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Biodegradable Quantum Dot Nanocomposites Enable Live Cell Labeling and Imaging of Cytoplasmic Targets

Betty Y. S. Kim,[†,‡,§,||] Wen Jiang,[†,‡,||] John Oreopoulos,[†,‡] Christopher M. Yip,[†,‡] James T. Rutka,[§] and Warren C. W. Chan*[,†,‡]

Institute of Biomaterials and Biomedical Engineering, University of Toronto, 164 College Street, Toronto, Ontario M5S 3G9, Canada, Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, 160 College Street, Toronto, Ontario M5S 3E1, Canada, and Division of Neurosurgery, The Hospital for Sick Children, University of Toronto, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada

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ABSTRACT

Semiconductor quantum dots (QDs) offer great promise as the new generation of fluorescent probes to image and study biological processes. Despite their superior optical properties, QDs for live cell monitoring and tracking of cytoplasmic processes remain limited due to inefficient delivery methods available, altered state or function of cells during the delivery process and the requirement of surface-functionalized QDs for specific labeling of subcellular structures. Here, we present a noninvasive method to image subcellular structures in live cells using bioconjugated QD nanocomposites. By incorporating antibody-coated QDs within biodegradable polymeric nanospheres, we have designed a bioresponsive delivery system that undergoes endolysosomal to cytosolic translocation via pH-dependent reversal of nanocomposite surface charge polarity. Upon entering the cytosol, the polymer nanospheres undergo hydrolysis thus releasing the QD bioconjugates. This approach facilitates multiplexed labeling of subcellular structures inside live cells without the requirement of cell fixation or membrane permeabilization. As compared to conventional intracellular delivery techniques, this approach allows the high throughput cytoplasmic delivery of QDs with minimal toxicity to the cell. More importantly, this development demonstrates an important rational strategy for the design of a multifunctional nanosystem for biological applications.

The ability to image and monitor molecular interactions within living cells is essential to the field of molecular biology and biomedical research.[1] Quantum dots (QDs) as contrast agents promise to accomplish this task by providing unprecedented advantages over conventional methods with their enhanced signal intensity, photostability, and multiplexed capability.[2,3] Although QDs have been used quite successfully in imaging studies of both fixed and permeabilized cells,[4] their use in live cell microscopy has been limited to staining and imaging of membrane-bound receptors and trafficking vesicles.[5,6] This drawback, which stems largely in part from difficulties in delivering large quantities of QDs into the cytoplasm without causing significant disruption to the plasma membrane integrity, has hindered the widespread use of QDs for tracking cytoplasmic proteins, organelles, and complex biomolecular processes in live cells.[7] Various invasive intracellular delivery methods including cationic lipid transfection, microinjection, and electroporation have been proposed to transport QD probes into cells. However, the limited transfer efficiency, complicated procedures, and more importantly, the potential for functional alterations due to disruption of the plasma membrane have limited the use of QDs for live cell imaging.[8,9] In addition, the relatively large size of QD-antibody conjugates and the heterogeneous composition of the plasma membrane in different cell types further complicates the transfer process and instrumentation requirements, which may prevent the intracellular delivery altogether (Table 1).[10] Recent efforts have demonstrated that chemical modification of the surface of QDs can facilitate their transport through the plasma membrane and into the cytosol. For example, studies have shown that QDs functionalized with cationic ligands, or the TAT peptide, can enter the cytosol of live cells.[11,12] However, attaching these ligands

* To whom correspondence should be addressed. E-mail: warren.chan@utoronto.ca.
[†] Institute of Biomaterials and Biomedical Engineering.
[‡] Terrence Donnelly Centre for Cellular and Biomolecular Research.
[§] Division of Neurosurgery, The Hospital for Sick Children.
[||] Co-first authors.

