Expert Opinion

Quantum dots: a powerful tool for understanding the intricacies of nanoparticle-mediated drug delivery

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Nanoparticle-mediated drug delivery (NMDD) is an emerging research area that seeks to address many of the pharmacokinetic issues encountered with traditional systemically administered drug therapies. Although the field is still in its infancy, recent research has already highlighted the potential for improved drug delivery and targeted therapeutics; however, the real promise lies in combining drug therapy with diagnostic imaging, nucleic acid delivery/gene therapy and/or biosensing applications all in one engineered nanoparticle vector. In this review, the authors discuss the unique contributions that luminescent semiconductor nanocrystals or quantum dots (QDs) offer for NMDD, how they can function as a powerful nanoscale platform to understand this process at its most basic level, and even provide drug-related properties in certain circumstances. Selected examples from the current literature are utilized to describe both their potential and the contributions they have already made towards the design and implementation of NMDD vectors. Important related issues such as QD biofunctionalization and toxicity are also discussed. The paper concludes with a perspective of how this field can be expected to develop in the future.

Keywords: biosensor, diagnostics, drug delivery, endocytosis, imaging, nanocrystal, nanoparticle, peptide, pharmacology, quantum dot, semiconductor

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1. Nanoparticle-mediated drug delivery

Although traditional pharmaceutical research has focused on new and improved formulations of drugs to treat disease, most have been designed for relatively few drug delivery methods. Effective doses are limited because most drugs are delivered systemically and cause unwanted toxicity to non-targeted cells and tissues. The rapid metabolism of drugs also limits their effectiveness, which requires multiple doses, leading to decreased patient compliance and increased cost. Emerging advances possible with nanoparticle-mediated drug delivery (NMDD) address some of these issues and may offer many mechanistic advantages over traditional drug therapies [1]. Although this field is still in its infancy, the results of recent research suggest many opportunities, including not only improved drug delivery, specificity and pharmacokinetics, but also combining drug/gene therapy with diagnostic imaging and/or biosensing applications in a single engineered nanoparticle (see **Figure 1**).

Nanoparticles are broadly defined as materials < 100 nm in size, although definitions vary. Technological advances now allow the high-quality synthesis of myriad nanoparticles in this size range from many different materials [2]. For biomedical applications these can be divided into five principal compositional categories:

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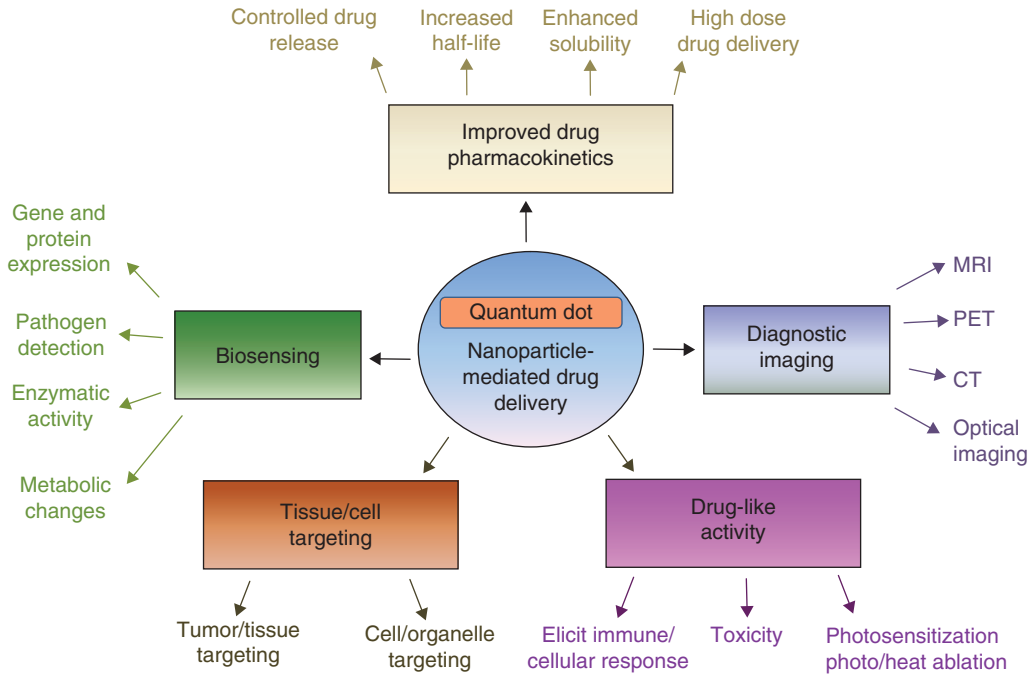

Figure 1. Potential of nanoparticle-mediated drug delivery. Schematic depiction of the potential properties offered by nanoparticle delivery systems. Ideally some or all could be combined into designed nanoparticle vectors. Multimodal nanoparticle systems would combine two or more of the potential functions in one platform. Semiconductor quantum dots with appropriately modified surfaces may be able to provide almost all of these activities; however, they are unique in that their properties can help in understanding the fundamental design, characterization and targeting issues that must first be overcome in creating these systems.
PET: Positron-emission tomography.

i) liposomes; ii) polymers; iii) carbon-based nanomaterials (buckeyballs/fullerenes, carbon nanotubes); iv) inorganic metallic or semiconductor nanocrystals; and v) hybrid nanomaterials that constitute a combination of two or more of the above. Although recent surveys show more than three dozen nanotechnology-enabled products on the market, commercialization and investment in new research is still growing at a rapid pace [3]. Examples of nanomedicine products in current use include polyethylene glycol (PEG) polymer coatings on protein therapeutics to reduce immunogenicity and increase plasma half-life, various cancer therapies, the use of colloidal gold for *in vitro* diagnostics of pregnancy, ovulation and HIV detection, magnetic particles for diagnostic cell sorting and iron oxide nanoparticles as a contrast agent for magnetic resonance imaging (MRI) [3]. However, about three-quarters of current NMDD research remains in drug delivery applications, with liposomes representing the most advanced in development as they have more optimal biocompatibility [4,5].

The most sought after NMDD advantage is the ability to improve the pharmacokinetic properties of a drug, allowing for controlled release and higher localized drug delivery. The small size of nanoparticles equips them with a high surface area-to-volume ratio (S/V), which means they can be loaded with a large 'cargo' on relatively few particles. Indeed, below 2 nm diameter, the S/V ratio of such nanoscale structures can exceed 50% [6]. By controlling targeting specificity, *in vivo* delivery and release of the drug, fewer doses are needed, thus decreasing cost and increasing patient compliance. Nanoparticles are being engineered with longer systemic half-lifes than existing drug therapies, and with engineered formulations that react to the surrounding environmental temperature or pH changes to induce drug release at the desired time and location [7]. In this way, drugs that were once deemed hard to use because of systemic toxicity issues can be used. A prominent example of this is encapsulating the chemotherapeutic agent doxorubicin in a biodegradable nanoparticle, which mitigates the severe cardiac toxicity associated with systemic dosing while still providing the same therapeutic efficacy [8]. Another intrinsic advantage is the ability to target specific cells and/or tissues. The size of most nanoparticles allows them to penetrate places inaccessible to larger molecules, such as the blood–brain barrier, the gastrointestinal tract and the central nervous system. Nanoparticles may also leach into the lymphatic system, small capillaries and tumor interstitium. Particular cell types can be targeted either passively or semi-actively by exploiting the enhanced permeability and retention (EPR) of tumors, that is, the dense leaky vasculature feeding into tumors [9]. An example of the latter is the chemotherapeutic agent Taxol, which has been paired with a nanoparticle form of albumin in Abraxane, which targets blood extravasation at

the tumor site, allowing for a 50% increase in drug delivery to the tumor [10]. Active targeting involves adding molecules such as antibodies, peptides or other ligands to the nanoparticle surface. These recognize/bind cell-specific proteins or receptors, thus targeting the nanoparticle complex to specific tissues or cell types [9]. This approach is most actively pursued in cancer treatment, as tumor cells often express unique proteins on their membranes that can be exploited for specific ligand, antibody or targeting-peptide binding [3,11-13].

Nanoparticles are also being developed as contrast agents for most forms of clinical/diagnostic imaging, including quantum dots (QDs) for fluorescent/optical imaging, magnetic particles for MRI and computed tomography (CT), and radionuclide-labeled nanoparticles for positron-emission tomography imaging (PET) [14]. Nanoparticle delivery of imaging agents has similar advantages to NMDD as the nanoparticles can be targeted with a large cargo of agent to the imaging region of interest and this can significantly improve contrast. It is also believed that by using nanoparticles that can provide multiple imaging modalities, physicians may be able to get a more complete 'multimodal' image of disease [15]. Concomitant with this, another potential nanoparticle application is biosensing, which, in this context, targets the real-time sensing of disease-related events *in vivo*. As part of imaging/diagnostics, these systems would, for example, give an amplified signal readout of cellular events related to a disease state, monitor treatment progress by reporting over/underexpression of a gene/protein of interest or enzymatic activity, and detect infectious agents, along with alterations in a metabolic or signal transduction pathway [16]. Although all of the above NMDD functionalities are being pursued individually, ultimately, multifunctional particles that combine two or more of these functions as desired in one nanoparticle vector and thus one treatment are the goal. Two complementary design strategies suggest that this may be readily feasible. The first exploits the high nanoparticle S/V ratio for creating mixed-surface multifunctional nanoparticles (our primary focus here), whereas the second relies on hollow nanocapsules designed to carry cargo and are engineered to respond to environmental influences, such as degradation or pH changes [7,17].

Before examining the role of QDs in this field, it is helpful to visualize the properties that an ideal NMDD system would have. These include: i) being chemically and photophysically robust, biocompatible and amenable to chemical conjugation; ii) the capacity to deliver a wide variety of drugs or other agents that are both soluble and insoluble in a large quantity; iii) the nanoparticle would provide unique properties to the system such as fluorescence or magnetism or potentially have drug-like properties of its own; iv) the ability to target the cells/tissue/or disease site of choice and potentially subcellular locations; v) combined therapeutic, imaging and/or biosensing capabilities; vi) allow real-time *in vivo* monitoring; and vii) proven to be non-toxic and cleared from the body in a timely manner to avoid chronic exposure. Although not all properties would be needed for every application, each capability still requires a great deal of the most basic design and engineering effort, let alone the combination of many into one system. It is here that QDs have the most to offer, and this is the focus for the subsequent discussion.

