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Quantum dots as a platform for nanoparticle drug delivery vehicle design

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Abstract

Nanoparticle-based drug delivery (NDD) has emerged as a promising approach to improving upon the efficacy of existing drugs and enabling the development of new therapies. Proof-of-concept studies have demonstrated the potential for NDD systems to simultaneously achieve reduced drug toxicity, improved bio-availability, increased circulation times, controlled drug release, and targeting. However, clinical translation of NDD vehicles with the goal of treating particularly challenging diseases, such as cancer, will require a thorough understanding of how nanoparticle properties influence their fate in biological systems, especially *in vivo*. Consequently, a model system for systematic evaluation of all stages of NDD with high sensitivity, high resolution, and low cost is highly desirable. In theory, this system should maintain the properties and behavior of the original NDD vehicle, while providing mechanisms for monitoring intracellular and systemic nanocarrier distribution, degradation, drug release, and clearance. For such a model system, quantum dots (QDots) offer great potential. QDots feature small size and versatile surface chemistry, allowing their incorporation within virtually any NDD vehicle with minimal effect on overall characteristics, and offer superb optical properties for real-time monitoring of NDD vehicle transport and drug release at both cellular and systemic levels. Though the direct use of QDots for drug delivery remains questionable due to their potential long-term toxicity, the QDot core can be easily replaced with other organic drug carriers or more biocompatible inorganic contrast agents (such as gold and magnetic nanoparticles) by their similar size and surface properties, facilitating translation of well characterized NDD vehicles to the clinic, maintaining NDD imaging capabilities, and potentially providing additional therapeutic functionalities such as photothermal therapy and magneto-transfection. In this review we outline unique features that make QDots an ideal platform for nanocarrier design and discuss how this model has been applied to study NDD vehicle behavior for diverse drug delivery applications.

Keywords

Nanoparticle; Drug delivery vehicle; Nanocarrier; Quantum dot; Fluorescence imaging; Traceable drug delivery; Nanomedicine

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1. Introduction

During the State of the Union address in 1971, President Nixon declared, “The time has come in America where the same kind of concentrated effort that split the atom and took the man to the moon should be turned to conquering this dread disease” [1]. That same year, Nixon signed the National Cancer Act of 1971, allocating an additional $100 million in funds for the discovery and testing of new compounds for cancer treatment, marking the beginning of the “War on Cancer” [2]. Ironically, beating cancer has proven trickier than nuclear physics and rocket science. After forty years and over $200 billion invested, the battle is still uphill [3]. Falling short of the National Cancer Institute’s prediction for a 50% reduction in cancer mortality by 2000 [4], mortality rates are now just 25% lower than their peak in 1991 [5]. Even more troubling is the realization that most improvements can be linked to earlier cancer diagnosis rather than advancements in therapeutics. Indeed, the same clinical treatments applied for over half a century – surgery, radiation therapy, and chemotherapy – have changed little [6, 7]. One of the most frustrating aspects for these therapies is the tendency for a tumor to grow or metastasize following a period of stability or tumor shrinkage. Such a tremendous resistance to treatment comes from the fact that malignant tumors are more than just masses of rapidly proliferating cells, but instead they represent complex tissues with substantial heterogeneity in proliferative, metabolic, immunogenic, angiogenic, and metastatic potential [8]. This phenotypic diversity provides ample opportunity for subpopulations of cancer cells to evade a single therapy and reconstitute a drug-resistant tumor [9]. Thus, the major challenge is to develop more specific and potent classes of therapeutics or therapeutic combinations that can reliably eradicate all cancerous cells within a patient [10].

Nanomedicine holds the key to addressing the challenges of tumor heterogeneity and adaptive resistance to therapy. The premiere advantage of nanocarriers is their potential for multi-functionality, which enables accommodation of drugs, affinity ligands, and imaging moieties within a single nanoparticle (NP) vector to achieve targeted and traceable drug delivery. Recently, there has been an explosion in the development of NDD vehicles composed of lipids, polymers, carbon materials, inorganic nanocrystals, and even hybrid combinations of those materials tailored towards not only dramatically improving pharmacologic properties of existing therapeutics, but also enabling delivery of new classes of potent anti-cancer drugs for gene- and immuno-therapies [11, 12]. Early on, the design of NP drug delivery vehicles was governed by the intrinsically poor pharmacokinetic (PK) properties of conventional chemotherapeutics. Low drug solubility, rapid metabolism and clearance, and most importantly a lack of selectivity regularly lead to therapeutic failure by causing severe systemic toxicity in healthy tissues, thus prohibiting the dose escalation necessary to eliminate tumor cells. Incorporating these drugs into nanocarriers offers exciting opportunities to redefine the PK properties, improving therapeutic efficacy and reducing side effects. Further incorporation of inorganic contrast agents, such as magnetic and gold NPs, within drug nanocarriers offers a unique combination of added therapeutic modality (*e.g.* magnetofection and photothermal therapy) with real-time reporting on the therapeutic efficacy for a theranostics approach to oncology. Initial advances in the development of NDD vehicles have already brought several chemotherapeutic-NP formulations, including Abraxane and Doxil, to the market. Featuring improved water-solubility (thus eliminating the need for toxic organic solvents), increased circulation half-life, and some size-dependent selectivity toward tumors by exploiting passive targeting via the enhanced permeability and retention (EPR) effect, the NP-drug formulations exhibit less toxicity than free drug alone; however, adverse side effects and only modest to no survival benefit achieved by these NPs for a number of clinical indications urge for development of more advanced NDD vehicles [13, 14]. Current research in nanocarrier engineering aims to harness the unique morphological and phenotypic features of cancer to achieve specific

tumor-targeted drug delivery, gain control over intracellular NP uptake and drug release, and, at the same time, avoid non-specific immunogenicity, toxicity, and entrapment by the body's defenses.

The success of developing specific and potent NDD vehicles for a variety of therapeutics heavily relies upon elucidating design guidelines for nanocarrier engineering, which, in turn, requires an in-depth understanding of NP behavior in complex biological systems, especially *in vivo* [15, 16]. In light of this, tracking the biodistribution, intracellular trafficking, and long-term fate of drugs and NP drug delivery vehicles represents a particularly valuable tool for characterizing PK. A number of contrast agents have been developed to facilitate detection of nanocarriers with magnetic resonance imaging (MRI), positron emission tomography (PET), ultrasound imaging, and optical imaging at varying levels of sensitivity and resolution. Pilot studies have already demonstrated that NP physical properties (*e.g.* size and shape) and surface chemistry (*e.g.* hydrophobicity, charge, density and orientation of surface ligands, distribution of ligands with formation of patches, etc.) have a critical impact on nanocarrier bio-distribution, drug delivery and release pathway, and interactions with different components of biological systems (*e.g.* defining the degree of toxicity and immunogenicity), manifesting in the challenge of fine-tuning many parameters simultaneously. Further work in this direction requires a systematic, comprehensive characterization of nanocarrier behavior at levels ranging from intracellular uptake and trafficking to whole-body biodistribution and clearance. Thus, a cost-effective NP platform for reliable and comparable evaluation of various nanocarrier designs at all stages of NP-based drug delivery is essential. In this review, we highlight the potential for fluorescent semiconductor nanocrystals, *quantum dots (QDots)*, to act as a model platform in shaping the intricate design criteria for engineering of NDD vehicles [17]. Complemented by the key advantages of optical imaging (high resolution, high sensitivity, multiplexing, and in particular low cost), QDots feature a combination of small size, versatile surface chemistry, and outstanding optical properties for real-time monitoring of otherwise "invisible" nanocarriers, while at the same time minimizing alterations in nanocarrier properties to authentically capture their behavior in biological systems. Furthermore, despite the potential for release of toxic chemicals (such as cadmium, Cd) [18, 19] and generation of reactive oxygen species [20], properly coated and passivated QDots do not exhibit acute toxicity *in vivo* (as shown in rodents [21] and rhesus monkey [22]), thus allowing short-term NDD vehicle monitoring without non-specific adverse effects (for further discussion on reducing long-term QDot toxicity see ref. [17]). Such properties have enabled use of QDot platform for elucidating mechanisms of NP targeting, intracellular uptake, and trafficking both *in vitro* and *in vivo*, facilitating examination of the nanocarrier behavior in various drug delivery applications and offering exciting opportunities for design of novel nanotherapeutics, such as NP-based antigen delivery vectors for immunotherapy.

2. Quantum dots as a platform for nanocarrier design

Fluorescent semiconductor nanoparticles, quantum dots (QDots), represent a versatile platform for design and engineering of NDD vehicles [14-16, 23]. With QDots, a combination of unique physical, chemical, and optical properties facilitates in-depth study of nanocarrier interactions with biological systems through real-time monitoring of NP biodistribution, intracellular uptake, drug release, and long-term nanocarrier fate (Table 1). At the same time, compact size and compatibility with a variety of surface modification strategies enables substitution of virtually any NP core with a QDot within single-NP drug delivery vehicles, or incorporation of QDot tags within larger multi-component vehicles. As a result, QDot labeled nanocarriers offer a powerful platform for studying the behavior of a diverse set of NDD vehicles, leading design and optimization of physicochemical properties and functionalities tailored to specific drug delivery applications, while permitting

subsequent removal of QDot or core exchange with a different NP of interest (*e.g.* gold NP for photothermal therapy) in nanocarriers without affecting overall physical properties and biological fate.