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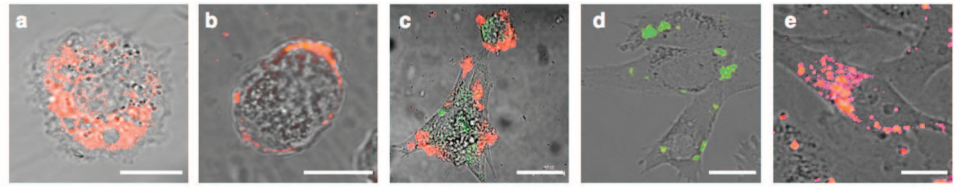
Table 1. Subcellular Delivery and Labelling Methods of QD Fluorophores

	Cell Fixation + Permeabilization[4]	Physical Approach		Chemical Approach		
		Microinjection[9,10]	Electroporation[9,10]	Lipofectamine*	TAT peptide[10,*]	QDNC*
Cells	Dead	Live	Live	Live	Live	Live
Biological Effects*	Cell death	Physical stress†	Membrane disruption†	Membrane disruption	Minimal	Minimal
Delivery Throughput	High	Low	Low	High	Medium	High
Delivery Efficiency	High	High	Low	Low	Medium	High
Specific Delivery	Yes	Yes	No	No	No	Yes
Subcellular Targeting‡	Yes	Difficult	No	No	No	Yes
Sample Images¶						

† 20% cell death for microinjection, 40% cell death for electroporation.
‡ Labelling of cellular structures other than membrane bound transport vesicles.
¶ Live cell QD delivery using a) Microinjection, b) Electroporation, c) Lipo-transfection, d) TAT peptide and e) QDNC.
* Experimentally determined.

to the surface of QDs to facilitate membrane translocation necessarily prevents coupling of other targeting ligands, such as those designed to enable binding to specific cytoplasmic organelles. These challenges suggest that in order to label subcellular organelles or proteins in live cells, a strategy to enable noninvasive delivery of bioconjugated QDs is necessary. In the present work, we propose the concept of cytosolic delivery of protein conjugated QDs in live cells to label subcellular molecular targets using a carrier system that is environmentally responsive. Utilizing the surface of biodegradable polymers for cell penetration, the chemical and structural properties of protein-coated QDs can be preserved, thus enabling them to actively seek specific intracellular targets within the cytosol. The biocompatibility of the delivery system coupled with cell entry capabilities without disturbing the plasma membrane could extend the use of QD bioconjugates to selectively label subcellular structures, monitor and track cytoplasmic processes, and elucidate protein interactions in live cells (Figure 1).[13]

Using a double microemulsion procedure, both free and protein-conjugated QDs were encapsulated within a poly(D,L-lactide-*co*-glycolide) (PLGA) nanosphere to create a hybrid nanocomposite (QDNC) (Supporting Information).[7] Size-selective fractionation using differential glycerol gradients yielded homogeneous QDNC populations (104.5 $\pm$ 7.8 nm mean diameter) (Figure 2a and Supporting Information Figure S1a). This procedure yielded a highly dense packing of the QD bioconjugates within the nanocomposite core while preserving their photophysical properties (Figure 2b and Supporting Information Figure S1b). The QDNC degradation kinetics were comparable to other PLGA nanoparticle systems with greater than 50% of encapsulated QDs released within the first 12 h of incubation in aqueous buffer at physiological temperature (Figure 2c).[14]

When directly incubated with cells, a significant increase in cellular uptake, possibly through nonspecific fluid phase pinocytosis of the QDNCs was observed, as compared to QDs lacking the surface targeting ligands.[15] The absence of free QD uptake by SNB19 cells could be attributed to the tendency of the QDs to form large aggregates that clustered on the plasma membrane thus preventing efficient cellular internalization (Figure 3a). In contrast, the QDNCs were rapidly internalized and sequestered within trafficking vesicles via a transport mechanism similar to that described for cell penetrating peptide-QD bioconjugates (Figure 3a,b).[12,16] With continued sorting of the internalized QDNCs into late-stage vesicles, progressive acidity of the endolysosomal environment enables them to actively escape from the trapped vesicles, thereby gaining entry into the cytoplasm (Figure 3b). The ability to evade endolysosomal sequestration is unique to QDNCs due to their pH-dependent surface properties. In the acidic environment of late endosomes and lysosomes, transfer of excess protons from the bulk solvent to the QDNCs results in their exhibiting a positive zeta potential. This in turn facilitates QDNC accumulation around the periphery of negatively charged vesicular membranes. (Figure 3c and Supporting Information Figure S2).[14] The now positively charged QDNCs promote local vesicular membrane destabilization and eventual cytoplasmic escape.[17] This hypothesis is further supported by the fact that similarly sized QD-polystyrene nanospheres, which possess a negative zeta potential in all pH conditions, were unable to undergo