2. Quantum dot properties and bioconjugation

Quantum dot nanocrystals are typically synthesized from binary combinations of a variety of semiconductor materials (ZnS, CdS, CdSe, InP, CdTe, PbS, PbTe) and provide a full range of emission wavelengths from the UV to the near IR. Owing to quantum confinement effects, QDs are characterized by the unrivaled synthetic ability to size-tune their narrow, symmetrical photoluminescence (PL) spectra (~ 25 – 40 nm full-width at half-maximum) as a function of core size, allowing the option of selecting a fluorescent emission (see **Figure 2**) [18-21]. For biological applications, QDs are usually used as core–shell structures as the wider band-gap semiconductor shell material improves fluorescent properties, passivates the core and prevents leaching [18-22]. High-quality QDs are most commonly synthesized in organic solution at high temperatures using pyrophoric precursors and surface-stabilized with hydrophobic organic ligands that lack aqueous solubility [18,22]. For bio-applications, QD surfaces require further modification with a variety of bifunctional surface ligands or caps that simultaneously interact with the QD surface while providing aqueous solubility [20,21]. The two major strategies for accomplishing this are either use of 1-amphiphilic polymers that interdigitate with the native organic surface or 2-wholesale cap-exchange with hydrophilic surface capping ligands by mass action; see **Figure 2D** for a schematic. For many applications, biologicals such as proteins, peptides or DNA must be attached to the QD surface to provide the necessary targeting or biorecognition. QD surface ligands can sometimes provide functional groups, for example carboxyls or amines, which can be chemically coupled to biomolecules [20,21,23]. Thiolated peptides or DNA can also directly interact with the QD surface or, alternatively, the authors have found that polyhistidine appended proteins, peptides and even DNA can be self-assembled to the QDs by coordinating to their surface by means of metal-affinity interactions [20,24-27]. Various commercial QD preparations are also available, some of which are provided preconjugated to biotin/or protein-G, thus allowing binding to cognate biologicals or antibodies [28,29]. The attached biologicals provide the QD conjugates with utility such as the ability to act as a biosensor and bind to targeted (bio)molecules or undergo cellular delivery [20,30]. The primary goal is obviously to retain the full activity of the biologicals once immobilized on the QD surface, although in many cases the chemistries used are heterogenous, resulting in mixed avidity. Pertinent reviews discussing many of these issues are available [18-21,31].

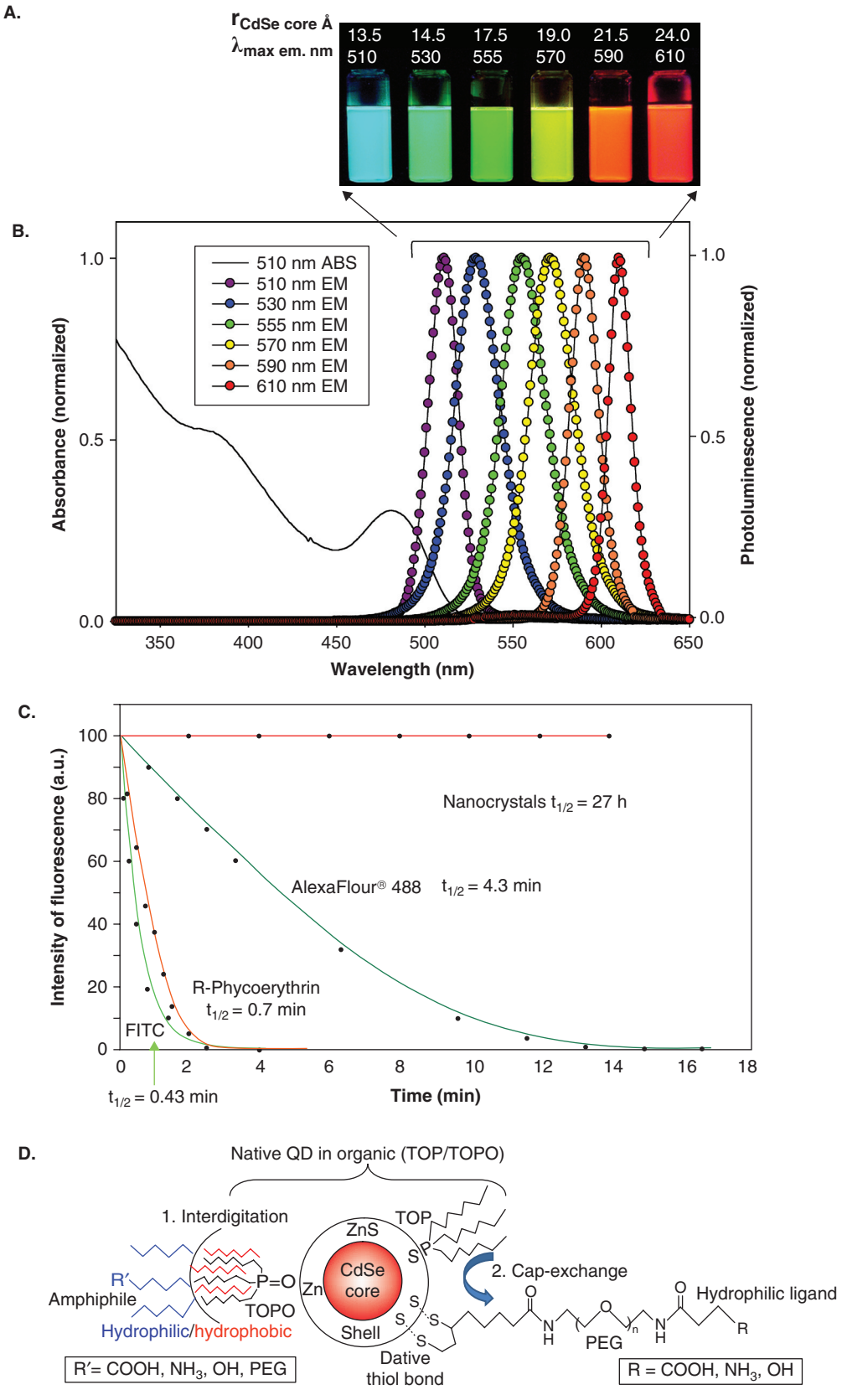

Figure 2. Select quantum dot photophysical properties (continued).

It is the unique QD physical and optical properties that are most relevant to understanding NMDD (see **Table 1** and **Figure 2**). From a photophysical perspective, perhaps the most important property is that they can be easily excited and visualized in the biological environments of cells and tissues. This arises from a combination of several intrinsic properties cumulatively unavailable to conventional organic dye or fluorescent protein fluorophores. Beyond size-tunable PL spectra, these include high molar extinction coefficients (~10 – 100 times that of organic dyes), which are coupled to broad excitation spectra that increase progressively towards the UV, high quantum yields, exceptional resistance to photodegradation, and two-photon action cross-sections that range up to almost two orders of magnitude larger than organic dye molecules (10,000 – 20,000 Goppert Meyer units at 800 nm) [28,32-34]. The latter allows access to the optimal tissue transparency window for fluorescent excitation where there is low background noise from water, proteins and other endogenous fluorophores. A direct consequence of combining these unique absorption/emissive properties is multiplexing; that is, multiple differentially-emissive QD populations can be excited at one wavelength far removed from their individual emissions (large effective Stoke's shift) potentially allowing for the monitoring of many simultaneous events [20,35].

These same properties allow the QDs to function as excellent fluorescence resonance energy transfer (FRET) donors. As compared with conventional fluorophores, QD donors provide unique photonic benefits, including the ability to optimize spectral overlap by size-tuning the QD PL, increasing intra-assembly energy transfer by arraying multiple acceptors around the central QD, which proportionally increases the FRET acceptor cross-section, reduced direct excitation of the acceptor through choice of QD excitation line, and access to both multiplex and multiphoton FRET configurations [34,36-39]. QDs have already been shown to be excellent donors for both organic dye and fluorescent protein acceptors [37,40,41]. These same FRET properties make QDs excellent potential photosensitizers for photodynamic therapy agents [42-45]. From a purely physicochemical perspective, QDs also have relevant properties. They can be prepared with surfaces that present different charges as desired or a variety of biocompatible polymers, including PEG, which is widely used in pharmaceuticals [20,21,23,46]. Combining control of surface chemistry with their high S/V ratios can allow for 'mixed-surface' functionalization, that is, controlled loadings of various different biologicals to the surface. Depending on surface preparation, they can also be resistant to chemical degradation and thus tolerate the sometimes harsh biological environments, for example the highly acidic endo/lysosomal compartments [30,47].

3. Quantum dots in nanoparticle-mediated drug delivery

The following sections highlight examples where QDs have either made direct contributions or alternatively have good potential for utilization in NMDD research. The unique information provided by the QDs in each configuration is discussed along with how this contributes to the overall understanding of NMDD.

3.1 Monitoring nanoparticle cellular uptake and determining intracellular fate

Perhaps the most important factors to understand for successful implementation of NMDD are the mechanisms of nanoparticle cellular delivery; namely, specific targeting, uptake and intracellular fate [48]. QDs have already made a significant contribution to this area, although, interestingly, this was probably not the originally intended purpose. Since the first report that CdSe/Zns core–shell QDs labeled with the iron-transport protein transferrin could bind the transferrin receptor and subsequently undergo cellular uptake [49], many other groups have tested a variety of similar QD cellular targeting strategies. Rather than focusing on understanding NMDD, this was instead motivated by an interest in testing and demonstrating the utility of new QD preparation or bioconjugation methods [50], or, conversely, using QD fluorophores to elucidate aspects of the cellular delivery machinery [51]. **Table 2** lists representative examples where the QDs were conjugated with disparate molecules that facilitated their subsequent cellular targeting, including sugars, vitamins, peptides, aptamers and proteins; their diversity is reflected by their molecular masses, which vary over 2 orders of magnitude from ~ 200 Da for the sugars to ~ 180 kDa

Figure 2. Select quantum dot photophysical properties (continued). A. Photo demonstrating the size-tunable fluorescence properties and spectral range of six QD dispersions versus CdSe core size. All samples were excited at 365 nm with a UV source. For the 610 nm-emitting QDs, this translates into an effective Stokes' shift of ~ 250 nm (r = radius). **B.** Emission spectra of the six different QD dispersions in **A**. The black line shows the absorption of the 510 nm-emitting QDs. **C.** Time-dependent photobleaching curves of QD– and fluorophore–antibody conjugates under continuous high-pressure mercury lamp excitation. Fluorophores include fluorescein isothiocyanate (FITC), the light harvesting complex R-phycoerythrin and AlexaFluor 488 dye. From this experiment, the QD–antibody conjugates were estimated to be 4500 times as photoresistant to photobleaching as the FITC conjugate. **D.** Schematic of the two principal methods for making QDs water soluble. In this example, CdSe-ZnS core–shell QDs are synthesized in organic using trioctyl phosphine/trioctyl phosphine oxide (TOP/TOPO) ligands. The QD is then made water soluble by either 1-interdigitating with amphiphilic polymers whose hydrophobic sections complex with the native surface or 2-cap exchange with hydrophilic ligands; a bidentate-thiol appended polyethylene glycol (PEG) ligand is shown [23]. Both ligands or caps can then be modified further for bioconjugation.
A, B. Reproduced with permission from [20]. **C.** Reproduced with permission from [127].

Table 1. Relevant quantum dot properties of interest.

