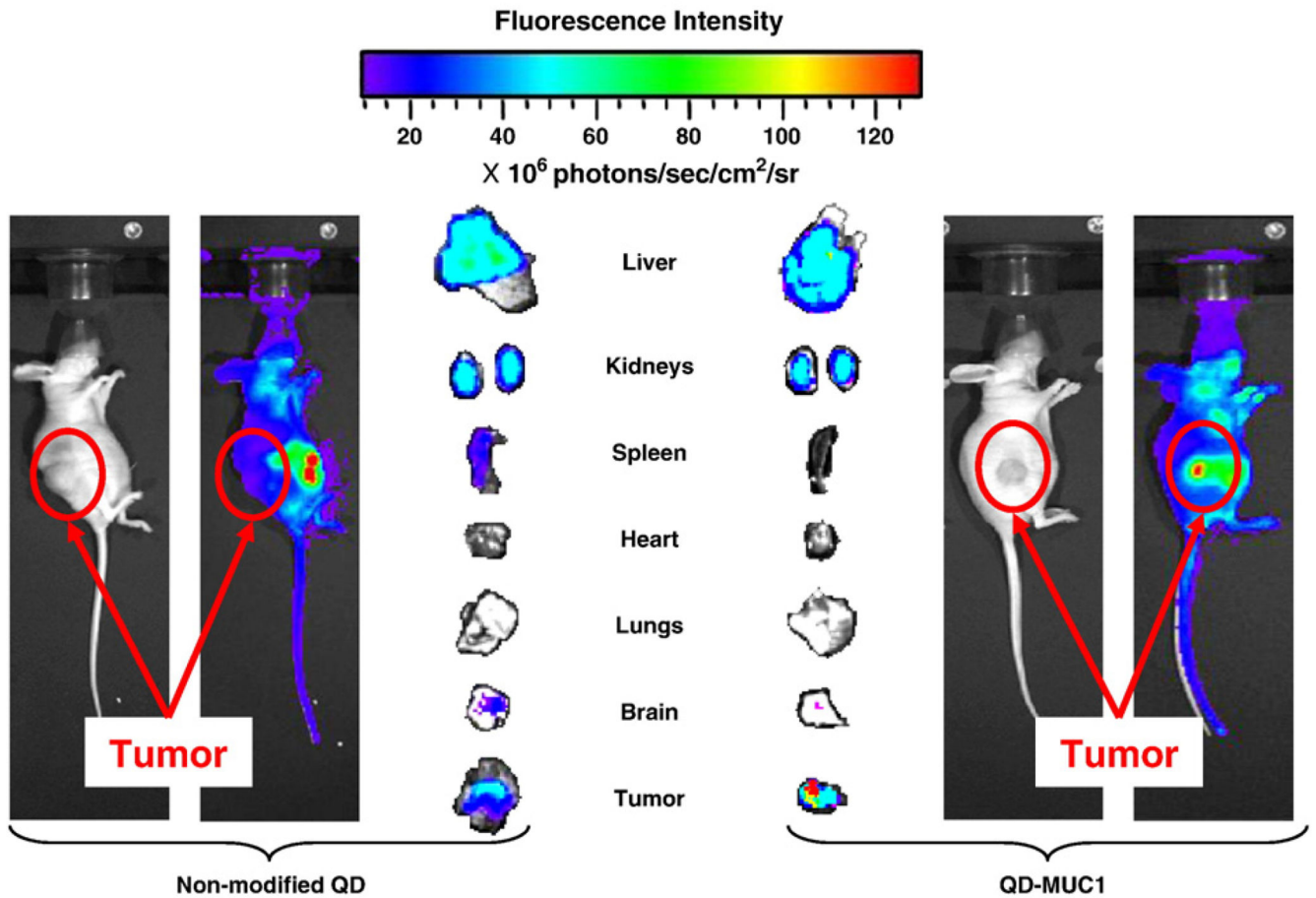
Figure 7.
Real-time *in vivo* imaging of non-targeted versus targeted QD biodistribution in a nude mouse model 24 hours after intravenous injection of QDs. Fluorescence intensity reflects accumulation of QDs; blue represents lowest intensity and red represents highest. Targeted QDs showed greater accumulation at the tumor site than non-targeted QDs. Adapted from [60] with permission from Elsevier.
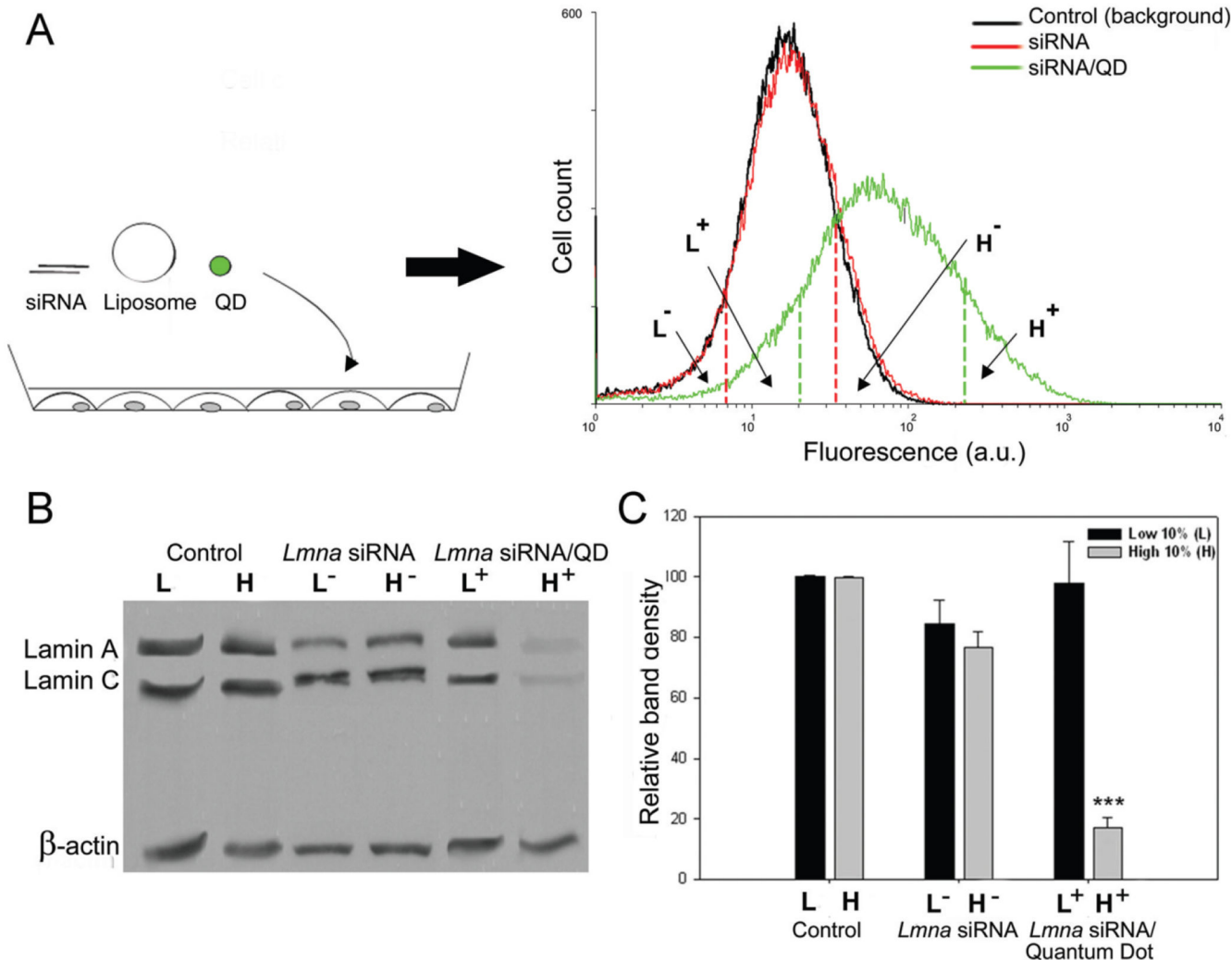
Figure 8.
Cd/Se core-Zn/S shell QD/siRNA complexes enable sorting of transfected cell populations. (A) A schematic representing the transfection of fibroblast cells with liposome/siRNA/QD complexes. Fluorescence was measured using flow cytometry, and the histograms show control cells, siRNA-treated cells, and QD/siRNA-treated cells. (B) Western blot of Lamin A/C expression after transfection (β-actin as loading control). Cells with a high level siRNA/QD fluorescence show knockdown of the Lamin genes. (C) Band densitometry analysis of the western blots. Adapted from [215] with permission from Oxford University Press.