2.1 Nanoscale dimensions for non-intrusive tagging of drug nanocarriers

Consisting of hundreds to a few thousand atoms, QDots possess an extremely small core size of only 2-10 nm in diameter, which ranks alongside the dimensions of large proteins and renders QDots the smallest platform for NP-based drug delivery vehicle engineering. In fact, only gold and magnetic nanocrystals approach such dimensions, whereas majority of polymeric, viral, and liposomal nanocarriers often measure several tens to hundreds of nanometers in size [24]. The benefits of QDot compactness are three-fold. Being smaller than other inorganic (*e.g.* gold) or organic (*e.g.* condensed DNA) NP cores of interest, QDots can be readily swapped with those cores for the purpose of studying nanocarrier behavior and optimizing nanocarrier properties. At the same time, QDots can be non-intrusively incorporated within larger drug delivery vehicles as tracers for monitoring intracellular trafficking and biodistribution. Finally, individual QDots released from larger carriers can mimic redistribution and eventual clearance of free drug or other NP components.

Theoretically, QDots can model NP cores as small as 2 nm in diameter, providing a foundation for design and testing of ultra-compact nanocarriers capable of rapid elimination from the body via renal clearance [25, 26]. In practice, however, most commonly used nanocarriers are of larger size. Since crystal dimensions directly define optical properties (as discussed later), QDot size cannot be arbitrarily increased to accommodate such discrepancy. Luckily, a number of surface modification methods developed by far offer suitable routes for depositing inert “spacers” on the surface of the QDot core, while preserving optical properties. For example, controlled deposition of a layer of silica provides tunable shell thickness through a straightforward procedure, facilitating achieving desirable NP dimensions [27-29]. This feature ensures consistency between the QDot model and final nanocarrier architecture, enabling complete development and characterization of the nanocarrier based on the QDot platform before substituting it with the NP core of interest.

Larger multi-component carriers, in general, offer greater versatility by accommodating a wider range of materials and providing more space for drug loading and integration of additional functionalities. In particular, liposomes represent a popular drug delivery vehicle due to their capacity to carry diverse cargo and tunable physical properties for achieving stability in blood circulation and on-demand drug release in response to intracellular or external stimuli [30-32]. Furthermore, liposomes are biocompatible, biodegradable, and clinically established, forming a majority of NP-based drug formulations currently on the market for cancer treatment [14]. Yet, the evaluation of liposome interaction with biological systems and, therefore, rational design of physical and chemical properties of this nanocarrier are hampered by the lack of tracing capacity. Linking QDots to the liposome surface [33], loading within the core [34], or embedding into the lipid bilayer [35], offer suitable routes for visualization of the nanocarrier, while exerting minimal to no alterations to the liposome properties (Figure 1). In one example, Al-Jamal *et al.* have investigated the effect of surface charge and composition on tumor penetration and cell uptake of 100 nm liposomes by incorporating QDot tags within the aqueous core [36]. Since QDots were sequestered inside, overall nanocarrier behavior remained unperturbed and was governed by the liposome properties alone. In another example, Sigot *et al.* have proposed a dual-labeling strategy for simultaneous tracing of the liposome envelope (by QDots linked to lipids) and the cargo (by QDots loaded inside) [37], which might provide insights into liposome interaction with cells and kinetics of cargo release and intracellular trafficking.

Polymeric NPs represent another promising drug delivery platform that offers high flexibility in tuning nanocarrier chemical composition and properties. Systematic evaluation of *in vitro* and *in vivo* performance of these drug delivery vehicles is complicated, as there is no single method suitable for the study of biodistribution as well as intracellular uptake. Co-loading QDots along with the cargo within the polymeric mesh can streamline this process by providing readily detectable signal for precise nanocarrier tracing at single-cell to whole-body levels, while keeping the surface properties intact [38-42]. Like with tagging of liposomes, QDots loaded within the polymeric NPs exert virtually no effect on the nanocarrier functions (*e.g.* targeting, intracellular uptake, and cargo release). For example, QDots co-loaded with a model small-molecule drug (MitoTracker dye) within 100 nm biodegradable PLGA NPs enabled nanocarrier tracing, while exhibiting no interference with pH-mediated charge reversal, polymer hydrolysis, cargo release, and drug functionality, as evidenced by successful intracellular delivery of MitoTracker dye and clear mitochondrial staining [38]. Similarly, QDots electrostatically condensed with plasmid DNA and positively charged polymer into quantoplexes showed no interference with the nanocarrier *in vivo* biodistribution and lacked adverse effects on plasmid DNA unpackaging and expression [43].

Setting the limit on the minimal core size of about 2-5 nm, QDots present unique possibility of tracing not only the drug carrier itself, but also monitoring bio-degradation and cargo release kinetics, tissue/tumor penetration, and clearance. A fairly tight fit within the 5.5 nm renal clearance threshold [25] and 5-10 nm blood vessel junctions [44] permits renal excretion and normal extravasation of only individual compact NP cores released from degraded drug delivery vehicles, while keeping intact nanocarriers in circulation and promoting accumulation within tumors via EPR mechanism. At the same time, high size-dependence of diffusive transport through an interstitial space hinders efficient delivery of large complexes to target cells even after accumulation within the tumor, requiring nanocarrier degradation and release of free drug [45, 46]. In this regard, QDots are well-suited for assessing site-specificity and kinetics of drug release, measuring degree of drug penetration within the tumor, and monitoring NP clearance. For example, Wong *et al.* have utilized QDots encapsulated within biodegradable 100-nm delivery vehicles to demonstrate enhanced diffusive transport and improved tumor penetration only upon protease-triggered nanocarrier degradation within the tumor microenvironment (Figure 2) [47].

2.2 Versatile surface chemistry for mimicking of various nanocarrier designs

QDot versatile surface chemistry complements the compact core size in framing a highly adaptable platform for drug nanocarrier engineering. In particular, metal atoms on the QDot surface present suitable anchor points for attachment of a variety of surface ligands via metal coordination chemistry, enabling deposition of application-specific surface coatings, linking of targeting and sensing moieties, and further build-up of additional layers in a manner analogous to that widely applied for surface modification of other inorganic NPs. For example, QDot surface can be directly decorated with monothiol ligands (*e.g.* mercaptoacetic acid, cystein) [48-51], di-thiol ligands (*e.g.* dihydrolipoic acid and its derivatives) [52, 53], polydentate polymers [54, 55], and dendrons [56], producing a wide variety of surface coatings with different shell thickness, stability, charge, and non-fouling properties. In a same way a number of targeting and sensing moieties can be linked to the QDot surface via free sulfhydryl groups or, most commonly, polyhistidine tag, yielding bio-functionality [57-60]. Considering growing interest in incorporation of inorganic NPs within multi-component drug delivery vehicles, technically simple and robust self-assembly governed by metal coordination might become a common method of choice in nanocarrier engineering, making use of QDot model system for comprehensive examination of such formulations. However, care should be taken to account for effect of nanocrystal properties

on the overall binding affinity and geometry of surface coating (*e.g.* in general thiol-mediated binding to gold surface is highly stable in physiological conditions, whereas same binding to semiconductor QDot surface is relatively weak [61]).

Introduction of an adaptor layer in between the QDot nanocrystal surface and the organic coating offers a useful approach to minimizing the material-related influence of the QDot core on the NP structure and properties. Since the adaptor layer, and not the nanocrystal itself, interacts with the surface coating and outside environment, a greater flexibility can be achieved in developing the universal design criteria for nanocarrier engineering independent of the NP (inorganic or organic) core used. One such approach involves formation of a polymerized silica shell on the QDot surface (Figure 3A), which shields the nanocrystal from the environment and enables multitude of chemical modifications developed for silica materials [27, 28, 62, 63]. Notably, engineering of silica-based drug nanocarriers represents one of the most promising directions in the field of nanotherapeutics due to abundance and biocompatibility of this material. As a result, we expect to see a growing number of studies using silica-coated QDots as traceable analogs for exploration of pharmacological properties of silica-based drug delivery vehicles. Alternatively, QDots prepared via organic-phase synthesis and already featuring a stabilizing layer of hydrophobic surfactants associated with the QDot surface [64-68] enable deposition of a wide range of molecules via hydrophobic interactions. Common examples of nanocarrier preparation via this route include encapsulation with amphiphilic molecules, such as polymers (Figure 3B) [69-71] and phospholipids (Figure 3C) [72], which intercalate the QDot surface ligands with hydrophobic hydrocarbon chains and expose hydrophilic functional groups on the interface with aqueous environment. Owing to abundance of hydrophobic small-molecule drugs and NPs, and the unique ability of hydrophobic surfactants to shield the NP core (essentially turning it into a reverse micelle and enabling easy exchange of the cores without affecting overall surface properties), spontaneous encapsulation driven by hydrophobic interactions might become the most resourceful platform for custom design of nanocarrier components.