Property	Relevance to monitoring drug delivery and biosensing	Ref.
Photophysical		
Broad absorption spectra	Effectively excited at wavelengths extending well into the UV	[20,21,31,128]
High multiphoton action cross-sections	Effectively excited deep within tissues without photodamage	[33,34]
Narrow, size-tunable photoluminescent emissions ranging from the UV to near IR	Provides choice in emission wavelength, possibility of multiplexing	[20,21,31,35]
High quantum yields	Bright, easily imaged in cells, tissues	[28,32]
High photostability	Strongly resistant to photobleaching, well-resolved signal	[28,32]
Long excited state lifetime	Access to time-resolved imaging	[38,129]
Effective FRET donors	Allows for photocommunication and biosensing with environment	[38,39]
Effective PDT sensitizers	Can effectively sensitize proximal PDT drugs	[42,44,45]
Physical		
Resistant to chemical degradation	Resistant to harsh intracellular environments	[130]
High surface-to-volume ratios	Can be functionalized with multiple different drugs/sensors/tags	[26,65]
Biocompatible	Can be functionalized and made pH stable with inert PEG molecules	[23,46,109,110]
Bioconjugation	Amenable to drug/protein/peptide/sensor attachment	[26,57,65]
Capable of high avidity	Can be functionalized with multiple replicates of a drug or sensor	[20,26,57,65]
Surface charge can be tuned	Control over net charge and subsequent electrostatic interactions	[23,46]

FRET: Fluorescence resonance energy transfer; PDT: Photodynamic therapy; PEG: Polyethylene glycol.

for the antibodies. This diversity in turn allowed a variety of ligand–receptor interactions to be exploited for QD delivery, ranging from aptamer binding of prostate-specific membrane antigen to Cholera toxin B interactions with ganglioside receptors. In some cases, QDs even allowed uptake to be monitored real time at single cell resolution [51]. Various different QD surface coating strategies, from small dihydrolipoic acid capping molecules [47] to multilayer polymeric encapsulation [51], are also represented in these conjugates.

Examining just these examples cumulatively provides important information about nanoparticles and their subsequent cellular uptake. Despite the diversity of the QD materials and the different cellular interactions, almost all the QD conjugates appear to undergo endocytic uptake and intracellular sequestration in endosomes or vesicles. QD endocytosis also holds true across many different cellular phenotypes, including several different cancer cell lines as well as liver and neural cells. This is not surprising as endocytosis is the primary mechanism by which almost all membrane-bound ligands and nutrients (excluding those that undergo facilitated or active uptake by dedicated transport proteins) are internalized and sorted by cells [48,52]. The only outlier in this list is the dopamine-facilitated uptake of CdSe/ZnS QDs in a dopamine-receptor transfected mouse A-9 cell line. This cellular configuration, for all intents and purposes, is an artificial recombinant construct designed to express a neuronal receptor in a non-neuronal cell line for research applications and thus is not expected to reflect true *in vivo* cellular responses. As many other types of nanoparticle of various sizes are made hydrophilic using similar surface functionalization strategies and are amenable to bioconjugation with the same ligands, it is not unreasonable to expect some similar results when using them. Indeed, cellular delivery of transferrin and TAT-peptide functionalized gold nanoparticles resulted in similar enhanced cellular uptake, confirming this supposition [53,54].

Importantly, these results suggest that should a particular NMDD application require cytoplasmic or specific subcellular organelle targeting, the conjugate/drug cargo may need to bypass endosomal uptake or alternatively manifest extra endosomal escape capabilities. From just these results it is readily apparent that QDs can provide a stable fluorescent platform, allowing the testing of surface functionalization chemistries both *in vitro* and *in vivo*, determination of intracellular fate at single-nanoparticle resolution, along with testing of endosomal escape strategies and NMDD efficacy before utilization with other nanoparticle materials that can be far harder to track if they are not intrinsically fluorescent.

3.2 Monitoring delivery of biologically active cargos

The principal function of nanoparticles (NPs) in this context is the delivery of drugs and other 'drug-like' biologically active cargos, including proteins, peptides and nucleic acids, the latter for gene therapy and RNA silencing in targeted cells. For these purposes, the NPs are required either to display or to encapsulate these cargos during administration, through systemic circulation, or through multiple membrane

Table 2. Examples of targeted intracellular delivery of quantum dots.

QDs functionalized with	Cognate receptor/ligand	Target cells	Fate (cell lines tested in)	Ref.
TAT peptide (< 2 kDa)	Heparin sulfate proteoglycans	Diverse eukaryotic cells	Endosomes (HEK293T/17, 17/COS-1)	[47]
Dopamine (< 1 kDa)	Dopamine receptor	Neural cells or those expressing dopamine receptors	Cytoplasm (Transfected A9)	[131]
Transferrin (~ 80 kDa)	Transferrin receptor	Diverse eukaryotic cells	Endosomes (HeLa)	[49,132]
Epidermal growth factor (EGF, 6 kDa)	EGF receptor*‡	Diverse eukaryotic cells	Endosomes (CHO, A431)	[51,110]
Anti-EGF receptor single domain antibody (< 15 kDa)	EGF receptor*‡	Diverse eukaryotic cells	Membrane-localized/endosomes (SK-BR3, MDA-MB468)	[133]
Cholera toxin B (12 kDa)	Ganglioside receptors	Diverse eukaryotic cells	Endosomes/cytoplasmic vesicles (fibroblast)	[134]
Anti-type I insulin-like growth factor receptor (IGF1R) antibody (~ 180 kDa)	IGF1R*‡	Overexpressed in breast cancer cells	Endosomes/some nuclear localization (MCF-7)	[135]
Folic acid (vitamin B9, ~ 500 Da)	Folate receptor‡	Overexpressed in certain carcinomas	Endosomes/endosomal intermediaries (nasopharyngeal epidermal carcinoma KB cells)	[136]
Nerve growth factor (NGF, 30 kDa)	TrkA receptor	Neurons	Endosomes/along neural processes (neural PC12)	[137,138]
RGD peptide (< 1 kDa)	$\alpha_v\beta_3$-integrins*	Diverse eukaryotic cells	Membrane-localized (Calvaria Osteoblasts, SKOV-3)	[30,139]
Prostate-specific membrane antigen (PSMA)-binding RNA aptamer (18.5 kDa)	PSMA*‡	Prostate cancer cells	Lysosome/endososme (LNCaP)	[57]
D-galactosamine, D-Mannose (~ 180 Da)	Asialoglycoprotein receptor	Liver cells	Endosomes	[111]

*Therapeutic target.
‡Upregulated or altered activity in certain cancers.

interactions both specific and nonspecific, all before final delivery at the required target site, that is, a tumor or infection. The issue of cargo attachment and selective targeted release intracellularly is complex and is concurrently being addressed by multiple biochemical strategies; for discussions, see [55,56]. Beyond mechanisms of NP delivery, QDs are now providing an understanding of many other basic properties of these systems, including NP delivery capacity, specific targeting, conjugate size, fate, intracellular stability and therapeutic efficacy.

In one example, Bagalkot *et al.* assembled a CdSe/ZnS QD-aptamer conjugate capable of targeting specific cancer cells for drug delivery [57]. A DNA aptamer that bound prostate-specific membrane antigen (PSMA) was coupled to amine functionalized QDs and incubated with the anthracycline antineoplastic drug doxorubicin (Dox), which intercalates with double-stranded G-C DNA base pairs. As Dox is also fluorescent it partially quenches the QD PL via FRET and is also self-quenched on DNA binding. The loaded conjugate was then exposed to both PSMA positive and negative prostate adenocarcinoma cell lines and tracking of the QD PL demonstrated significant uptake only in the receptor-expressing cell line. Intracellular delivery of the Dox cargo was signaled by a concomitant increase in both the QD and Dox fluorescence correlating with the reduction of FRET. The targeted cell line was thus fluorescently labeled in two colors (QD and Dox) as a result of this delivery strategy and monitoring cellular viability over time showed a QD–Dox conjugate cytotoxicity equivalent to that of free Dox in solution [57]. In this configuration, the QD provides multiple roles, including nanoscaffold for aptamer and drug attachment, fluorescent marker in the conjugate, and part of the FRET signal in drug release. As mentioned previously, the ability specifically to target delivery of Dox may also significantly mitigate systemic toxicity issues [8].

Wang's group took advantage of a similar FRET signaling mechanism to monitor the intracellular unpacking of CdSe/ZnS QD–DNA complexes [58]. Plasmid DNA coding for green fluorescent protein (GFP) was chemically modified with biotin and bound to streptavidin-functionalized 605 nm-emitting QDs. The plasmid–QD conjugates were then encapsulated with Cy5 dye-labeled chitosan polymers in a nanocomplex, which also established FRET between the QD and dye (see **Figure 3**). The

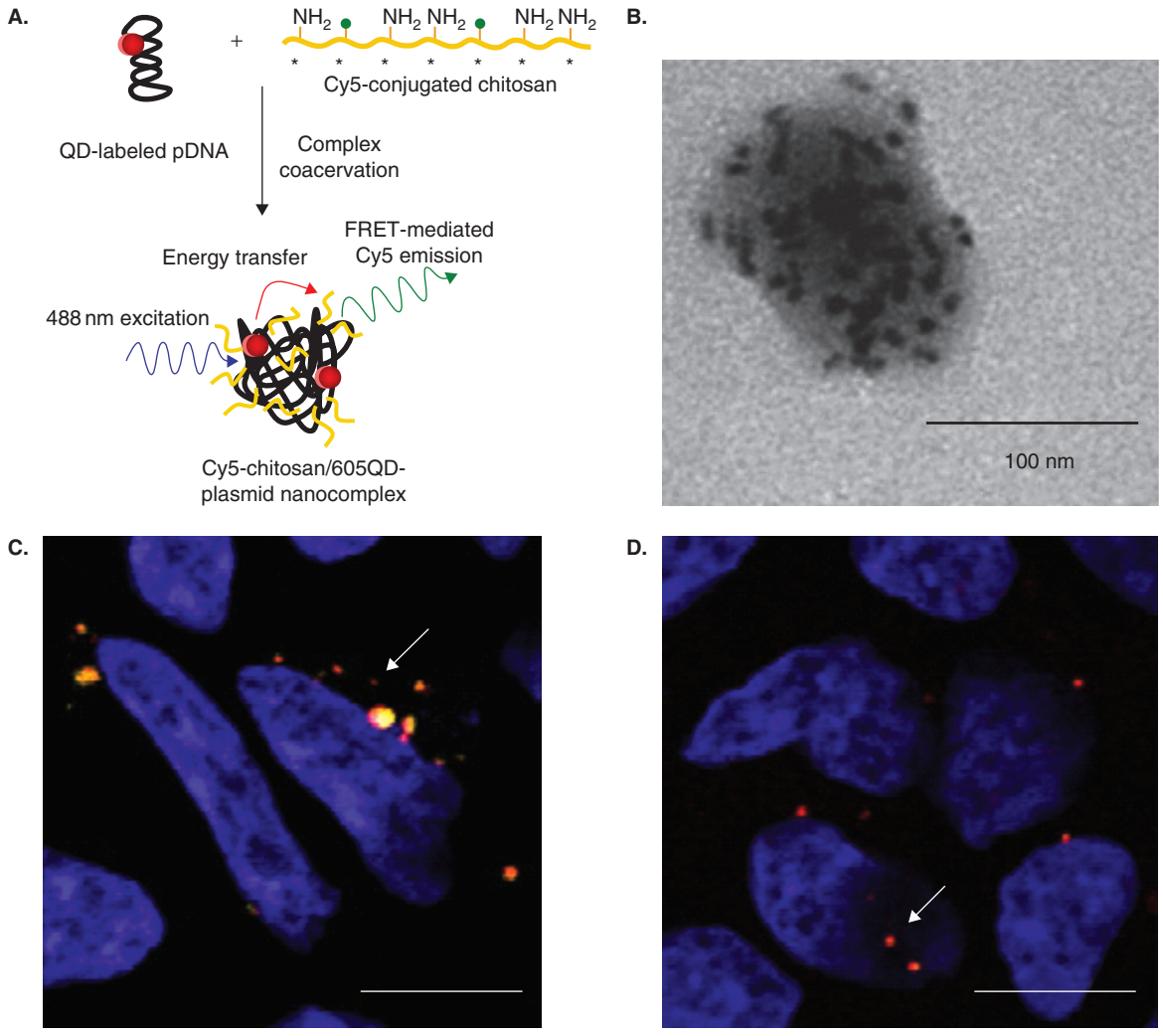

Figure 3. Monitoring intracellular unpacking of QD–DNA complexes. A. Plasmid DNA and chitosan were labeled with 605QD and Cy5, respectively, and allowed to interact forming a FRET complex. On excitation at 488 nm, QD-FRET-mediated Cy5 emission (pseudo-colored green) indicates a compact and intact nanocomplex. **B.** Transmission electron microscope image of a typical nanocomplex with the electrondense QDs conjugated to multiple encapsulated plasmids. **C.** HEK293 cells at 24 h post-transfection. Most nanocomplexes remained intact as indicated by Cy5 emission (yellow/orange). The nuclei are counterstained with DAPI (blue). The onset of QD-labeled DNA release (red) was detected starting at this time point. **D.** At 48 h post-transfection, most nanocomplexes have unpacked and released DNA, as indicated by the QD-only signal (red) in the perinuclear region.