Table 1.

Quantum dots available commercially from companies in the U.S.

Company	Product Line	Composition	Excitation (nm)	Emission (nm)	Solvent	Formulation	Product Notes
Cromoz	CDot20				water	dry powder	conjugated with cancer therapeutic agents
	CDot10					dry powder	
Crystalplex	Trilite ™	CdSeS		490-665	water	solution	composition rather than size dependent; carboxyl, amine, hydroxyl, alkyl functionalized surface
Nanoaxis	AxiCad ™	CdTe/ZnTe	500-700	530-740	water	dry powder or solution	conjugated with antibody and peptide
NN-Labs, LLC	Line 10	CdTe	620	640	organic	solution	photovoltaic application
	Line 9	InP/ZnS		530-650	nonpolar (chloroform, hexane, toluene)	solution	heavy metal free
	Line 4	CdSe/ZnS	520-640	530-670	organic	solution	
	Line 3	CdSe/ZnS	520-640	530-660	water	solution	
	Line 2	CdSe	480-640	485-670	organic	solution	
	Line 10	CdS	360-460	365-490	organic	solution	
NanoTech Ocean	QSP	CdSe/ZnS		520-630	nonpolar (chloroform, hexane, toluene)	dry powder	electronic grade
Sigma-Aldrich	Lumidot ™	CdS		380-480	toluene	solution	
	Lumidot ™	Cd/Se		480-640	toluene	solution	
Sun Innovations	PhosphorDots ™	doped Yttrium	300-980	486-650	aqueous or organic	solution	particularly suited for life sciences; NIR

Table 2.

Comparative analysis of quantum dot properties versus conventional organic fluorophores in biomedical applications.

Property	Quantum Dots	Conventional Organic Fluorophores
Size	Tunable (2-10 nm)	Typically <0.5 nm (protein or small molecule)
Absorption Spectra	Broad, discrete bands	Narrow
Emission Spectra	Narrow (symmetric, size-tunable)	Broad (asymmetric with red-shift tail)
Molar Absorptivity (ε)	Large (typically on the order of 0.5 to 5×10^6 cm^{-1}M^{-1})	Small (typically on the order of 0.5 to 2.5×10^5 cm^{-1}M^{-1})
Emission Spectra	Narrow (symmetric, size-tunable)	Broad (asymmetric with red-shift tail)
Quantum Yield	High (0.1-0.8 in visible range and 0.2-0.7 in NIR)	Low (0.5-1.0 in visible range and 0.05-0.25 in NIR)
Fluorescence Excited State Lifetime	Long (10 to 100 nanoseconds with multi-exponential decay)	Short (< 10 nanoseconds with mono-exponential decay)
Two-Photon Cross Section	Large (typically on the order of 0.2 to 5×10^{-46} cm^4 sec photon^{-1})	Small (typically on the order of 1×10^{-49} cm^4 sec photon^{-1})
Detection Sensitivity	High	Low
Resistance to Photo-bleaching	High	Low
Aqueous Solubility	Low (need for surface coating or functionalization)	High (control via chemistry)
Tissue Penetration Depth	Medium (size dependent)	High
Spectral Multiplexing Capability	High	Low (spectral overlap)
Quantification Capability	High	Low
Toxicity	Potential for accumulation and heavy metal leaching, typically slow metabolism	Chemistry dependent, typically rapid metabolism
Key Strengths	Ease of spectra tunability	Typically stable, water-soluble materials
	Quantum yield (up to 100 times brighter)	Ease of bioconjugation (many available commercially)
	High photostability	Deep tissue penetration
	Potential toxicity *in vivo*	Lack of tunability
Key Challenges	Poor aqueous solubility and stability	Prone to photo-bleaching
	Difficult to alter biodistribution	More difficult to enable *in vivo* multiplex imaging