The versatility of QDot surface chemistry further manifests in the fact that, once the first-layer coating is deposited, linking of subsequent layers or individual ligands is governed by the properties of the first-layer coating, and not the chemical composition of the QDot core itself. This makes a multitude of bioconjugation approaches commonly used in NDD vehicle engineering fully adoptable to QDot model nanocarriers. For example, a variety of methods for covalent bond formation between reactive functional groups on biomolecules and organic coating (*e.g.* primary amines, carboxylic acids, alcohols, and thiols) have been successfully applied for preparation of bio-functional QDots. In particular, many biomolecules contain primary amine groups that can be linked to carboxyl-coated QDots via carbodiimide-mediated amide formation. Alternatively, a wide selection of cross-linking reagents enables conjugation of biomolecules to amine modified QDots, such as active-ester maleimide-mediated amine and sulfhydryl coupling using genetically engineered or endogenously present sulfhydryl groups on proteins [73-75]. Electrostatic interactions between charged QDot coatings and biomolecules have also been utilized for non-covalent deposition of engineered proteins. For example, avidin, a highly positively charged glycoprotein, was deposited on the surface of negatively charged QDots for further conjugation to biotinylated antibodies [76], while chimeric fusion protein was used for indirect coupling of native unmodified immunoglobulin G antibodies [77]. As a result, universal compatibility of QDots with a rich set of surface modification and bio-functionalization strategies commonly employed in NP-based drug delivery vehicle engineering enables accurate modeling and characterization of nearly any nanocarrier, where utilization of adaptor coatings and common self-assembly mechanisms opens access to design of more general vehicles.

2.3 Photo-physical properties for traceable drug delivery and drug release sensing

Besides rendering QDots a compact fluorescent tracer and versatile scaffold for design and optimization of universal surface coatings, nanometer-scale dimensions directly define a set of unique photo-physical properties that make QDots well suited for sensitive real-time tracking of individual nanocarriers and monitoring of drug release (Table 1). Specifically, high-quality monodisperse QDots (*e.g.* CdSe/ZnS core-shell NPs) emit light with high intensity in a narrow spectral range, and the wavelength of emitted light directly depends on the size of the nanoparticle core [62, 78-80]. At the same time, QDots efficiently absorb light over a wide spectrum from UV up to emission wavelength of the particle [81]. Unique combination of these features enables simultaneous imaging and spectral identification of multicolor QDots using single-source excitation (*e.g.* UV lamp), thus providing an exciting opportunity for simultaneous tracking of multiple nanocarriers within the same biological system and direct comparison of their behavior under identical experimental conditions. For example, Kobayashi *et al.* have utilized 5 multicolor QDot tracers (emitting light in the range from 565 to 800 nm) to visualize NP transport through lymphatic system and accumulation within sentinel lymph nodes of 5 distinct lymphatic drainage basins in a mouse [82]. Popovic *et al.* used intravital microscopy and QDots of 3 distinct colors to simultaneously monitor extravasation of 12 nm, 60 nm, and 125 nm nanocarriers within the same vascular network in real time, demonstrating unhindered extravasation and diffusion through extracellular matrix for smaller 12 nm NPs, but not for larger carriers [29]. Delehanty *et al.* have taken advantage of QDot multicolor labeling potential for highlighting different routes of NP intracellular delivery, such as peptide-mediated endocytosis, polymer-based transfection, and cytosolic delivery via microinjection, within the same live cells *in vitro* (Figure 4) [83]. Taken together these studies represent a full range of inquiries, from whole-body biodistribution to single-cell uptake and trafficking, that can be addressed within the framework of multicolor imaging. What remains to be demonstrated through this added functionality is the study of interaction between different nanocarriers and behavior of multicomponent drug delivery vehicles *in vivo*.

Combination of superior brightness and resistance to photo-degradation represents another set of QDot properties highly useful for long-term nanocarrier tracking. While organic fluorophores, commonly used as optical tags for visualization of a variety of drug delivery vehicles, are too dim to enable high-sensitivity detection and get quenched quickly under continuous illumination, thus compromising long-term monitoring, properly passivated and coated QDots retain fluorescence intensity at nearly the same level for more than 30 minutes, allowing reliable imaging of individual NPs [71, 84, 85]. Ample of studies have utilized this property for real-time tracking of membrane-bound receptor diffusion [86, 87], visualization of cargo translocation along microtubules [88, 89], study of receptor-mediated signal transduction and endocytic uptake [90-92], monitoring of NP exchange between cells [93], and visualization of the virus uptake and behavior within living target cells [94], nicely highlighting the power of QDot imaging for comprehensive interrogation of the mechanisms of nanocarrier interactions with cells *in vitro* and *in vivo*. Recent development of nanowire-based single-cell endoscopy [95] opens new exciting opportunities of precise nanocarrier delivery to intracellular compartments and detailed visualization of the nanocarrier behavior with high spatial and temporal resolution via QDot fluorescence.

In vivo imaging and study of nanocarrier biodistribution greatly benefits from the large QDot Stokes (red) shift of up to 300-400 nm [96, 97]. As biological molecules tend to emit most of light within the blue-green spectral range, shifting of QDot emission toward red and near-infrared (NIR) region yields clear contrast between tissue autofluorescence and QDot signal, while still permitting efficient excitation by the blue-green light (Figure 5). Furthermore, substantial absorption and scattering of visible light by biological tissues encourages use of NIR QDots (emitting light within 700-1000 nm range) for *in vivo*

fluorescence imaging [98, 99], warranting recent efforts on synthesis of compact biocompatible NIR QDots [100-102]. Featuring small dimensions along with favorable optical properties and an extremely large Stokes shift, such QDots promise to become a model nanocarrier of choice for biodistribution and real-time intravital tracking studies.

Sensing of the microenvironment might provide essential information necessary to uncover complex changes correlated with nanocarrier cell binding, uptake, and intracellular drug release. At the same time, sensing of the drug concentration within the nanocarrier enables evaluation of the drug loading efficiency and facilitates real-time monitoring of drug release. QDots enable such sensing functionality through fluorescence resonance energy transfer (FRET). Using fluorophore FRET pairs, close-proximity (typically < 10 nm apart) physical interactions between two fluorescently labeled components are detectable by energy transfer from a higher-energy donor to a lower-energy acceptor fluorochrome, which manifests in quenching of the donor and enhancement of the acceptor dye fluorescence. While FRET pairs consisting of organic dyes suffer from instability and photobleaching, QDots have proven to be exceptional donors [103, 104]. As a result, QDot-dye and QDot-quencher pairs have emerged as superior alternatives for dynamic studies of changes in microenvironment [105], nanocarrier degradation [40, 106], and drug unloading [42, 107-109]. Notably, nanocarrier degradation and cargo release directly translates into changes in QDot and organic dye fluorescence levels, thus enabling not only tracking of intracellular drug distribution, but also facilitating real-time measurement of the drug release kinetics.

While favorable optical properties make QDots highly amenable to different types of fluorescence imaging, semiconductor nanocrystalline core itself enables use of alternative imaging modalities tailored toward achieving higher imaging resolution or more accurate quantitative analysis of nanocarrier abundance in a particular sample. High electron density of the QDot core, for example, facilitates detection of individual NPs within the intracellular compartments using transmission electron microscopy (TEM). Interestingly, similar to multi-color fluorescence microscopy, different QDot types can be distinguished by size with TEM [110] and by chemical composition with electron spectroscopy imaging (ESI) [111], enabling multi-NP studies at high resolution (< 200 nm, the diffraction limit of optical microscopy). At the same time, being composed of chemical elements showing low abundance in biological specimens (such as Cd^{2+}), QDots can be detected by elemental analysis. In one study, inductively coupled plasma mass spectrometry (ICP-MS) was used to accurately measure concentration of QDot-containing nanocarriers accumulated within different organs of tumor-bearing mice even in cases when fluorescence-based analysis might be unreliable (*e.g.* due to QDot quenching or shallow depth of imaging) [36].

3. Evaluation of nanocarrier interaction with biological systems

Major interest in engineering of NP-based drug delivery vehicles is driven by the powerful capability of nanocarriers to completely re-define pharmacokinetic properties of virtually any drug, ranging from small-molecule therapeutics to large proteins and DNA plasmids. Encapsulation of the drug within the NP keeps it shielded from the biological environment until the moment of carrier degradation and drug release, thus minimizing non-specific and potentially adverse interactions *en route* to the target. Consequently, all the interactions with biological systems are now governed by the physical and surface properties of the nanocarrier itself. Rich selection of the NP materials, sizes, and surface coatings along with the capability to incorporate a range of targeting moieties and biodegradation mechanisms for controlled drug release provides ample flexibility in NDD vehicle design [12, 14, 23, 46]. At the same time, this flexibility presents a great challenge to elucidate general relationships between nanocarrier properties and its behavior in biological systems in order to define a set of design parameters that could lead further engineering efforts in NDD. To

address this challenge, model QDot nanocarriers are being extensively used for the study of many steps of the NP-based drug delivery, including critical mechanisms of intracellular uptake and trafficking as well as overall *in vivo* biodistribution.