Reproduced with permission from [58].

FRET: Fluorescence resonance energy transfer.

resulting nanocomplexes were delivered to HEK293 cells with the aid of Lipofectamine, a common transfection reagent. DNA unpacking, as monitored by loss of FRET over time, began at 24 h post-transfection and appeared complete within 48 h (**Figure 3C, D**). A relatively low level of GFP expression (< 10%) was found after complete DNA unpacking and this was ascribed to the random nature of the biotin-labeling chemistry, which may have significantly compromised the plasmid viability. A later study using this same FRET mechanism with essentially the same combination of materials demonstrated that use of higher molecular mass chitosan with the QD–DNA complexes resulted in a higher dissociation rate and higher transfection efficiency at the lower pHs found in endosomes [59]. This suggests a possible mechanism for selectively unpacking and delivering plasmid DNA as the endosomal pH drops during its cycling process. Similar GFP expression vectors have been conjugated to more complex phospholipid QD coatings and also provided monitoring of delivery along with high transfection efficiencies, confirming that the linkage chemistry used to form these sensors is an important issue [60]. Overall, the ability to monitor intracellular gene-delivery kinetics in real time will be a powerful tool in developing this technology.

Recently there has also been much work on the development of QDs to deliver and image small-interfering-RNAs (siRNAs)

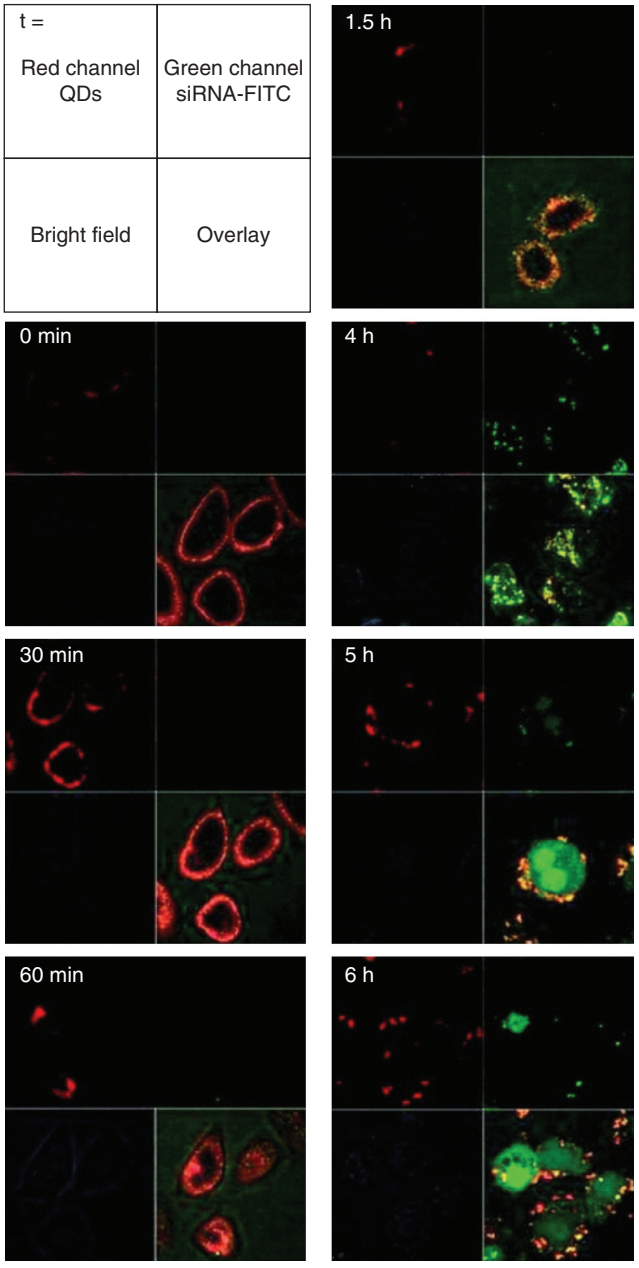

Figure 4. Monitoring intracellular unpacking of QD–siRNA complexes. Time lapse fluorescence imaging of QD–siRNA complexes and their transport in living cells. QD–siRNAs adsorbed onto cell surface immediately after they were added into the cell culture. QD–siRNAs entered cells in < 1 h incubation time. The green fluorescence from FITC-labeled siRNA started to appear at incubation time of 1.5 h, indicating siRNA separation from QD. The green fluorescence increased over time, and at ~ 5 h, the siRNAs were distributed evenly in cells instead of showing a punctuate structure, suggesting efficient endosomal escape.
Reproduced with permission from [64].

for therapies based on RNA interference (RNAi) [61]. Combining siRNA with a targeted NP delivery strategy has the potential to limit the nonspecific cytotoxicity and 'off-target' effects that are associated with systematic siRNA delivery. In one prominent example, Bhatia's group used aminolated CdTe/ZnS QDs as a scaffold to which siRNA and a tumor-homing peptide were attached for delivery to a HeLa cervical cancer cell line expressing GFP as a model protein for knockdown [62]. The siRNA was attached to the QDs by means of a disulfide linker, which was expected to hydrolyze in the highly reducing intracellular environment. The authors demonstrated efficient uptake of the silencing siRNA but tracking of the QD–siRNA conjugates by fluorescence microscopy showed that they were largely sequestered within endosomes. To achieve efficient silencing, further cationic liposomal encapsulation of the conjugate was required as this allowed efficient endosomal escape and eventual GFP knockdown. In another example of targeted siRNA delivery, Tan *et al.* used chitosan NPs with encapsulated CdSe/ZnS QDs for the targeted delivery of siRNA to breast cancer cells [63]. An antihuman epidermal growth factor receptor 2 (HER2) antibody was chemically conjugated to the chitosan for targeting and HER2-specific siRNA was then allowed to complex electrostatically to the chitosan NPs. The fully 'loaded' complexes were then exposed to cell lines expressing the HER2 receptor along with a negative control, and specific targeting and uptake, as monitored by QD fluorescence, was seen only for the receptor-expressing cell line. The authors noted a subsequent two- to three-fold decrease in HER2 receptor expression in the targeted cell line following siRNA delivery. Qi and Gao used a nonspecific approach to deliver siRNA targeted to the same HER2 receptor [64]. They used amphipols (linear polymers of alternating hydrophilic and hydrophobic side chains) to encapsulate the electrostatically assembled CdSe/ZnS QD–siRNA complexes. Analysis with gel electrophoresis allowed the authors to estimate a loading of ~ 10 siRNA per QD. Again by tracking the QDs, they found that the complexes were efficiently taken up by human breast adenocarcinoma cells, underwent endosomal release, and HER2 silencing was achieved with minimal toxicity (see **Figure** 4). Also, the amphipols appeared to provide a protective function to the siRNA by minimizing intracellular enzymatic degradation. It is not clear, however, whether this latter nonspecific delivery strategy would result in delivery to all exposed cells if translated to more complex biological systems or animals. From just these examples, we again see the QD utilized as a central attachment/delivery scaffold and the ability to track the QD conjugates visually contributing directly to iteratively improving overall performance.

The authors have recently evaluated the capacity of CdSe/ZnS QDs to deliver protein cargos intracellularly [65]. For this study, multiple copies of two structurally diverse fluorescent

proteins, the 30 kDa monomeric yellow fluorescent protein (YFP) and the 240 kDa multichromophore b-phycoerythrin light-harvesting complex (b-PE) were attached to QDs using either metal affinity-driven self-assembly or biotin–avidin interactions, respectively, as the b-PE was conjugated to streptavidin. Cellular uptake of these QD–protein complexes was found to be dependent on the additional presence of a self-assembled cell-penetrating peptide (CPP), although it is only a fraction of the size of the fluorescent proteins (< 3 kDa) [47,65]. Despite this size difference, the strong positive charge provided by the polyarginine TAT peptide sequence still facilitated the initial interactions of the QD complex with the cellular membrane for subsequent uptake. On delivery, the QD conjugates were found to be distributed almost exclusively within endolysosomal compartments, as confirmed by counterstaining with the endosomal marker transferrin (see **Figure 5A**). This indicates that intracellular delivery of both QD assemblies was driven primarily by endocytotic uptake, consistent with previous results [47]. Following endosomal uptake, the integrity of the QD–b-PE conjugate was verified by taking advantage of the intrinsic QD resistance to photobleaching. Continuous UV illumination of the intracellular conjugates resulted in a selective bleaching of the b-PE component, but not the QD, in the otherwise completely superimposable emissions, confirming attachment (see **Figure 5B**). As the average number of proteins/peptides assembled per QD in the ensemble was known, the conjugate size and molecular mass of protein cargo delivered per QD was easily estimated. When utilizing ~ 10 YFPs plus a nominal 50 CPPs per QD, intracellular delivery of conjugates with a spatial extension of ~ 150 Å and protein cargos with molecular masses of at least ~ 300 kDa was achieved. The size estimate takes into account both the nanocrystal and protein/peptide dimensions. With an average of 4-Streptavidin-b-PE per QD conjugate, assembly size increases to a molecular mass that potentially exceeds ~ 1.2×10^3 kDa and overall dimensions approaching ~ 500 Å. One important inference from this latter result is that CPP-facilitated endosomal uptake of nanoparticles has a size range and carrying capacity probably far larger than previously anticipated. In this case, the intrinsic fluorescent properties of the protein cargos in combination with the QDs provided a unique tool to estimate the size of a 'cargo', test the ability of NPs to mediate intracellular delivery, and verify cargo delivery along with intracellular conjugate integrity.