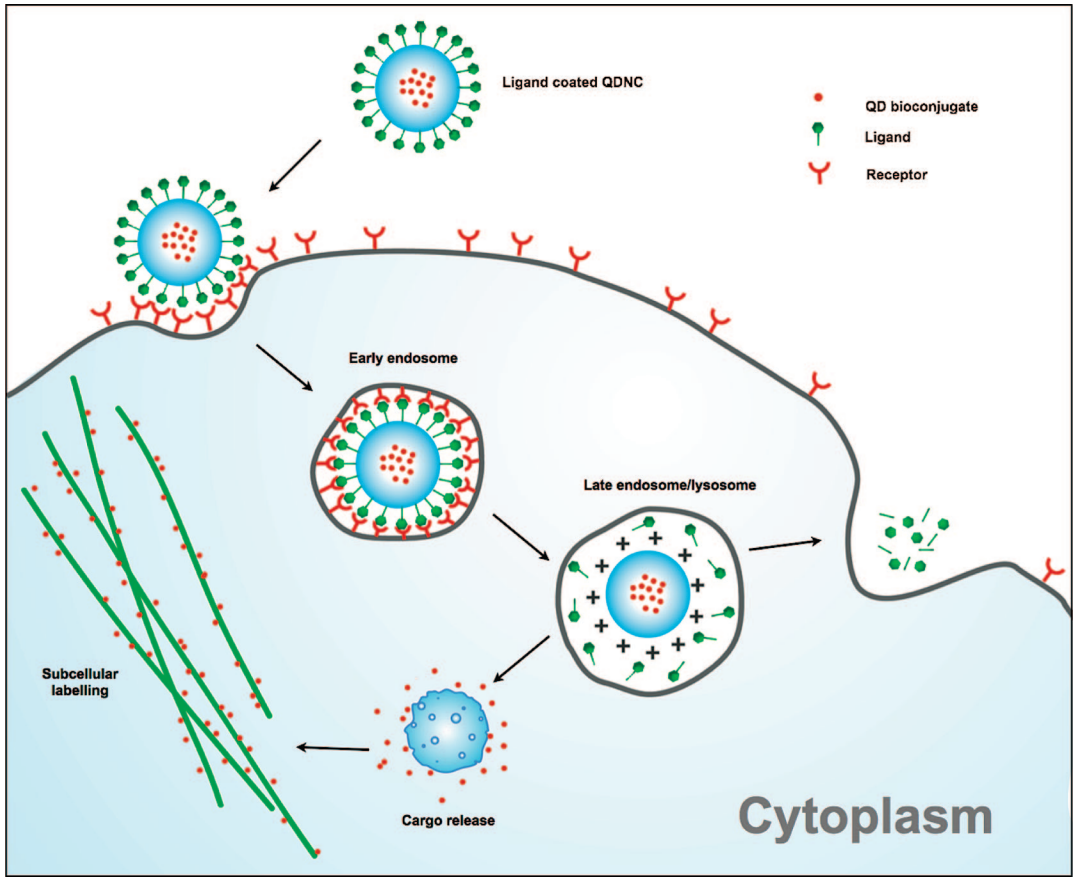


Figure 1. Mechanism of cytosolic delivery and subcellular targeting of QDNCs. Schematic representation depicting QDNC escape from the endolysosomal compartment upon cellular internalization with cytosolic release of the encapsulated cargo. Antibody-conjugated QDs can be delivered in this manner to allow the labeling of subcellular organelles or other molecular targets. Note: The actual charge distribution on the QDNC may be heterogeneous inside the acidic vesicles with partial cleavage of the peptide bonds on the QDNC surface.