3.1 Visualizing intracellular uptake and trafficking in vitro

Unlike many small-molecule nutrients, signaling agents, and therapeutics, NPs cannot easily cross intact cell membrane and freely diffuse within the crowded intracellular environment. To overcome this barrier, physical disruption of the membrane (*e.g.* with electroporation or microinjection [112-115]) has been successfully employed for cytosolic NP delivery. Direct dispersion of QDots within the cell cytoplasm enables study of intracellular NP trafficking mechanisms mediated by surface ligands as well as evaluation of the organelle-targeting capability of short peptides. For example, NP diffusion within cell nucleus and cytoplasm could be monitored for 30 minutes using QDots injected by nanotubes [114]. Active NP transport through the nuclear pore complex was observed with 25-nm QDots functionalized with nuclear localization sequence (Figure 6A), and QDot decoration with mitochondrial localization sequence led to active transport into the mitochondria (Figure 6B), whereas neither of these organelles was accessible through passive diffusion [113]. Such studies provide a straightforward route for design and evaluation of targeting functionalities in directing trafficking of nanocarriers or released drugs towards specific intracellular compartments.

Physical methods, however, suffer from very low throughput and poor utility for *in vivo* drug delivery needs. Instead, active transport mechanisms mediated by molecular interactions between cells and NPs must be employed for efficient targeting, uptake, and intracellular trafficking of nanocarriers by large cell populations. While a number of natural mechanisms for transport of macromolecules exist, high complexity and selectivity (*e.g.* by size, chemical composition, secondary structure) of these transporters limit their use to only a few specific nanocarrier designs. Dependence of nanocarrier-cell interaction on NP size [116-119], surface charge [120-122], cell cycle [123], and cell phenotype [124] further complicate development of more versatile drug delivery strategies. As discussed previously, QDot model nanocarriers represent a powerful tool for untangling such intricate relationships through consistent evaluation of different parameters on a single NP platform. Furthermore, being well-suited for interrogation of live cells with fluorescence microscopy, QDots enable direct visualization of nanocarrier transport at sub-cellular resolution. For example, taking advantage of QDot compatibility with a variety of surface ligands, Al-Hajaj *et al.* explored the effect of compact negatively-charged (mercaptopropionic acid and dihydrolipoic acid), zwitterionic (cysteine), and positively-charged (cysteamine) coatings on the modes of NP cellular uptake and elimination, assigning predominant role to lipid raft-mediated endocytosis in this process [125]. Zhang *et al.* utilized negatively-charged polymer-coated QDots to systematically study the molecular mechanism of cellular uptake for larger (20-30 nm) NPs [126]. Again, interaction of carboxylic acid groups with lipid rafts, but not with clathrin or caveolae, was found to be responsible for triggering NP endocytosis. In contrast, Jiang *et al.* coated QDots with zwitterionic D-penicillamine and observed clathrin-mediated endocytosis followed by active transport toward perinuclear region and maturation into lysosomes, highlighting all steps of intracellular processing of such nanocarriers [127].

In general, prior studies have demonstrated that most nanocarriers can interact with cells and trigger uptake to some extent regardless of what surface coating is used (with exception of NPs shielded by non-fouling materials, such as poly(ethylene glycol), or PEG, which resist binding to cell surface). However, since the degree of uptake and intracellular processing vary significantly, a myriad of targeting and cell-penetrating moieties has been developed with the focus on improving efficiency and selectivity of nanocarrier uptake [128, 129].

Interestingly, mechanism of cellular uptake of NPs, proteins, and small molecules linked to the same targeting ligands might be completely different, which warrants careful investigation of functionalized NP behavior even for well-studied ligands. For example, through a systematic study of internalization pathway of nanocarriers functionalized with HIV-derived TAT peptide using QDot model, Chen *et al.* demonstrated the primary role of lipid raft-dependent macropinocytosis in NP uptake, whereas individual FITC-tagged peptides entered cells in major part via clathrin-dependent endocytosis [130]. Another study used TAT-conjugated QDots and dynamic confocal imaging to examine different steps of TAT-mediated intracellular processing of nanocarriers, highlighting macropinocytosis assisted by the cytoskeleton, active transport along microtubules toward perinuclear region, and even shedding of NP-containing vesicles from the tips of cell filopodia [131]. In contrast to TAT peptide-mediated delivery, Anas *et al.* have found that clathrin-dependent endocytosis plays a major role in cellular uptake of nanocarriers functionalized with an insect neuropeptide allatostatin [132]. Similarly, other cell-penetrating moieties might employ different mechanisms for cell uptake, requiring systematic comparison of various ligands on the same NP platform, which can be achieved with QDot model nanocarriers. Conveniently, once suitable targeting and cell-penetrating ligands are identified, they can be directly applied to other NP-based drug delivery vehicles and expected to exhibit behavior identical to that of QDot model.

Besides intracellular uptake, endosomal escape represents another obstacle for drug delivery with NP-based vehicles. Since majority of strategies for trespassing cell membrane employ some type of endocytosis, sequestration of nanocarriers within endosomes and subsequent degradation in lysosomes might significantly reduce or completely block delivery of active drug to intracellular space. Therefore, integration of endosome escape mechanisms constitutes a substantial portion of current nanocarrier engineering efforts. Fluorescence imaging with QDots aids in straightforward evaluation of this functionality, as diffuse QDot distribution throughout cell characteristic to cytosolic release can be easily distinguished from the grainy pattern characteristic to QDot sequestration within endosomes. For example, Delehanty *et al.* used this strategy to examine a number of intracellular NP delivery methods, including polymer-mediated and peptide-mediated endocytosis, and identify an amphiphilic peptide capable of QDot cytosolic release over a 48-hour period through endosome membrane destabilization (Figure 7) [129]. Similarly, endosome escape via the proton sponge effect (rupture of the endosome due to build-up of osmotic pressure) has been demonstrated for QDots coated with cationic (*e.g.* polyethylenimine [133]) or zwitterionic [109, 134] polymers capable of absorbing large amounts of protons upon endosome acidification. In addition to evaluation of single-NP systems, QDots have also been used to study intracellular behavior of larger multi-component nanocarriers. In one example, 100 nm biodegradable poly(D,L-lactide-co-glycolide) (PLGA) particles were loaded with QDots as a model cargo. Upon cellular uptake and endosome acidification, PLGA underwent charge reversal, causing endosome membrane destabilization and cytosolic release of QDots [38]. Therefore, QDots have proven to be quite suitable and resourceful for study of the two major obstacles to intracellular drug delivery – cellular uptake and endosomal escape – both for single-NP and larger multi-component nanocarriers.

3.2 Tracing nanocarrier biodistribution in vivo

Complex interactions and barriers encountered *in vivo* impose another set of criteria and constraints for NP-based drug delivery vehicle design. Extending circulation time in blood, achieving targeting and extravasation within areas of interest (*e.g.* tumor), while efficiently evading clearance and uptake by the mononuclear phagocyte system (MPS, previously known as reticulo-endothelial system, or RES) represents a particular challenge and, at the same time, a unique niche for engineering of “smart” nanocarriers. As virtually all nano-

sized particles are recognized as foreign objects by the body's defense and accumulate within MPS-containing organs [135], optimization of surface coatings, which provide an interface and define interactions between the NP core and the environment, becomes of critical importance. In realizing this opportunity, QDots have already demonstrated utility for nanocarrier biodistribution studies as sole imaging agents (when combined with whole-body fluorescence imaging, intravital microscopy, and post-mortem evaluation of tissues and organs [97, 136, 137]) or in combination with other tracers (*e.g.* labels for positron emission tomography and scintigraphic imaging [138, 139]). As such, several studies evaluated the effect of a popular non-fouling coating, PEG, on the NP circulation in blood and clearance by the MPS, noting a general correlation between increasing PEG chain length and extended circulation time, but failing to cease eventual NP sequestration within the liver and spleen [140-142]. Nonetheless, PEG-coated QDots have been successfully used for the study of nanocarrier *in vivo* behavior, in particular for evaluation of targeting functionality.

Pioneering work on *in vivo* NP targeting utilized post-mortem imaging of whole organs or microscopy of tissue sections for evaluation of QDot biodistribution, as shallow imaging depth, poor QDot quality, and significant interference from tissue autofluorescence precluded from whole-animal fluorescence imaging. For example, Akerman *et al.* demonstrated good targeting specificity of two affinity peptides developed through phage display to bind lung endothelium and tumor vasculature by detecting selective accumulation of red and green QDots within respective tissues [143]. Recent studies, in contrast, take advantage of high-quality PEG-coated red and NIR QDots for sensitive *in vivo* imaging along with advanced instrumentation (*e.g.* hyper-spectral imaging and two-photon microscopy) for efficient removal of the background signal. This way, targeting of nanocarriers to integrin $\alpha v\beta 3$ in tumor vasculature by cyclic RGD (arginine-glycine-aspartic acid) peptide [136], to prostate-specific membrane antigen on prostate cancer cells by specific primary antibody [97], to epidermal growth factor receptor (EGFR) of EGFR-overexpressing tumors by epidermal growth factor (EGF) [144, 145], to liver by hyaluronic acid derivatives [146], and to the brain by wheat germ agglutinin [147] has been demonstrated. With further advancements in intravital microscopy and QDot nanocarrier design, real-time observation of all steps of NP-mediated drug delivery process might become accessible at unprecedented detail, providing exciting insights on the interaction of nanocarriers with complex biological systems *in vivo*. Feasibility of this prospect has already been demonstrated by Tada *et al.*, who used high-resolution confocal real-time intravital microscopy to monitor tumor targeting by antibody-conjugated QDots [148]. Over the course of 24 hours post *iv* injection, vascular transport, extravasation, binding to cancer cells, and cellular internalization could be observed at a single-NP level with 30 nm spatial resolution, enabling quantitative determination of the nanocarrier transport kinetics (Figure 8).