3.3 Biosensing

Ultimately, one of the desired functionalities of NMDD is the ability to monitor and report on disease-related or therapeutic events at the site of interest *in vivo*, including, for example, detecting the presence/level of a drug or alternatively the enzyme or metabolic process that is being targeted. Resonance energy transfer-based sensors are now the most powerful and popular technology for real-time fluorescent monitoring of cellular events [66,67]. However, the photophysical liabilities of the dye and fluorescent protein fluorophores used in these sensors, including pH sensitivity, low quantum yields, long maturation times, sensitivity to photobleaching, broad absorption and emission profiles, and so on [68], substantially hinder their utility in this technique. Thus, many researchers are interested in exploiting the unique QD properties for FRET-based sensing [37,39]. The authors, and others, have already demonstrated a variety of FRET-based QD sensors specifically targeted to detecting pH changes [69], HIV-related peptides [70], nucleic acids [25,71,72], nutrient sugars such as glucose [73] or maltose [27] and antibiotics [74]. Enzymatic processes have also been monitored and include the proteolysis of thrombin, caspase-3, collagenase and various other proteases [26,41] along with β-lactamase activity [75]. In almost all these sensing configurations, the central QD is biofunctionalized with an acceptor-dye labeled 'sensing' receptor (protein, peptide, DNA) that samples the local environment. The physical location or photophysical properties of this receptor are altered (i.e., hybridization, displacement or cleavage) when it interacts with a target molecule/process and this forms the basis for FRET signal transduction [37,39]. Many of these sensors already target ligands or processes that are directly pertinent to NMDD. For example, many malignant cells are known to have altered intracellular pH environments along with localized pH changes in the extracellular milieu [76]. Alternatively, cancer extravasation and metastasis exploits the activity of matrix metalloproteinases such as collagenase for tissue remodeling [77].

Rao and co-workers prototyped a sensor for monitoring β-lactamase activity that can be considered emblematic of what these sensors have to offer [75]. β-lactamase (Bla) is an enzyme of bacterial origin that specifically hydrolyzes antibiotic drugs of the structural class that includes penicillin and the cephalosporins. Its overexpression or mutation is primarily responsible for bacterial antibiotic resistance, which is a serious, growing health issue. To target this enzyme, the authors synthesized a Bla-recognized lactam chemical substrate that was labeled at one end by a Cy5 acceptor dye and expressed a biotin at the other terminus. This enabled the self-assembly of this substrate to streptavidin-functionalized 605 nm-emitting CdSe/ZnS QDs (see **Figure 6**). Addition of lactamase enzyme to the QD-substrate solution resulted in changes in FRET efficiency and transduced enzyme activity over time [75]. The authors reported that they needed to iteratively modify the substrate design to express a longer spacer between the QD and the Bla-binding site to provide unhindered enzyme access for optimal function. This highlights how the design of each conjugate may require refining to provide optimal function or accommodate a particular sensing constraint. Nevertheless, the potential of a sensor able to monitor antibiotic resistance *in situ* is clear and directly suggests related utility for monitoring chemotherapeutic resistance in recurrent cancers.

For this application, QDs and their use in FRET-based sensing have been demonstrated almost exclusively *in vitro*. Three reasons principally account for this status. First, much

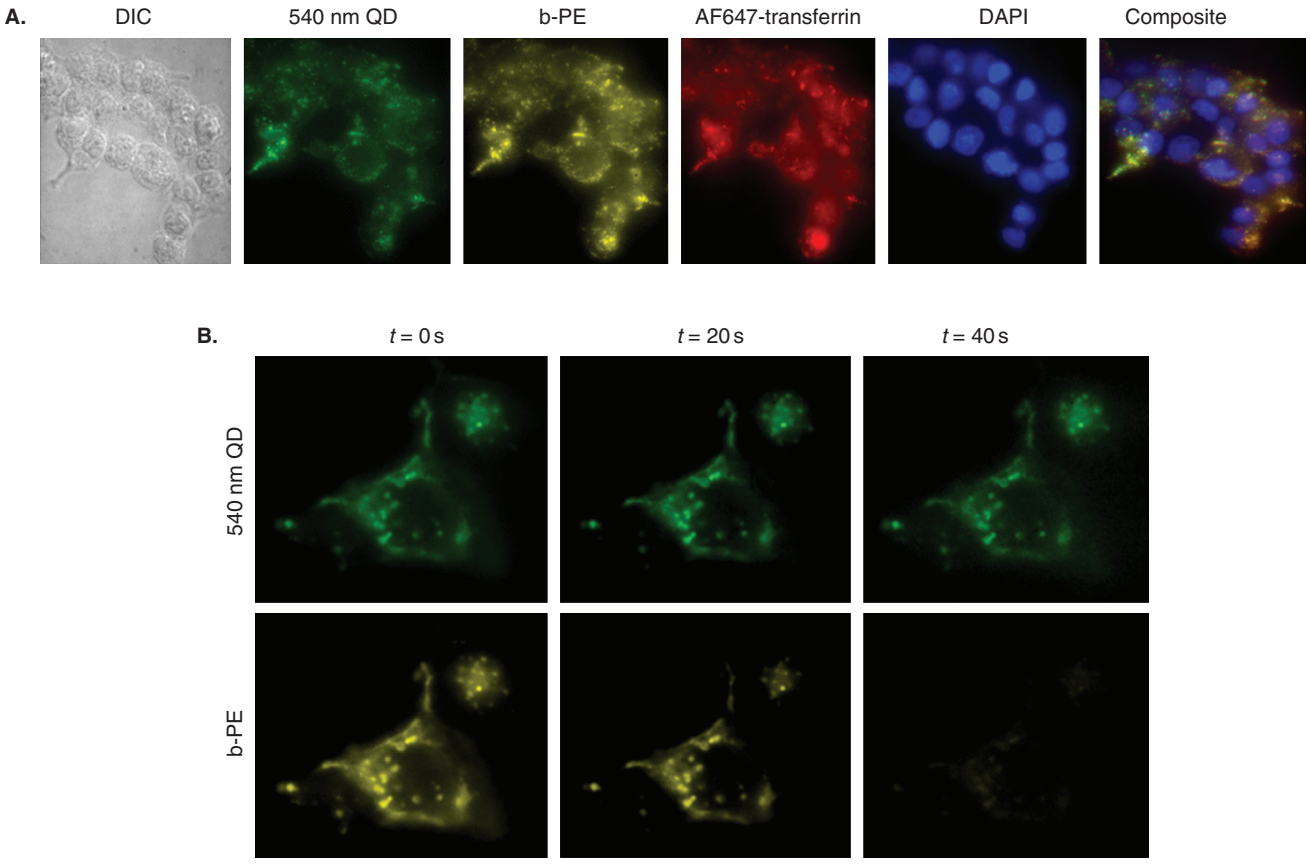

Figure 5. Quantum dot delivery of fluorescent protein cargos. A. Representative images of HEK293/T17 cells incubated with 540 nm-emitting QDs conjugated to both b-PE and cell-penetrating peptides. The corresponding DIC, fluorescence images of 540 nm QD, b-PE, AF647-transferrin, DAPI and merged composite fluorescence images are shown. **B.** HEK293/T17 cells incubated with the same conjugates and continuously illuminated with 488 nm laser excitation. Progression of fluorescence intensity over time collected from the spectrally separated QD and b-PE fluorescence emission channels. Scale bar is 10 μm.
Reproduced with permission from [65]
DIC: Differential interference contrast.

of the initial research was focused on understanding basic QD-FRET processes [38]. Second, considerable effort was then expended in engineering the first generation of *in vitro* sensors. Finally, there are still very few facile methods available for directly delivering sensor-functionalized QDs to targeted tissues or cellular substructures (see below). However, the consensus is that the next major advancement for these materials in this field will be their use in a cellular environment. Thus, FRET sensing based on the unique benefits provided by QDs can be considered to have great potential for NMDD and should soon transition to providing information about how *in vivo* biosensing can be accomplished.

3.4 Photodynamic therapy

Photodynamic therapy (PDT) is based on a photochemical process whereby excitation energy from a photosensitizing agent is transferred to a nearby oxygen molecule generating reactive oxygen species (ROS), which are cytotoxic [42]. These toxic species oxidize and degrade neighboring biomolecules and subcellular structures (e.g., DNA and mitochondrial biomembranes) and ultimately trigger apoptosis and cell death. In principle, this therapy can be highly selective as only targeted diseased cells or tissues simultaneously exposed to the PDT agent and photoexcited by incident laser or UV light in the presence of available oxygen will be treated. Although PDT has become an established chemotherapeutic treatment for several types of topical cancer, conventional PDT agents are sometimes administered systemically leading to low final concentrations in target tissues after biodistribution, tend to be poorly excited owing to low extinction coefficients and have poor solubility in biological media, all of which can mitigate some of the therapeutic benefits [42].

In contrast to just providing an understanding of the mechanism involved, in this case it is believed that QDs can contribute directly to achieving efficient PDT as an integral part of the functional PDT drug complex. Owing to their unique FRET properties, including large absorption cross-sections and some of the highest two-photon action cross-sections available,