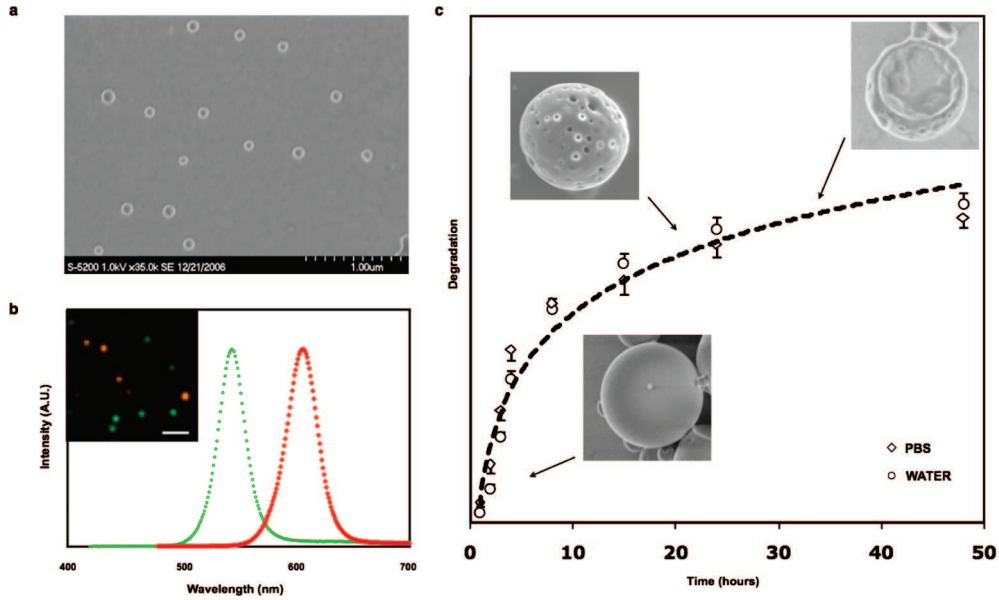


Figure 2. Characterization of QDNCs. (a) Homogeneous sample observed with scanning electron microscopy (SEM) showing QDNC populations with 104.5 ± 7.8 nm mean diameter. (b) Total internal reflection fluorescence (TIRF) microscopy images and emission spectra of QDNC sample prepared using green (λ_{em} 560 nm) and orange (λ_{em} 605 nm) emission QDs. (c) Degradation profile and corresponding SEM images of 100 nm QDNCs incubated in aqueous solvents at physiologic temperature. Scale bar, 2 μm.

endolysosomal escape (Figure 3d and Supporting Information Figure S3a). Typical of materials trapped inside organelles and vesicles en route to the microtubule organization center, distinctive vesicular accumulation near the perinuclear region was observed for QD-polystyrene nanospheres (Figure 3d).[18] These findings not only confirm that QDNCs experience