4. Study of NP-mediated drug delivery mechanisms

Complete characterization of NDD behavior in biological systems can ultimately predict how they will affect the body, both in terms of therapeutic benefit and adverse effects, ultimately guiding the development of more effective NDD vehicles. QDots, featuring small size, versatile surface chemistry, and excellent optical properties, have emerged as a primary tool for addressing this challenge. In this section, we discuss the recent contributions of QDots for design of NDD vehicles for diverse classes of chemical and biological drugs, placing particular emphasis on dynamic and quantitative studies achievable through QDots alone. Also, we briefly discuss how QDots might be uniquely suited to study nanoparticle-mediated antigen delivery for immunotherapy, an area of growing interest in oncology.

4.1 Small-molecule drug delivery with traceable nanocarriers

Because most chemotherapeutics fail due to poor PK properties, incorporating these drugs within nanocarriers to alter their PK properties presents the most straightforward approach to improving existing cancer treatments. Though nanocarrier localization has been evaluated *in vitro* and *in vivo* using label-free methods, such as high-performance liquid chromatography (HPLC), or relying on the intrinsic fluorescence of some small molecule drugs, these methods are time consuming, and analysis is only possible post mortem [30, 149].

Diverse imaging reagents, including magnetic NPs, gold NPs, radioisotopes, and organic fluorophores, have been used to track NP drug delivery vehicles at both subcellular and systemic levels. Among these contrast agents, QDots have proven increasingly useful for developing advanced small-molecule nanocarriers that achieve targeted and traceable drug delivery, especially for cellular studies [33, 42, 150]. For example, QDots were recently used to evaluate *in vitro* cellular uptake of a complex nanocarrier featuring cancer cell specific folic acid targeting ligand, mesoporous silica core for high drug loading, and PEG-conjugated phospholipids for enhanced NP uptake and improved biocompatibility [151]. Drugs were covalently attached to the nanocarrier via an acid-sensitive linker that destabilized only at intracellular pH, reducing systemic toxicity caused by premature drug release in circulation. Though such multifunctional nanosystems have the potential to improve upon the performance of early chemotherapeutic-NPs several times over, this carrier only yielded modest improvements in cancer-specific cytotoxicity (39.1% increase compared with the commercial formulation). Taking advantage of real-time and quantitative QDot analysis, nanocarrier optimization might be achieved by comparing cellular uptake kinetics across several formulations.

Though covalent conjugation of drug to NP surface enables on-demand drug release using semi-stable linkers, loading hydrophobic drugs onto the surface of porous or solid nanoscaffolds remains difficult, and limits space for incorporating additional functionalities. Thus, a more traditional approach has been to incorporate drugs within the NP core during formulation, reserving the surface for conjugation of targeting or sheathing moieties. For example, liposomes and micelles have been widely used as drug delivery vehicles because of their versatile size, charge, and surface properties [152]. QDots have been used to trace these nanocarriers by surface attachment or loading within the liposome core. For example, Weng *et al.* achieved surface labeling of targeted liposomes by conjugating hydrophilic QDots and HER-2 ligands to PEG-phospholipids, which were then displayed on NP surface following hydration [33]. After Dox loading within the lipid bilayer, fluorescence cytometry detection of QDots showed that HER-2 liposomes achieved excellent selectivity for tumor cells with a 900-1800 fold increase in cellular uptake compared to non-targeted liposomes and an excellent cytotoxicity profile with a half maximal inhibitory concentration (IC_{50}) threefold lower for HER-2 positive cells and two times higher for HER-2 negative cells compared to free Dox. Also, *in vivo* fluorescence imaging demonstrated that QDot-tagged liposomes accumulated in tumors and showed good circulation half-times (~3 hours). Alternatively, covalent conjugation of QDots can be avoided through passive loading during liposome formulation [36], shielding QDots from the physiological environment and improving circulation half-time to ~5 hours.

In addition to NP tracing, QDots provide the unique opportunity to monitor drug release kinetics in real-time by fluorescence resonance energy transfer (FRET). In one example, a Bi-FRET mechanism was developed using a QDot-aptamer conjugate for targeted delivery of a small-molecule drug to cancer cells [153]. A DNA-based aptamer for prostate-specific membrane antigen (PSMA) was conjugated to QDots and incubated with doxorubicin (Dox) chemotherapeutic, which intercalated within aptamer. As Dox is fluorescent, FRET between

QDot/Dox and Dox/aptamer quenched both Dox and QDot signal, rendering the NP in the 'off' state when Dox was bound (Figure 9A). Consequently, intracellular release of Dox was visualized by a simultaneous increase in both QDot and Dox fluorescence (Figure 9B). Monitoring cell viability over time showed that cytotoxicity induced by QDot-Dox was comparable to free Dox. In this setup, the QDot provides multiple functionalities including scaffold for drug and targeting attachment, fluorescent marker for NP localization, and drug-release sensor. Recently, a similar QDot-FRET system was applied for ovarian cancer cells using MUC1 aptamer, and attachment of Dox to QDot by acid cleavable linker showed higher toxicity to ovarian cells compared with free Dox, thus demonstrating the usefulness of FRET studies for optimizing novel nanocarriers [154].

4.2 Real-time monitoring of siRNA and DNA delivery

In recent years, DNA and in particular short-interfering RNA (siRNA) therapies have emerged as useful tool for probing gene function in biological research and hold great potential for the treatment of human diseases [155]. Where conventional chemotherapeutics act universally against both normal and cancerous cells, gene-based therapies offer greater potency and selectivity by targeting specific oncogenes and aberrant signaling pathways in cancer. Moreover, targeting multiple molecular targets in a single tumor has the potential to overcome adaptive resistance common to monotherapies [9]. However, because DNA and siRNA are rapidly degraded and cleared in the body, the development of non-viral gene delivery methods has been the major bottleneck for clinical application of these therapies [156]. In the past decade, substantial progress towards non-viral gene delivery has been made, resulting in a better understanding of the gene delivery process along with a plethora of methods for siRNA/DNA delivery via complexation/conjugation with a variety of nanocarriers (cationic lipids and polymers, carbohydrates, inorganic NPs) [157]. Still, significant challenges remain because of the great challenge of simultaneously addressing many systemic (circulation half-times, extravasation though vasculature, immunogenicity, entrapment by the mononuclear phagocyte system) and cellular (cellular entry, endosomal escape, unpacking) barriers [158].

In recent years, QDots have been used to correlate NP intracellular and systemic distribution with gene knockdown efficiency, cytotoxicity, and immunogenicity [109, 159-162]. SiRNA/DNA drugs can be loaded within a NP core through formulation with cationic lipids (lipoplexes) and polymers (polyplexes), or attached to the surface of a nanoscaffold through direct conjugation or electrostatic complexation. Though the loading efficiency of small-molecule hydrophobic drugs on NP surfaces is low, the case is different for biological drugs, such as siRNA, that achieve high potency and catalytic activity even at moderate concentrations. Because the nanocarrier can protect the siRNA/DNA from degradation by nucleases, it is critical that complexed siRNA/DNA remains stable in circulation, but then disassociates (unpacks) from the nanocarrier within the cell for efficient gene knockdown/expression. As with small-molecule drug delivery, quantitative QDot-based FRET measurements have been applied within the field of siRNA/gene delivery to strike a balance in the drug-nanocarrier affinity *in vitro*. For example, Chen *et al.* harnessed this feature to build a first-order three-compartment mathematical model for unpacking kinetics of quantoplexes [40]. Quantoplexes are large nanocarriers (~100-200 nm) prepared through electrostatic condensation of negatively charged QDots, negatively charged DNA, and positively charged polymer. By labeling DNA and polymer with QDots and fluorescent Cy5 dye respectively, FRET was used to measure the disassociation of DNA from polyplexes within the endosome/lysosome, cytoplasm, and nuclear compartments. Note that intracellular distribution studies are particularly important for siRNA delivery because endosomal escape and cytoplasmic delivery are critical steps for achieving high transfection rates. Using three different cationic polymers, the model yielded biologically relevant steady

state and kinetic rate constants that correlated intracellular trafficking dynamics with their overall transfection efficiency. Unlike previous approaches using fluorescent dye FRET pairs, quantitative analysis enabled by high brightness of QDots yielded new insights, for example showing that the disassociation of PEI polyplexes occurred much more rapidly than previously reported. More recently, QDot-FRET systems have been used to study intracellular delivery of siRNA in polyplexes [108] and plasmid DNA in lipoplexes [42], establishing itself as a universal tool for understanding the intracellular disassociation and trafficking behavior of nanocarriers.