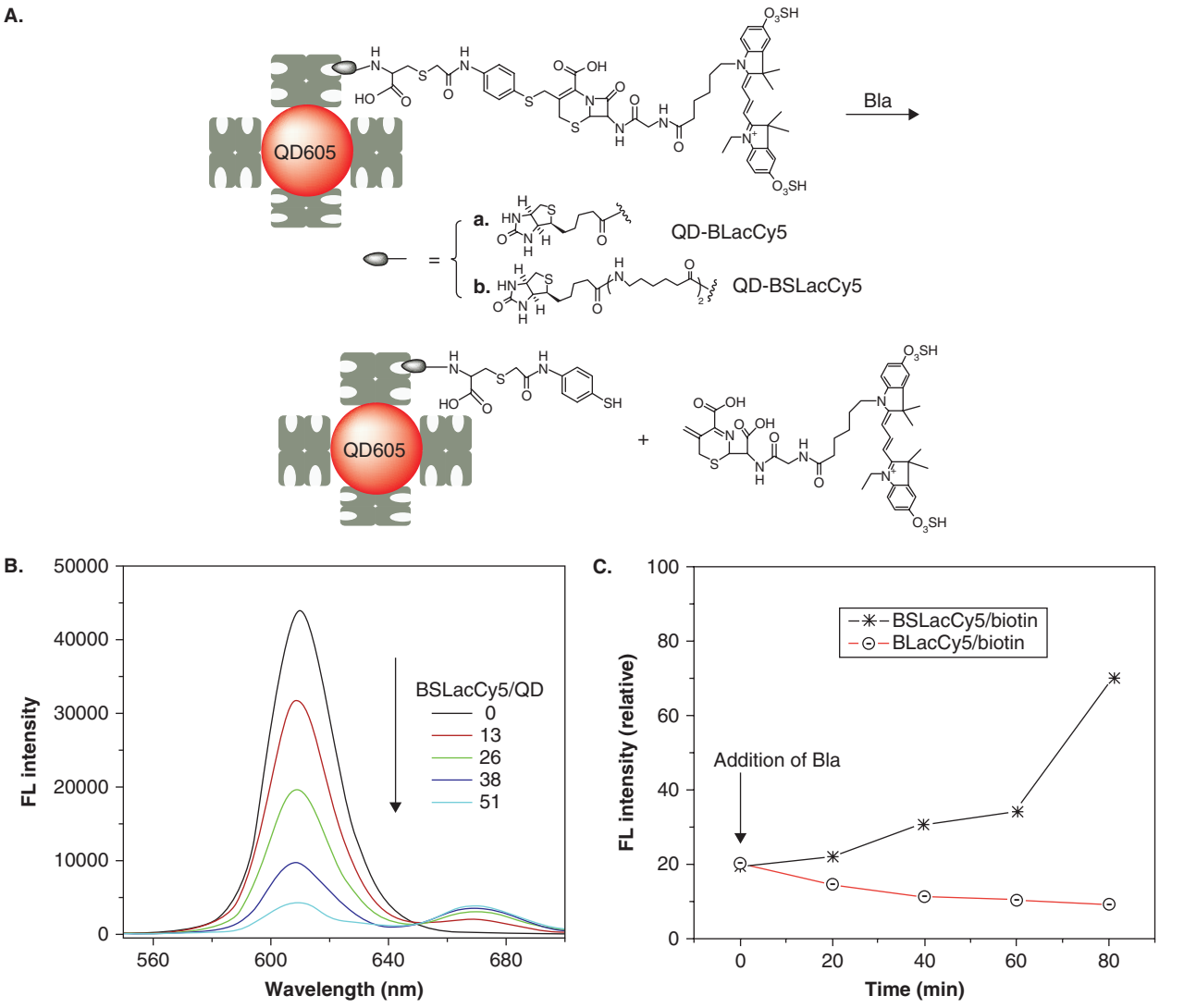

Figure 6. QD-FRET sensor for detecting β-lactamase activity. A. The Bla substrate is labeled with the acceptor Cy5, and immobilized to QD by means of the biotin and streptavidin binding. Bla activity cleaves the lactam ring and releases Cy5 to restore QD fluorescence. BLacCy5 and BSLacCy5 indicate the original substrate and the longer optimized substrate, respectively. **B.** Binding of Cy5-labeled β-lactamase substrate induces FRET quenching of QD emission. The ratios of BSLacCy5/QD605 are as indicated. **C.** Activation of the QD probes by 0.03 mg/ml BLA over time. Note the improved function of the longer substrate.
Reproduced with permission from [75].

QDs can be excited using near-IR multiphoton sources in deep tissues and then efficiently sensitize a proximal PDT agent, thus effectively functioning as the 'energy-harvesting antenna' for this therapy. Interestingly, singlet oxygen is not the only reactive species that can be generated using QDs in an energy transfer-based PDT strategy. Other compounds can induce similar damaging effects, and can also be induced by photosensitization, including nitric oxide species (NOS), ROS, hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), superoxide (O_2^-) and hydroxyl radical (OH), reviewed in [78]. Conjugation with anticancer antibodies or targeting peptides can enhance targeting of the QD-PDT agent complex to specific tumors and enhance localized concentration [42]. With their high S/V ratios, the QDs can accommodate and sensitize several PDT molecules simultaneously, which may also improve therapeutic efficiency.

Several studies have already proved the ability of QDs to act as effective sensitizers and have also demonstrated specific cytotoxic effects. For example, Tsay *et al.* showed that the quantum yield of singlet oxygen was greater when generated from a CdSe/CdS/ZnS multilayered-QD/photosensitizer construct compared with when a photosensitizer was used alone [79]. Neuman *et al.* showed that the coupling of CdSe/ZnS QDs to *trans*-$Cr^{III}(cyclam)(ONO)_2^+$, a PDT agent specific for the conversion of light energy into NOS, greatly increased the production of NOS by an order of

magnitude over the use of the agent alone [80]. Bakalova and co-workers used CdSe core only QDs coupled to an antibody targeted to leukemic cells to demonstrate specific cytotoxicity [81]. After the QD–antibody complexes bound the leukemic cells, the culture was diluted with primary lymphocytes and the addition of trifluorperazine and sulfonated aluminum phthalocyanine, two common PDT agents. Subsequent UV irradiation of the mixed cultures demonstrated that the QD–antibody conjugates specifically sensitized the leukemic cells and resulted in their selective destruction. It was not clear, however, whether FRET from the poorly emissive QD cores enhanced the photosensitization in this case or whether the results were a result of direct toxic effects from the core-only QDs. There are indications that QDs alone are capable of producing free radical species under UV excitation in aqueous solution, and Niemeyer and co-workers have demonstrated that this behavior may potentially be controlled through the choice of the QD material and capping ligand [82]. QD-based PDT may also offer an alternative therapy to the increasingly toxic drugs required to treat antibiotic-resistant bacterial infections. For this application, the QD sensitizes a PDT agent but the reactive radicals generated are used as a light-activated antimicrobial agent. Indeed, preliminary testing of QDs in the presence of the PDT agent toluidine blue O showed enhanced killing of the pathogenic bacteria *Staphylococcus aureus* and *Streptococcus pygenes* [83].

3.5 Quantum dot-based multimodal imaging

Quantum dot fluorescent attributes have already established them as robust fluorophores for long-term cellular and *in vivo* imaging in animal models [32,33,51,84]. The benefits of exploiting this utility for visual tracking of NP conjugates *in vivo* are readily apparent (partially covered in Section 3.1 earlier). QDs can also potentially contribute other useful imaging attributes as part of a multifunctional nanoparticle conjugate. So *et al*. elegantly demonstrated that CdSe/ZnS or CdTe/ZnS QDs coupled to the enzyme luciferase could function as 'self-illuminating' *in vivo* imaging agents [85]. Their designer conjugates consisted of a highly mutated *Renilla reniformis* luciferase (Luc8) optimized for functioning in serum that was chemically coupled to carboxylated QDs. When the luminescent substrate coelenterazine was added to the conjugate solution, Luc8-catalyzed oxidation generated a bioluminescent emission at 480 nm; however, the proximity to the attached QDs resulted in a bioluminescent resonance energy transfer (BRET)-based excitation of the nanocrystals (see **Figure 7A, B**); in essence, an *in situ* chemically catalyzed local photonic excitation of the nanocrystals. They further demonstrated that similar conjugates formed with various different QD colors extending well into the near IR could be injected intramuscularly into mice and the resulting multiplex fluorescence emissions individually visualized with appropriate filters after a second tail-vein injection of the coelenterazine substrate (**Figure 7C**). The ability to track NP fate *in vivo* even in deep tissues without the need for multiphoton or other excitation sources can be enormously beneficial to this field. It is also important to note that this BRET-based imaging configuration is specifically enabled by the unique QD absorption properties and may not be achievable with other fluorophores.

A related realm in which QDs are beginning to contribute is in the development of multimodal imaging agents. This seeks to combine multiple imaging techniques (e.g., fluorescence imaging, MRI, CT and PET) to produce a final imaging product of much greater resolution and informative value than could otherwise be obtained with any single technique alone, each of which has inherent advantages and disadvantages. In particular, multimodal imaging aims to visualize with different levels of spatial resolution and depth using different modalities. For example, fluorescence imaging has submicrometer resolution but is restricted to objects or tissues not thicker than a few millimeters. PET imaging, on the other hand, allows for whole body imaging but with a much lower resolution (several millimeters). The incorporation of QDs into multimodal platforms offers the potential for both high resolution and high sensitivity image acquisition, with the QDs contributing fluorescence and in most cases also functioning as the platform for conjugate formation. A recent report by Duconge *et al*. coupling fluorine-18 (the most common PET imaging agent) to phospholipid-encapsulated QD micelles to generate a combination nuclear-fluorescence imaging agent highlights this approach [86]. Combined PET/fluorescent studies of this untargeted QD conjugate in mice showed prolonged circulation in the blood before uptake by the reticuloendothelial system and allowed further quantitative analysis of the *in vivo* NP distribution at both the whole body and cellular scales. These are exactly the types of studies and pharmacokinetic parameters that will be essential to developing the many facets of NMDD.

QDs have also undergone preliminary evaluation with other imaging modalities. Bakalova's group developed a multimodal probe using both the nanocrystals and the MRI contrast agent gadolinium [87]. The probe incorporated gadolinium complexes into a silica shell surrounding the QD or alternatively conjugated them to the surface of the silica sphere. They found the QD complex to have both high quantum yield and good MRI contrast along with low cytotoxicity. Intravenous administration in rats did not significantly alter any physiological parameters and allowed *in vivo* monitoring. This type of untargeted probe can allow tracing of blood circulation with several modalities, including fluorescence confocal microscopy, multiphoton microscopy and MRI. Cormode *et al*. reported on a slightly different type of fluorescent-MRI probe [88]. They synthesized a high-density lipoprotein (HDL) micellular NP with a CdSe/ZnS QD core using a mixture of gadolinium-labeled and unlabeled phospholipids. The primary apolipoprotein A-I protein constituent was then allowed to absorb into the NP's lipid corona, forming the final HDL mimic.

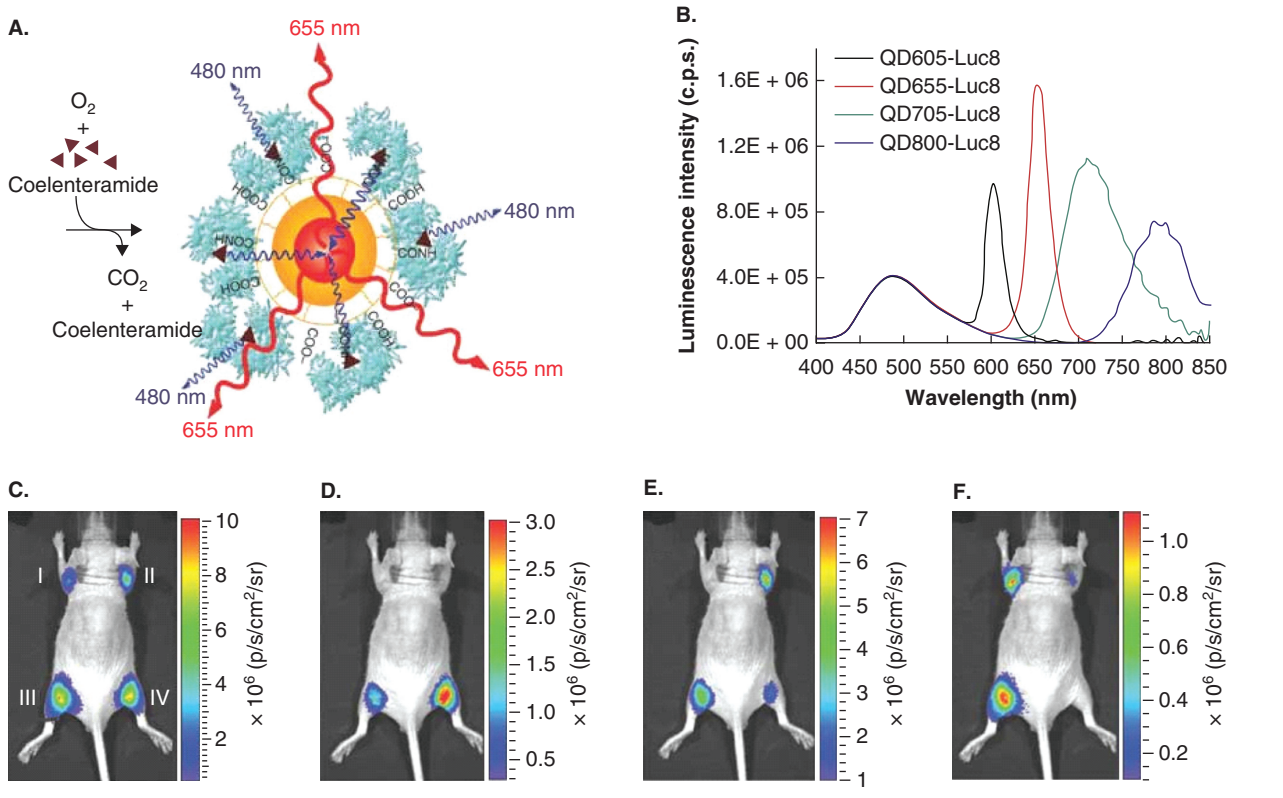

Figure 7. Self-illuminating quantum dot probes. A. Schematic of a QD that is covalently coupled to a BRET donor, Luc8. The bioluminescence energy of Luc8-catalyzed oxidation of coelenterazine is transferred to the QDs, resulting in QD emission. **B.** Bioluminescent emission spectra of QD–Luc8 conjugates with Luc8 emission centered at 480 nm. **C – F.** Multiplexed *in vivo* bioluminescence imaging of the following conjugates intramuscularly injected at the indicated sites: (I) QD800–Luc8, 15 pmol; (II) QD705–Luc8, 15 pmol; (III) a mixture of QD665–Luc8, QD705–Luc8 and QD800–Luc8; and (IV) QD655–Luc8, 5 pmol. Images were collected with the following emission filters: **C** without any filter, **D** with 575 to 650 nm filter, **E** with x-Cy5.5 filter (680 – 720 nm) and **F** with ICG filter (810 – 875 nm). The acquisition time for each image was 30 s.
Reproduced with permission from [85].