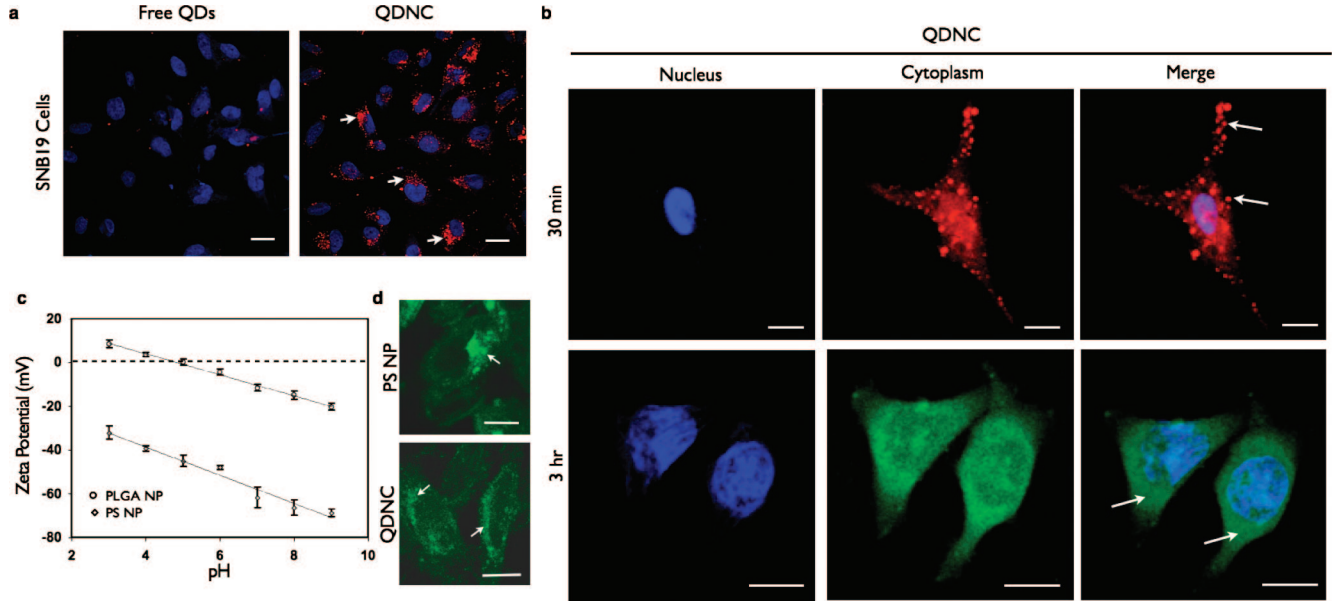


Figure 3. Intracellular delivery and escape of QDNCs from trafficking vesicles. (a) QDNCs (arrows) typically experience enhanced cellular uptake as compared to free QDs under similar experimental conditions. (b) Time-lapsed tracking of QDNCs demonstrated distinct spatial localization with vesicular sequestration observed 30 min postincubation with diffusive distribution within the cytoplasm after 3 h, suggesting the release of QD cargo (arrows = QDNCs). (c) The change in ζ-potential of QDNCs from negative to positive within acidic environments enable their escape from the endolysosomal compartment. (d) Escaped QDNCs (arrows) often have distinct cytoplasmic distribution pattern that is homogeneous, diffuse, and delocalized. In contrast, QD-doped polystyrene nanoparticles (PS NP as indicted by an arrow) does not possess the ability to change their ζ-potential polarity, which prevent their escape from the perinuclear endolysosomal confinement. Scale bars, 10 μm.

significant enhancement in cell uptake, but that they can also undergo translocation from endolysosomal vesicles into the cytoplasmic space. By selectively modifying the QDNC surface charge properties, the release and distribution of QDs can potentially be controlled in a temporal and spatial manner.

To minimize nonspecific uptake, targeted delivery of QDNCs using molecular recognition ensures efficient and site-specific accumulation.[19] Carboxylic acid functionalized QDNC enables the use of carbodiimide derivatives to couple biomolecules for labeling specific cell types. This "global" targeting capability allows for differentiation between non-relevant cell populations and those of interest. The attachment of biological ligands to the QDNC surface not only enhanced their specific interactions with cell-surface receptors, it also minimized particle uptake due to nonspecific processes (Figure 4a and Supporting Information Figure S3b). We demonstrated cell-specific labeling using QDNCs conjugated with Herceptin (QDNC-Herceptin), a monoclonal antibody that targets ErbB2 cell membrane receptors.[20] QDNC-Herceptin incubation with ErbB2-positive SKBR3 breast tumor cells showed distinct staining of the plasma membrane (Figure 4a), an effect that was absent in ErbB2-negative HeLa cells (Supporting Information Figure S3b). In addition to ErbB2 receptors, we examined whether QDNCs can be used to target cell receptors that undergo intracellular trafficking. Incubation of epidermal growth factor conjugated QDNCs (QDNC-EGF) with SNB19 cells revealed that immediately following binding to the EGF receptors, QDNC-EGF was internalized along the same trafficking pathway as that of soluble EGF ligands (Figure 4a and Supporting Information Figure S2).[21] However, unlike the internalized EGF, QDNC-EGF were capable of translocating into the cytoplasmic space (Figure 4a). On the basis of this observation, we speculated that although the presence of EGF or other targeting molecules could potentially alter the surface properties of QDNCs, enzymatic activities within the late degradative vesicles readily cleave the covalently attached proteins. In the absence of the covalently bound EGF, the QDNCs were prone to cationic conversion, which catalyzed their eventual escape through a charge-mediated transport mechanism.