To complement *in vitro* studies, *in vivo* imaging of siRNA delivery vehicles will be useful for evaluating their performance based on systemic criteria including favorable blood half-life and adequate tissue distribution. Preliminary studies with NIR dyes have already demonstrated feasibility and utility of such characterization. For example, siRNA-loaded dual NIR dye/magnetic NP probes haves been used to monitor siRNA delivery non-invasively by MRI and fluorescence imaging [163]. While being a particularly attractive imaging modality due to high sensitivity, excellent resolution, rapid acquisition times (seconds to minutes), and convenience [29, 164], *in vivo* fluorescence imaging is inherently not quantitative due to signal attenuation at increased penetration depths. To address this limitation, fluorescence molecular tomography (FMT) systems capable of generating quantitative three-dimensional images based on sophisticated algorithms have been developed [165]. In an excellent study by Leuschner *et al.*, FMT was used to study *in vivo* biodistribution of a liposomal siRNA nanocarrier labeled with NIR dye. As prior NP formulations have required high siRNA doses (~ 1 mg/kg), which are expensive and potentially cytotoxic, combinatorial synthesis and screening was first applied to identify a liposomal formulation with efficient knockdown in mice and primates with doses as low as 0.01-0.03 mg/kg [166]. This formulation achieved efficient knockdown of chemokine receptor CCR2, which inhibited recruitment of inflammatory monocytes to the diseased tissue site and attenuated progression of atherosclerosis, myocardial infarction, diabetes, and cancer in diseased mouse models. However, the low brightness of NIR dyes required elevated dosing in the 0.5-2 mg/kg range to achieve sufficient signal-to-noise ratios, which can significantly affect the physical and chemical properties of the nanocarrier as well as their biodistribution. Therefore, for tracking NDD vehicles at physiologically relevant doses, QDots represent an attractive alternative to organic dyes for *in vivo* studies, as high brightness provides excellent contrast even at low concentrations.

QDots have been utilized for a number of *in vivo* biodistribution studies, including lineage-tracing experiments for embryogenesis [72] and molecular imaging of cancer [97]. However, most of these studies focused on steady-state or long-term biodistribution, and little is known about how these biodistribution patterns are initially established. Recently, Zintchenko *et al.* used QDots as tracers to measure the rapid dynamics of quantoplexes (QDot-DNA-polymer) *in vivo* [43]. Fluorescence images were obtained every 15 seconds to measure the rapid biodistribution kinetics of four different quantoplex formulations with different combinations of core (linear polyethylenimine (LPEI) and branched PEI (BPEI)) and N/P ratios (6 and 10 relative ratio of nitrogen in PEI to phosphate in DNA). Remarkably, they discovered that short-term biodistribution patterns for BPEI and LPEI differed dramatically. BPEI quantoplexes were immediately routed to the liver, where LPEI quantoplexes first accumulated in the lung (Figure 10A) and partially redistributed to the liver within 5 minutes post injection. The authors attributed this redistribution to reversible aggregation of quantoplexes with blood platelets. Importantly, all quantoplex formulations had similar charge and size, indicating that changes in redistribution patterns reflected other relevant *in vivo* parameters such as the binding strength between quantoplex and biological substrates. Moreover, an *in vivo* luciferase assay was applied 6 hours post-injection of ‘bare’ polyplexes and quantoplexes to demonstrate that the QDot-label in quantoplexes did not

adversely affect the activity of encapsulated DNA. Interestingly, although the main fluorescence signal (QDots) was found in the liver area at this time (Figure 10B), luciferase activity was exclusively found in the lung area (Figure 10C). This stresses that early distribution patterns of nanocarriers largely define where they elicit their therapeutic effect. Overall this study indicates that QDot tracers are an excellent platform for detecting early and rapid distribution events, which can provide mechanistic insights into the PK and function of siRNA/DNA nanocarriers and assist in the development and improvement of new formulations.

4.3 Future focus: NP-antigen delivery for immunotherapy

For the vast majority of NP-based delivery applications to date, a primary consideration has been to design NPs that avoid interaction with the immune system. NPs are readily taken up by phagocytic macrophages and dendritic cells (DCs) and sequestered within the mononuclear phagocyte system (MPS), reducing circulation half-times and potentially causing undesirable immunostimulation that leads to inflammation, hypersensitivity, anaphylaxis, or autoimmunity [167]. Of course, proper immunostimulation can also lead to positive outcomes, as with vaccination, for example. In these cases, NP uptake into immune cells becomes a tremendous advantage. NP-based vaccines can strengthen immunostimulation in comparison to free antigen delivery by enhancing antigen uptake, improving *in vivo* stability, and acting as an adjuvant [167]. Consequently, a variety of NP based vaccines (using polymeric NPs, liposomes, solid lipid NPs, nanoemulsions, and gold NPs [168, 169]) have been developed for vaccination with weakly immunogenic antigens, some of which are now in Phase III clinical trials for indications including malaria, HIV, hepatitis A, influenza, prostate cancer, and colorectal cancer [170]. Though NP-mediated antigen delivery for immunotherapy has received less attention than small-molecule drug delivery or gene delivery in the past, this is quickly changing. The recent FDA approval of two therapeutic cancer vaccinations (Provenge for metastatic prostate cancer in 2010 [171], Ipilimumab for metastatic melanoma in 2011 [172]), though offering modest therapeutic benefit in themselves, have reignited interest in the development of vaccines and immunotherapies for cancer, including NP-based approaches. As an example, Cho *et al.* recently demonstrated that multifunctional iron oxide/zinc oxide core/shell NPs could effectively deliver carcinoembryonic antigen into DCs *in vitro*, which, when transplanted *in vivo*, reduced tumor volumes in mice compared to free antigen delivery [173].

Unlike siRNA, where the delivery criteria are challenging but at least understood, development of effective cancer vaccines remains one of the greatest “black boxes” in research today, though three major steps are clearly known. First, the vaccine must deliver antigen to DCs and stimulate their maturation; secondly, the DCs must home to lymph nodes to activate antigen-specific T-cells; finally, these T-cells must then migrate to the tumor to elicit their cytotoxic effect [174, 175]. Currently, very little is known about how to effectively deliver antigen within DCs, though there is good evidence pointing to potential issues in this critical step. It is in this arena we feel QDots hold particular promise. First, as DCs lose their antigen-processing capacity as they mature, the rate of NP uptake is probably an important parameter for the strength of DC activation [173]. Second, as generation of a cytotoxic CD8+ T-cell response is critical for antitumor effects [174], antigen should be presented on DCs via the MHC-I (cytosolic) pathway. As previous NP vaccines have accumulated in lysosomes, likely resulting in MHC-II peptide presentation [173, 176], incorporating mechanisms for NP endosomal escape and cytosolic delivery of antigen could potentially favor a MHC-I response (Figure 11). Third, as protein degradation into peptides (by nucleases in the endosome and proteasomes in the cytosol) is necessary for antigen processing and presentation [177], and because attachment of antigen to NP can potentially protect the antigen from degradation by these cellular machineries, mechanisms for antigen

release from NP are likely necessary. QDots, capable of quantitative characterization of NP uptake kinetics, visualization of intracellular distribution, and monitoring of antigen release through FRET, could provide a powerful tool in elucidating these fundamental design criteria, as has already been successfully demonstrated within the field of siRNA delivery.

A few examples of QDots for immunotherapy-related studies have emerged in recent years. For example, Mackay *et al.* tagged DCs with dual QDot-magnetic imaging probes for tracing DC migration to lymph nodes in mice using two-photon optical imaging and MRI [178]. In order to study the major steps in vaccine-induced immune responses, Sen *et al.* delivered QDots complexed with antigenic ovalbumin to DCs and tracked QDot dynamics *in vitro* and *in vivo* [179]. Dynamic fluorescence microscopy was used to follow intracellular QDot fate in real time, showing that DCs first internalized QDots via pinocytosis, and QDots were then sequestered within endosomes and finally trafficked along microtubules in an actin-dependent process. *In vivo*, fluorescence microscopy demonstrated that DCs also internalized QDots in mice, which stimulated DC migration to lymph nodes where DCs then formed stable clusters with T-cells (suggesting antigen presentation). Additional functional studies for evaluating T-cell activation, including proliferation and cytokine secretion assays, showed that antigen delivery with QDot-ovalbumin achieved CD4+ T-cell priming through the MHC-II pathway at 5-20 fold lower dose concentrations than free ovalbumin. Thus, QDots represent a comprehensive tool for studying the major cellular events characteristic of effective vaccine responses and could prove useful in future studies for the optimization of each step.

5. Conclusions and perspectives

NDD is leading the way toward overcoming the fundamental limitations of simple free-drug formulations, providing means to change their pharmacological properties and also understand their biological fate in great detail. Among many contrast agents for studying NDD vehicles, QDots are particularly suitable. It is their unique amalgamation of useful features, such as small size, versatile surface chemistry, and exquisite optical properties, that make QDots an ideal platform for the comprehensive characterization of NDD vehicle behavior across single-cell to whole organism levels. In this new field, QDots have already made substantial contributions, enabling dynamic monitoring of nanocarrier cell uptake, intracellular distribution, circulation half-times, and biodistribution. Past research has yielded methods to reliably prepare QDots with a wide array of physical properties and functionalities, paving the path toward exploiting their vast potential in NDD research. In the future, we envision QDots will facilitate systematic evaluation of NDD vehicles across a wide range of materials and diseases, and new mechanistic insights regarding NDD design criteria will continue to emerge from such studies. It is perhaps the field of immunology, in the development of NP-based vaccines that can confront the most challenging diseases, that remains the most unexplored but exciting frontier for QDots.