Injection of the probe into apoE knockout mice (used as a model for artherosclerosis) allowed both MRI imaging of artherosclerotic plaques and QD fluorescent visualization in aortic sections. Beyond fluorescence, there may also be other types of imaging modality that the QDs themselves can provide. A recent report utilized nanosecond pulsed laser excitation to induce photoacoustic and photothermal bubble formation with CdTe/ZnS QDs in an experimental set up [89]. These phenomena can potentially be used to improve contrast in addition to acting as a sensitizer or as a thermal/ablating agent.

3.6 Nanoparticle-enhanced cellular responses

Several studies have demonstrated the usefulness of QDs for eliciting specific cellular responses, thus illustrating an extra utility for potential *in situ* therapeutic applications. These strategies typically involve the conjugation of QDs to immunogenic peptides or antibodies directed against specific cellular receptors. In one such study, Anikeeva *et al.* used CdSe/ZnS QD constructs to stimulate and understand the mechanisms involved in CD8-dependent T-cell responses [90]. QDs displaying an engineered major histocompatibility complex (MHC) were able to induce a cellular response by binding to T-cell receptors and its co-receptor CD8. T-cell receptors recognize both polymorphic and non-polymorphic peptide structures on MHC class I (MHC-I) and class II (MHC-II) complexes. CD8 is also able to bind non-polymorphic domains of MHC proteins, such that $CD8^+$ populations of T cells interact even more strongly with MHC-I complexes, making CD8 important in the amplification of the T-cell response. The authors developed FRET-based QD nanostructures consisting of QDs conjugated to proximal, dye-labeled MHC-I peptides. Each MHC–peptide complex contained polymorphic peptide domains of either cognate (viral) or non-cognate (self) origin and each complex was self-assembled stoichiometrically onto the QD, allowing the number of complexes per QD to be controlled. In this way, the interaction of MHC-I complexes with T-cell receptors (TCRs) could be observed by microscopy, quantified using flow cytometry, and the subsequent cellular responses measured by calcium flux. By introducing mutations within

the non-polymorphic, CD8-binding domain of both cognate and non-cognate MHC-I complexes, the authors were able to demonstrate that a cooperative binding effect between TCRs is the driving force behind the spread of signal from just a few productively ligated TCRs to many other TCRs (see **Figure 8F**).

Sen *et al*. used a similarly structured CdTe/ZnS QD/antigenic peptide complex to induce a T-cell response by means of another important antigen-presenting cell type: dendritic cells (DCs) [91]. DCs are important in the lymphatic adaptive immune response where they endocytose small molecules from the surrounding tissue. After traveling to the lymph nodes, the DCs present the molecules within the context of an MHC-I or MHC-II complex. The authors were able to deliver and track the QD–peptide construct in real time both *in vitro* and in an *in vivo* model. Twenty-four hours post-injection, DCs that had taken up the QD constructs were localized within lymph nodes and had induced T cells to form stable clusters around them. Peptide-conjugated QDs have also initiated cellular responses in other cell types. Vu and co-workers coupled the homodimeric 26 kDa β subunit of nerve growth factor (βNGF) to QDs and exposed them to PC12 cells, which are used as a model system to understand NGF signaling cascades [92]. βNGF contains all the biological activity arising from NGF and binds with high affinity to the tyrosine kinase A (TrkA) receptor. The authors observed the βNGF-QD binds to cell surface receptors and becomes internalized over a few hours and then triggers a change in phenotype by stimulating neurite outgrowth over 3 – 5 days. Recently, Roberti *et al*. have reported that CdSe/ZnS streptavidin QDs functionalized with the amyloid protein α-synuclein can act as potent inducers of aggregation both *in vitro* and in cells even at nanomolar concentrations [93]. This process may constitute a sensitive model system for studying pathological amyloid formation characteristic of Parkinson's disease and Alzheimer's disease. In these conjugate configurations we see the QD function as more than just a fluorescent marker and nanoscale scaffold. Rather, it is able to present or deliver protein/peptide complexes that stimulate specific responses *in vivo*. It is not hard to speculate that in some of these instances the QD surface is mimicking the localized three-dimensional protein presentation architecture found on a cell membrane. The potential of arraying complex biomolecular structures on NP surfaces such that they elicit a specific cellular response in a targeted cell line or tissue is a relatively new concept in NMDD. Adding this activity as desired into the context of a multifunctional NP conjugate would indeed be an exciting achievement.

4. Toxicity

The field of NMDD does have many major hurdles to overcome. Of primary concern is the characterization of nanoparticle toxicity *in vivo*. Toxicity profiles will vary not only by material, but also by the shape and size of the nanoparticles along with the chemistry and functional modalities attached at their surface [94]. Their small size also allows them to move into small normally inaccessible areas (e.g., the respiratory system) or to be absorbed through the skin, which, although helpful for treatment in some cases, may cause undesired side effects in others. Of particular importance to NMDD is how nanoparticles are metabolized and cleared by the body or whether they aggregate and/or accumulate in the lungs, liver or spleen. The initial interactions and the subsequent short-term biological effects of NPs with cells and in the body will be almost completely dictated by the NP surface chemistry and size. Depending on NP fate and clearance rate, core materials can then become more pertinent over time. Thus, a full understanding of these aspects has to encompass both acute short-term and chronic long-term effects and each NP bioconjugate will need to be tested individually to evaluate its specific toxic effects [95].

Although the focus here has been on using QDs primarily as a tool to understand NMDD, their *in vivo* toxicity is also relevant. The fact that some of their constituent semiconductor materials (cadmium, tellurium, selenium) are toxic is not in dispute; rather, the issue is toxicity in the context of NMDD. It was initially believed that toxicity could arise from either leaching of the materials in the body over time or excitation-induced generation of free radicals in the manner described above for PDT. A consensus of accumulating results suggests, however, that initial QD cytotoxicity appears to be more directly dependent on core–shell structure, surface chemistry and dosage or exposure time. The presence of a core–shell structure can significantly mitigate toxicity arising directly from QD core materials. For example, Cho and co-workers compared the toxicity of CdSe/ZnS core–shell QDs with CdTe core-only QDs in a human breast cancer cell line (MCF-7) [96]. The core–shell QDs capped with cysteamine were found to be non-toxic, whereas the CdTe core-only QDs capped with cysteamine, mercaptopropionic acid, or *N*-acteylcysteine showed toxicities as great as 90% relative to the untreated control. The observed toxicity was caused by reactive oxygen species formed through $Cd2^{+}$-specific cellular pathways. Chemical modification of the QD surface can also significantly impact cytotoxicity. Duan and Nie demonstrated in HeLa cells that CdSe/CdS/ZnS capped with polyethyleneimine (PEI, used for endosomal escape properties) was extremely toxic (~ 10% viability), but this toxicity could be markedly reduced when the PEI was grafted to PEG (~ 90% viability) [97]. Related to this, QD surface charge is also often a determinant of cellular toxicity. Ryman-Rasmussen *et al*. found that commercially available QDs coated with carboxylic acids or PEG-amine both elicited significant toxicity when exposed to primary human epidermal keratinocytes [98]. By contrast, little toxicity was observed for similar PEG-capped QDs. In terms of dosage, Delehanty *et al*. showed that short 1-h exposure of TAT-peptide-conjugated QDs to both HEK and COS-1 cell lines resulted in significant labeling with

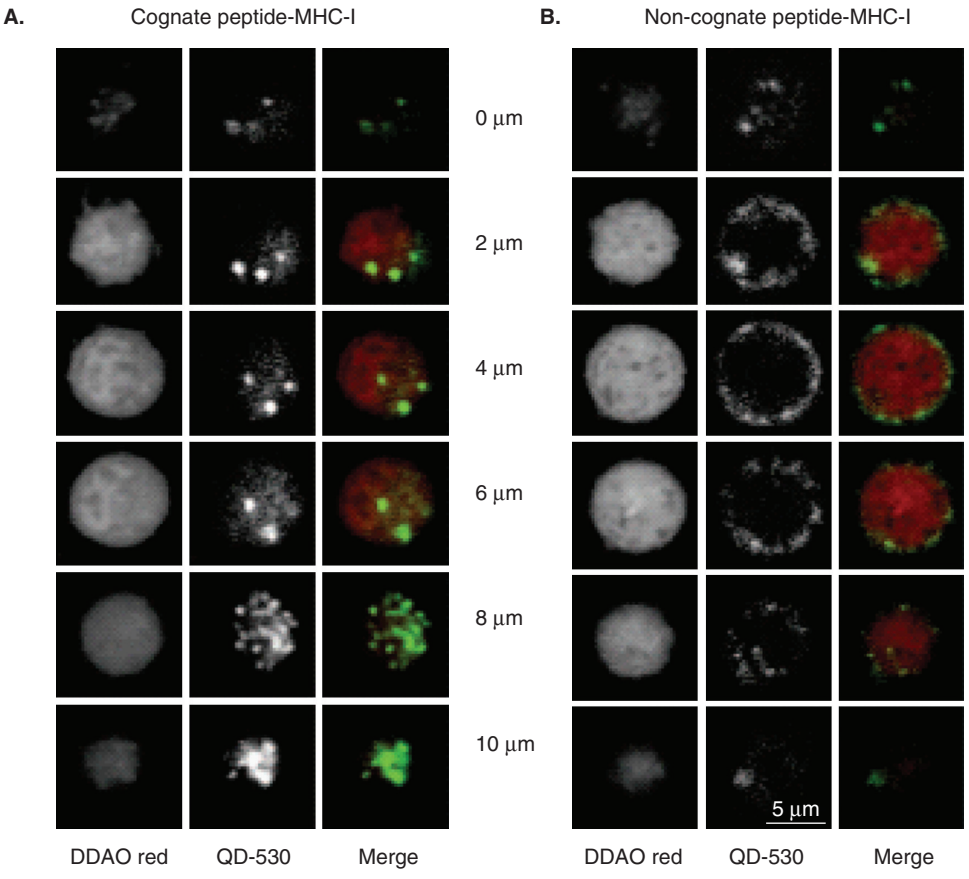

Figure 8. QD/pMHC binding to the surface of live T cells. Binding of cognate (QD/GL9-HLA-A2) **(A)** but not non-cognate (QD/IV9-HLA-A2) **(B)** conjugates to CER43 CTL leads to internalization of the QD conjugates. Images of the distribution of QD conjugates (green) in various z sections of CTL are shown.
Reproduced with permission from [90].

almost no toxicity, regardless of dosage [47]. However, 24-h chronic exposure of the same cell lines to the same QD concentrations decreased cellular viability considerably.