Aside from selectively identifying distinct cell populations, "local" targeting of specific subcellular organelles is necessary for the elucidation of fundamental process in live cells. The very same QD surface that is modified to enable intracellular delivery is also needed for the attachment of subcellular targeting biomolecules.[12,22] Therefore, achieving both tasks simultaneously by relying on surface chemistry of QDs alone will remain difficult. However, encapsulating QDs already coated with targeting molecules inside a capsule with a modifiable surface aimed at cytosolic delivery can circumvent such limitations. Once inside the cytosol, the capsule must release the QD bioconjugates in order to label subcellular structures through molecular recognition. By engineering QDNCs to meet these design specifications, we have successfully delivered antibody conjugated QDs into the cytosol and demonstrated live cell labeling of cytoplasmic structures, including actin microfilaments through the incorporation of anti-actin antibody conjugated QDs (AA-QDs) within the nanocomposite core (Figure 4b). The characteristic filament structures of actin labeled by AA-QDs indicate the

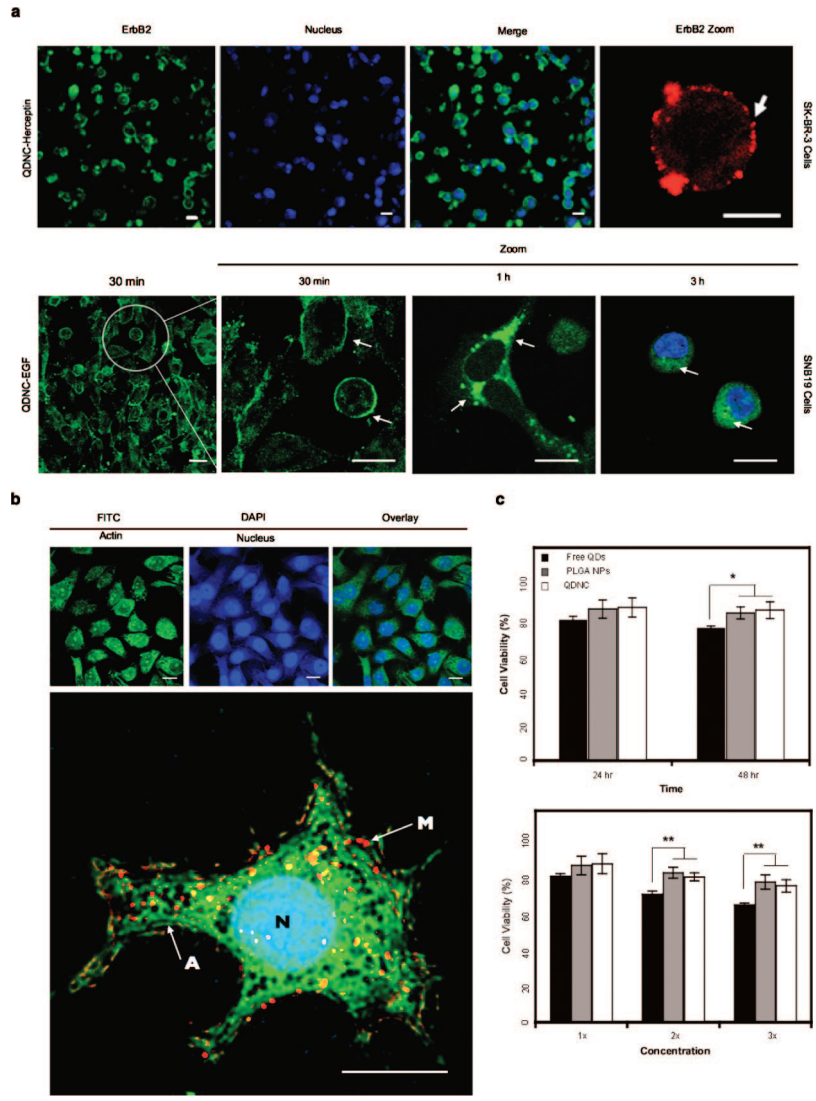


Figure 4. Targeted intracellular delivery and labeling of molecular markers using QDNCs with reduced cellular toxicity. (a) Membrane-bound receptor labeling (arrow) using QDNCs conjugated with anti-ErbB2 antibody. Membrane receptor binding (arrow, 30 min), endocytosis and vesicular accumulation (arrow 1 h), and subsequent labeling of the cytoplasm (arrow 3 h) using EGF conjugated QDNCs (QDNC-EGF). (b) Specific labeling of Actin filaments (A) and mitochondria (M) using intracellularly delivered QD-antibody conjugates. Nucleus (N) is stained with DAPI. (c) Reduced cytotoxic effects observed in QDNCs incubated in cell samples as compared to free QDs. This enhanced QDNCs biocompatibility was maintained at higher administered dosages. ($1\times$ = 50 nM for QDs, 100 μg/mL for QDNCs and PLGA NPs) All error bars = ±S.D., * denotes $p = 0.05$, ** denotes $p = 0.025$. Scale bars, 10 μm.