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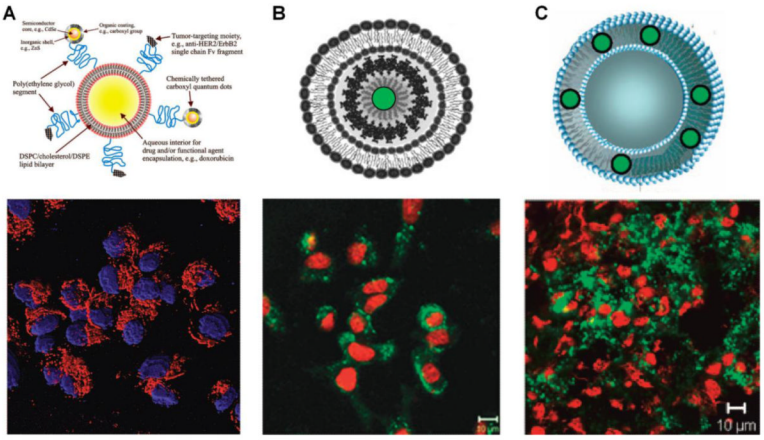

Figure 1.

Tagging liposomes with QDots offers one route to study liposome-based drug delivery vehicles. Linking QDot to the liposome surface (A) [33], loading inside the aqueous core (B) [34], or embedding within the lipid bi-layer (C) [35] enable detection of internalized nanocarriers with fluorescence microscopy (bottom row, red QDots in (A) and green QDots in (B) and (C)).

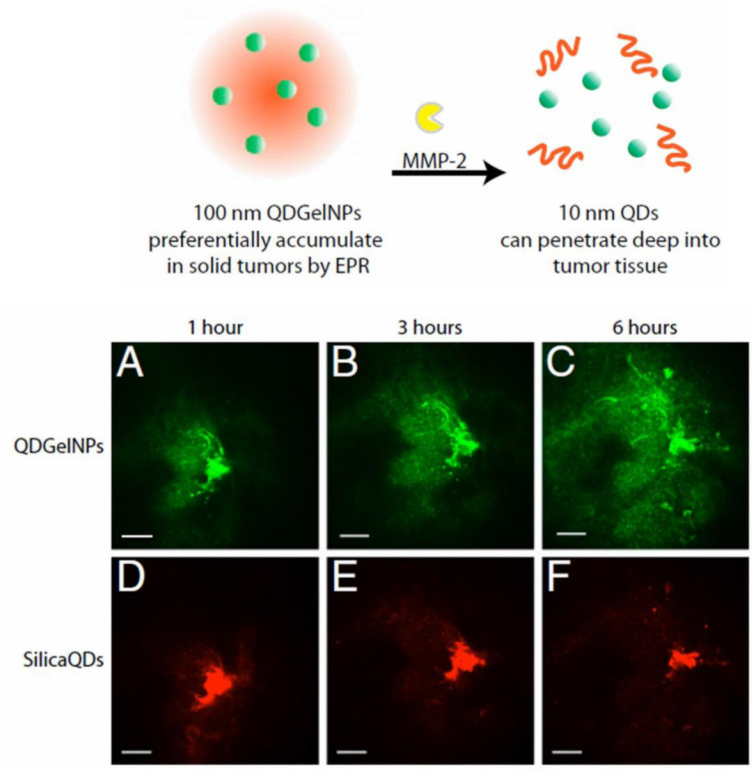

Figure 2.

Study of cargo release and tissue penetration. (Top) Degradation of QDGelNPs by MMP-2 protease triggers a change in their diffusivity. (A-C) Enzyme-mediated QDGelNP biodegradation releases individual 10-nm QDots, enabling cargo diffusion throughout the tumor. (D-F) At the same time, non-degradable 105-nm SilicaQDs fail to penetrate tumor tissue and remain at the injection site. Adapted from [47].

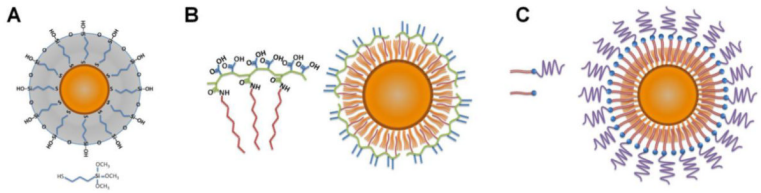

Figure 3.
Encapsulation with “adaptor” coatings shields the NP core from the environment, providing a flexible interface for further functionalization and enabling application of the same surface coatings to variety of nanocarriers. NP encapsulation within a silica shell (A) and utilization of hydrophobic interactions for NP coating with amphiphilic polymers (B) and lipids (C) represent most common examples of this approach. Adapted from [17].

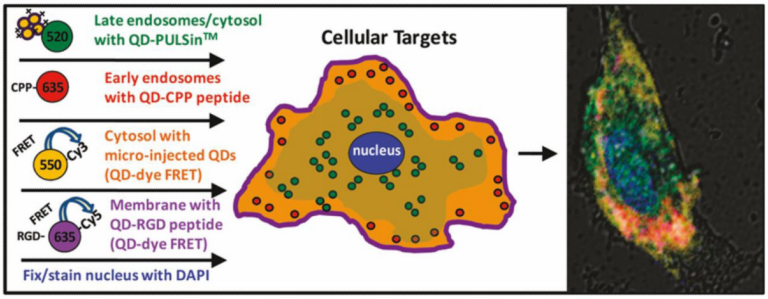

Figure 4.
Multi-color labeling with QDots highlights intracellular distribution of nanocarriers delivered via different uptake mechanisms. Adapted from [83].

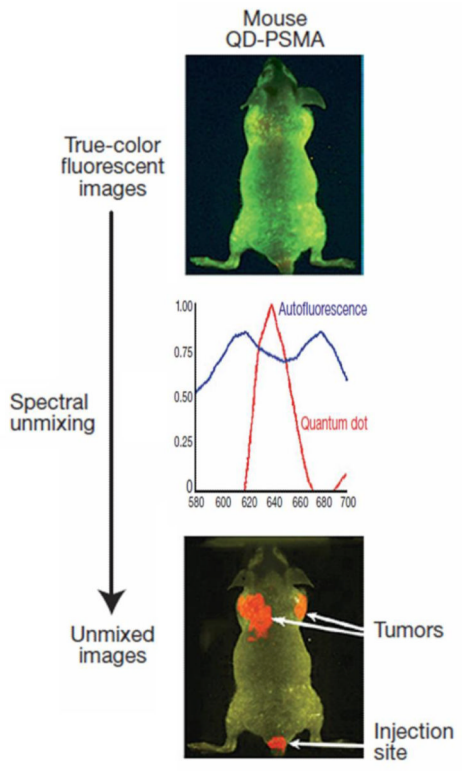

Figure 5.
Visualization of nanocarrier accumulation within implanted tumors in live mouse is achievable through clear separation of red-shifted QDot signal from tissue autofluorescence. Adapted from [97].

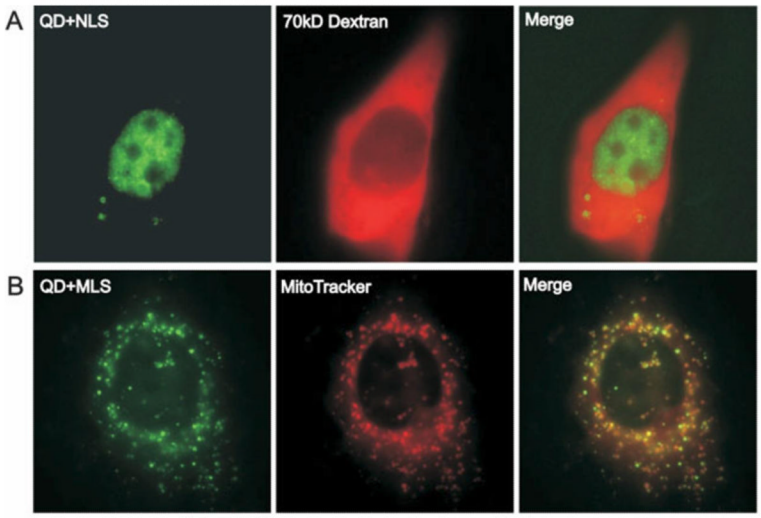

Figure 6.
Study of NDD vehicle targeting to specific organelles with affinity ligands using QDots as model nanocarriers. (A) NP functionalization with nuclear localization sequence (NLS) resulted in active QDot transport into the cell nucleus, whereas mitochondria localization sequence (MLS) routed QDots to mitochondria (B). Adapted from [113].