In vivo studies of QD toxicity have focused largely on tissue distribution and clearance of QD materials injected into mice or rats. A repeated finding throughout these studies is that the QD surface coating is often a key determinant of particle distribution and associated toxicity. Ballou *et al.* reported CdSe/ZnS QD deposition in the liver, spleen and bone marrow of BALB/c and athymic nude mice injected with poly(acrylic acid) polymer-coated QDs [99]. They found that QD circulation time could be increased significantly and nonspecific organ accumulation decreased when increasingly longer PEG coatings were added to the QDs. In one of the most important *in vivo* studies of this topic so far, Choi and co-workers systematically determined the requirements for renal filtration and urinary excretion of CdSe/ZnS QDs injected into the rat circulatory system [100]. Inhibiting the adsorption of serum proteins to QDs through the use of either zwitterionic (cysteine) or neutral organic coatings (PEG) kept the particle hydrodynamic diameter (HD) < 15 nm. This is the key size above which renal excretion does not occur. When the final HD of the QDs was tuned to < 5.5 nm, rapid and efficient urinary excretion and elimination from the body was observed, resulting in overall minimal toxicity. Concomitant with this are findings that varying the length of the PEG coating on a QD can change its biodistribution and clearance rates. Choi and co-workers utilized InAs/ZnS near IR QDs capped with PEGylated molecules of varying length and tested their biodistribution in Sprague–Dawley rats following intravenous injection [101]. Increasing PEG chain repeat length from 2 to 22 units resulted in unpredictable targeting to several organs, including the liver, kidney and pancreas. Shorter PEGs retain a more hydrophobic character and are rapidly taken up by the liver, whereas longer more hydrophilic PEGs remain vascularized for longer periods. Bruchez's group has also shown that CdSe/ZnS 655 nm QDs capped with amphilphilic polymers displaying PEG can persist in BALB/c mice for up to 2 years, although the fluorescence is blue-shifted, which is indicative of some surface coating and QD degradation *in vivo* [102].

Surveying just these representative results suggests that QDs can be engineered to elicit minimal toxicity in the context of NMDD through a combination of surface chemistry and control over conjugate size. The use of PEG as a solubilizing and biocompatibility agent borrows directly from a long, well-established pharmaceutical track record, but this too must be chemically tailored for optimal function. Again, as a tool to understand NMDD, QD usage would be focused primarily on cellular culture or small animal *in vivo* systems where understanding targeting, initial cell/organ interactions and drug-delivery pharmacokinetics are the principal focus. The long-term *in vivo* effects of these materials are still almost completely unknown. Paradoxically, QD-elicited cytotoxicity could actually be beneficial, for example, in the context of targeting tumor cells. This would still require engineering the QD conjugates to be both specifically targeted to only those cells and efficiently cleared from the body following use.

5. Expert opinion

It is readily apparent that QDs can enhance our understanding of this burgeoning field. Intrinsic QD properties such as enhanced fluorescent tracking, selective multiphoton excitation in tissues, FRET donor and/or PDT sensitizer (**Table 1**) can be directly exploited to improve our understanding of the mechanistic issues associated with NMDD. These may even expand as further properties such as inherent redox sensitivity along with photoacoustic and photothermal QD effects are fully characterized and exploited [89,103-105]. In almost all the examples examined here, we see similar aspects and utility replicated. The QD most often serves as a central nanoscaffold around which the final conjugate is built. Size, three-dimensional structure and spatial presentation of biological molecules on the QD can provide an impressive 'cargo' carrying capacity or even be combined to elicit specific cellular responses.

The incorporation of QD-based FRET sensing formats into several NP applications may be useful to providing an understanding of fundamental NMDD processes. In one scenario, functionalizing QDs with appropriately labeled fluorescent 'cargos' (drugs, peptides, proteins, gene sequences) affords the unparalleled ability to exploit QD FRET to track both NP location and fate intracellularly while simultaneously monitoring conjugate integrity until the point of dissociation or 'delivery'. This concept has been suggested by others for monitoring gene delivery, in particular [106]. Alternatively, the QD can function as part of a FRET biosensor for monitoring relevant processes such as proteolysis or pH changes. Although not discussed in depth here, such QD sensors have the potential to be designed such that they can be simultaneously tracked in a multiplex manner. This arises from the ability to excite multiple differentially emissive QDs with the same wavelength (**Figure 2A**, **B**) and would allow correlation of complex hierarchal cellular phenomena or monitoring of separate processes or different QD conjugates. Utilizing such sensing systems will certainly provide crucial information on targeting and drug delivery efficacy over time in preliminary cellular and animal model systems and may also spur development of sensitive imaging equipment capable of subtly monitoring multicolor *in vivo* fluorescent signals.

So what can be expected in the near term for QDs and NMDD? The short answer is a lot more basic research, engineering and testing. As incorporating QDs (and most other NPs for that matter) into biological applications is still such a relatively new endeavor, almost all aspects of engineering and applying the conjugates still need significant development. Three areas will continue to receive the most focus in the near future: surface functionalization for hydrophilic applications (water solubility) coupled to bioconjugation, cellular delivery and toxicity. As mentioned already, QDs are made hydrophilic using various different chemical strategies; however, each of these comes with both benefits and limitations. For example, cap exchange with monothiolated ligands results in QDs with short usable half-lifes owing to the dynamic ligand off-rate [107]. Alternatively, encapsulation with amphiphilic or charged triblock copolymers can provide QDs with high quantum yields but the overall penalty in size can preclude *in vivo* clearance or certain FRET applications [100,108]. No single QD functionalization strategy will suffice for all applications; however, ligands attached to the QDs with high affinity that provide 'small'-sized QD conjugates while still maintaining high quantum yield, long usable lifetimes and salt/pH stability are clearly the goal. Third-generation QD ligands combining multidentate thiols (strong QD surface interactions) and PEG molecules (pH and salt stability) appear to address some of these issues [23,109-111] and have even extended the same benefits to other NPs, such as gold [46].

Along with limited functionalization comes a limited set of bioconjugation chemistries. This results primarily from the small set of functional groups or 'handles' available on both the QDs and most biological molecules (usually amines, carboxyls and thiols) along with a need to perform almost all the reactions in aqueous environments. Although amines and carboxyls are ubiquitous on proteins and can be linked by means of carbodiimide chemistry [112], insolubility and crosslinked QD–protein macrostructures are common and often result in precipitation or heterogeneous protein orientation on the QD, which impairs optimal avidity [20,113]. Improved NP and biomolecule chemistries allowing for orthogonal attachment of proteins and other (bio) molecules as desired with control over number and orientation need to be pursued [114]. Beyond what is currently utilized, several established and developing chemistries are available to draw from, including self-assembly driven by polyhistidine or other peptidyl sequences [24,115,116], various chemoselective ligations [117,118], and the family of 'click' cycloaddition and related chemistries [119] to name but a

few. Another exciting prospect is exploiting post-translation modification chemistry in the context of targeting specific designer groups presented on the surface of QDs, and several such approaches have already been demonstrated [120]. Success here will most probably arise from using a combination of chemistries in a selective order to create a multifunctional QD conjugate.

In terms of targeted cellular delivery, the main issue to overcome is QD endosomal escape. Although a variety of endosomal escape peptides and reagents are available [121,122], none seems to consistently provide cytoplasmic delivery of all NPs, let alone just QDs [30]. Clearly a fuller understanding of endocytosis itself may provide some necessary insight. Alternatively, taking advantage of facilitated uptake mechanisms that bypass the endosomes, such as those provided by specific transport proteins (e.g., glucose or amino acid transporters) or specific chemicals such as the hydrophobic counterion pyrenebutyrate [123], may allow direct delivery to the cytoplasm. This is a complex problem that will have to be tackled on an individual basis as it is still not clear how different NP materials, sizes, surfaces, charges, and so on, can alter these processes.

The continuing issue of toxicity is perhaps the most complicated challenge. Several recent reviews have summarized some of the data collected so far for QDs and other types of nanoparticle material [95,124,125]. The main point to be made is that we know far too little about this important issue. Although some generalities can be made about differences between core and core–shell materials or PEG and non-PEGylated coatings (see Section 4), much of the other published data are almost un-comparable because of multiple differences in core–shell materials, coatings, sizes and even shapes. The studies that have appeared often use material concentrations that exceed the projected *in vivo* dose for a particular application anywhere from a hundred to several thousand times. In comparison, almost all the current dyes and probes used in biological research and imaging are toxic to some extent depending upon dosage, yet this is never mentioned as a hindrance to their utility in cellular or small animal studies. Furthermore, most of the cell-based toxicity results reported so far have used immortalized cancer cell lines and thus the observed response (to inappropriate dosages) may not even be realistic. The context for how the QDs will ultimately be used is another critical element of this equation, yet this is hardly discussed appropriately. For example, utilizing the QDs as part of a sensor in a transformed cell-based assay over a 1 – 2 day time span in an experimental environment versus utilizing them as an imaging agent for sentinel lymph node mapping in humans during surgery [84] raises vastly different toxicity concerns. The state of the art for the latter involves injecting a 'hot' radioactive tracer and using a Geiger counter to map the lymphatic pathway, a procedure that would make any potential patient apprehensive. Even within humans, using a targeted probe during localized surgery or for finding malignancies in a late-stage elderly patient versus using a QD probe as a generalized screening agent in a healthy individual are very different scenarios with vastly different associated risk/benefit comparisons. If *in vivo* diagnostic or other drug-like utility in humans are the goal, then the appropriate regulatory bodies (i.e., FDA) will mandate complete and pertinent toxicological studies for that material and, as with all other drugs, a decision will ultimately be made based on efficacy and the risk/benefit potential for that particular application. Well-crafted toxicological studies that examine *all* the pertinent variables are clearly needed, not only for QDs but also for all nanomaterials, and the leadership approach that the National Cancer Institute's Nanocharacterization Laboratory [126] has taken on this issue is an important start in the right direction. These issues and the utility of QDs as a potent tool to understand many of the fundamental molecular processes of NMDD should not be mutually exclusive.

Overall, the improvement of QD solubilizing techniques in conjunction with expanded bioconjugation chemistry, control over cytoplasmic delivery and a better understanding of the toxicology will make these nanocrystals far more useful for biological applications in general. Many of the lessons learned from applying QDs in biology will in turn contribute to the overall understanding of NMDD. Finally, rather than focusing on developing each NMDD-related aspect individually, it is also important to begin testing configurations where the QDs are simultaneously functionalized with poorly soluble drugs or proteins, peptides, imaging agents, sensors, and so on, as part of a mixed-surface multifunctional conjugate. Of all the NP materials now available, it is perhaps only QDs that have all the requisite physical and photonic properties to allow direct testing of all the functionalities ultimately desired in such constructs.

Declaration of interest

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