preservation of protein function through QDNC preparation and endolysosomal environment. Furthermore, multiplexed staining can be performed through the simultaneous delivery and detection of multiple subcellular markers. For example, costaining of actin filaments and mitochondria of live cells was achieved by delivering AA-QDs and MitoTracker Red encapsulated QDNCs into the cytoplasm (Figure 4b). Similarly, imaging of multiple cellular markers using various wavelength-emitting QDNCs also was achieved (Supporting Information Figure S4).

Finally, issues of cytotoxicity using cell penetrating strategies are ultimately dependent on the degree of membrane disruption. Both direct carrier injection and transient permeabilization of cell membrane using solution-based transfection agents have been reported to cause membrane damage.[23,24] The latter rely on positively charged macromolecules to interact with negatively charged plasma membrane thus disrupting subsequent intracellular signal transduction processes.[25] To examine their cytotoxic effects, we incubated SNB19 cells with QDNCs for up to 48 h. Overall, cell viability was not affected by QDNC incubation, and in some cases, we observed reduced cytotoxicity relative to free QDs. At a concentration of 100 μg/mL, QDNCs were found to enhance cell viability by 5−8% compared to free QDs. This is likely due to the biocompatible PLGA polymer shell that encapsulates the QDs that minimizes their tendency to aggregate and cluster around the plasma membrane (Figures 3a, 4c).[26] It is further possible that the enhanced biocompatibility of QDNCs is complemented by their environment-specific surface charge. In contrast to other transfection agents, QDNCs are positively charged only in low pH environments, such as in lysosomes, which renders them less capable of disrupting transmembrane processes.[17] Furthermore, cationic vectors such as polyethylenimine undergo

translocation by facilitating the buildup of osmotic pressure across the lysosomal membrane via the "proton sponge effect" to the point of membrane rupture, which drives molecules into the cytoplasm.[25] Such processes are extremely destructive to cells since highly degradative enzymes are released upon lysosomal rupture. In contrast, minimal cytotoxic effects suggest that the mechanism for QDNC endolysosomal escape likely differs from that of cationic lipids and lysosomotropic agents involving large-scale rupture of vesicular membranes. The extrusion of slightly cationic nanoparticles from organelles is thought to arise from localized destabilization of the lipid bilayer at the point of contact,[17] ensuring that the majority of endolysosomal membrane remains largely intact. The lack of significant disruption to membrane structures could help explain the minimal cytotoxicity of these QDNCs.

We have successfully demonstrated the efficient noninvasive delivery of bioconjugated QD fluorescent probes for live cell labeling and imaging through the design of a QD nanocomposite. Compared to conventional methods, delivery of these QDNCs induces minimal stress on the cell plasma membrane resulting in reduced cytotoxicity. The environment-specific surface charge of the QDNCs enables their escape from the endolysosomal compartments upon cellular internalization, where controllable release of the functionalized QD probes within the cytoplasmic space can be achieved through hydrolysis of the polymeric capsule. More importantly, we have developed a facile solution-based method for the targeted, multiplexed labeling of subcellular structures in live cells without the need for plasma membrane disruption or cell fixation. The ability to monitor multiple organelles and intracellular processes with QDNCs opens up the possibility of studying complex biological processes and molecular interactions in live cells. Furthermore, our system can be easily expanded to incorporate other types of nanostructures thereby providing a universal intracellular nanoscale delivery platform with significant potential in disease diagnostics, imaging and therapeutics.

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Supporting Information Available: Contains details of materials and methods for QD synthesis and QDNC formulation, QDNC characterization and degradation using SEM, TIRF, and TEM with EDX measurements, dynamic light scattering and ζ-potential measurements. Bioconjugation of QDNC and QD surface functionalization are included. Detailed methods for TAT peptide and Lipofectamine 2000 transfection, QDNC electroporation and microinjection are given. Multiplexed subcellular labeling and spectroscopic analysis and cytotoxicity measurements are included in the Supporting Figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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