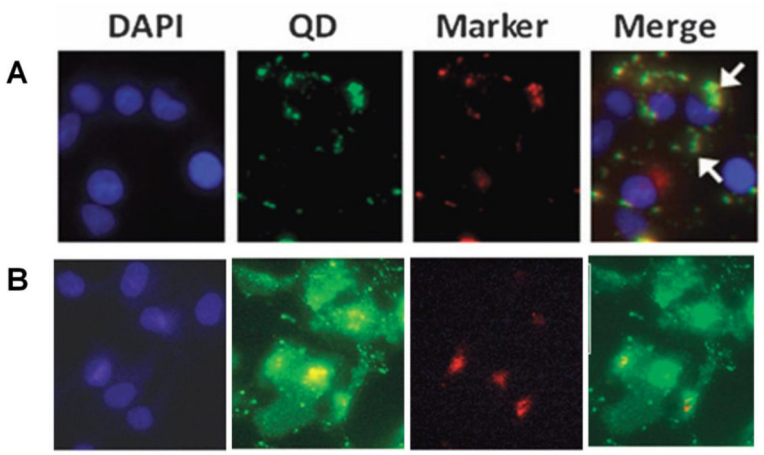

Figure 7.

QDot-aided evaluation of endosomal escape capability of different cell-penetrating peptides. (A) Characteristic punctate QDot appearance (green) and co-localization with endosomal marker (red) highlight endosomal sequestration of nanocarriers functionalized with HIV-1 Tat peptide-derived ligand. (B) Diffuse cytosolic distribution of QDots decorated with amphiphilic Palm-1 peptide indicates efficient NP escape from endosomes, identifying Palm-1 as a promising ligand for intracellular drug delivery. Adapted from [180].

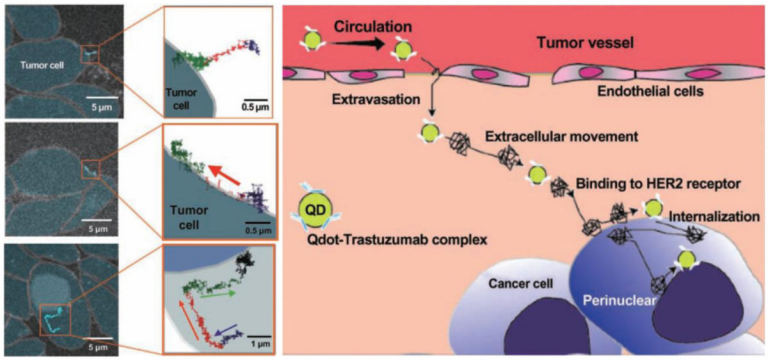

Figure 8.

Real-time monitoring of NDD vehicle vascular transport, extravasation, binding to cancer cells, and cellular internalization in a living mouse. Intravital confocal fluorescence imaging of antibody-functionalized QDots enabled direct observation of nanocarrier transport towards the cell surface (top panel), along the cell surface following target binding (middle panel), and intracellular trafficking toward perinuclear region (bottom panel). Furthermore, quantitative determination of the nanocarrier transport kinetics enabled identification of the six major delivery phases (right schematic) and rate-limiting steps for NDD *in vivo*. Adapted from [148].

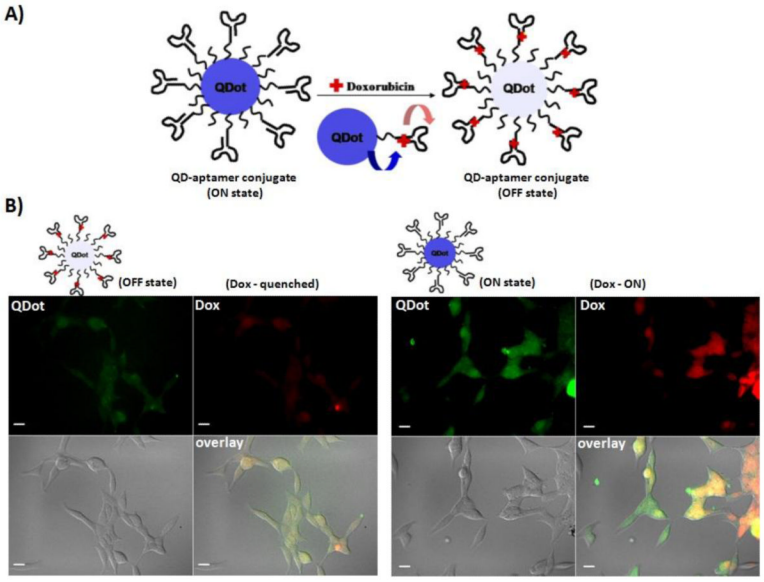

Figure 9.

Traceable drug delivery and release sensing with QDots. (A) Dox binding to QDot-aptamer conjugate quenches both Dox and QDot fluorescence in a Bi-FRET mechanism. (B) QDot cellular internalization and Dox unloading are detected through a concomitant increase in QDot and Dox fluorescence at 1.5 hours. Adapted from [153].

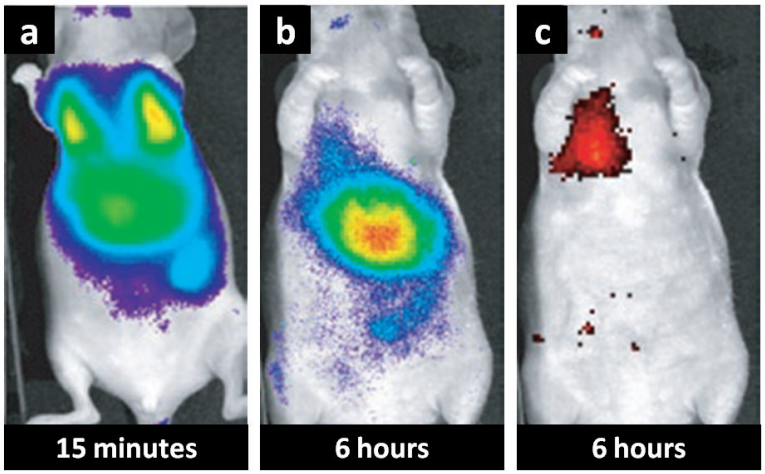

Figure 10.
Monitoring of dynamic biodistribution of DNA-polymer nanocarriers using QDot tags. Intravenously delivered QDot-polyplexes are first localized in the lung (A) but redistribute to the liver within 6 hours (B), while luciferase assay at 6 hours shows the majority of DNA transfection has occurred in the lung (C). Adapted from [43].

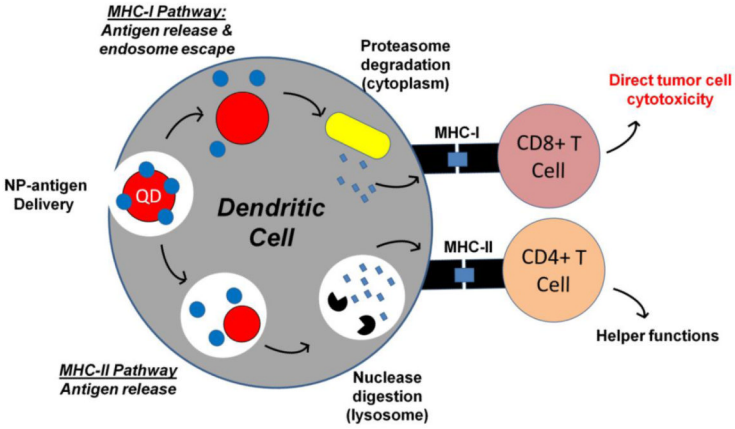

Figure 11.

Schematic of DC antigen processing and T-cell presentation. QDot serves as a tracer for monitoring internalization, antigen release, and intracellular distribution of NDD vehicle. Incorporating mechanisms for endosomal escape may shift T-cell response from MHC-II to MHC-I type for direct anti-tumor cytotoxicity.

Table 1

QDot properties for studying nanocarrier behavior in biological systems

QDot property	Description	Application
Small size	QDot core size of only 2-10nm enables tagging of various drug carriers with minimal effect on carrier properties	Labeling of single-NP and larger multi-component delivery vehicles for tracing and monitoring of vehicle degradation
Versatile surface chemistry	Compatibility with a variety of surface coating methods facilitates QDot integration within a wide range of NP carrier systems	Use of QDots as traceable analogs of other nanocarriers of interest with unperturbed carrier architecture, dimensions, and surface properties
Narrow emission profile	Sharp distinct emission peaks facilitate identification of individual QDot populations	Simultaneous observation of multiple NP carriers within the same model system
High brightness	Superior brightness enables detection of individual QDot probes within the specimen	Study of individual nanocarrier behavior within cells during uptake and intracellular trafficking; tracing of nanocarriers *in vivo*
High photostability	Resistance to photobleaching upon extended illumination enables long-term QDot tracking	Real-time monitoring of nanocarrier uptake and intracellular trafficking
Large Stokes shift	Large separation between excitation wavelength and emission peak reduces contribution of autofluorescence	Study of NP biodistribution and pharmacokinetics *in vivo*
Sensitivity to microenvironment	Dependence of QDot fluorescence on pH, surface passivation, and presence of quenchers/drugs yields microenvironment sensing capability	Monitoring changes in local microenvironment during different stages of nanocarrier intracellular uptake and trafficking, and drug release
Electron-dense inorganic core	High QDot contrast under TEM enables precise localization of NP carriers within cells at sub-cellular resolution	Study of nanocarrier trafficking and sequestration within different intracellular compartments