Delivering quantum dots to cells: bioconjugated quantum dots for targeted and nonspecific extracellular and intracellular imaging

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Bioconjugated nanomaterials offer endless opportunities to advance both nanobiotechnology and biomedical technology. In this regard, semiconductor nanoparticles, also called quantum dots, are of particular interest for multimodal, multifunctional and multiplexed imaging of biomolecules, cells, tissues and animals. The unique optical properties, such as size-dependent tunable absorption and emission in the visible and NIR regions, narrow emission and broad absorption bands, high photoluminescence quantum yields, large one- and multi-photon absorption cross-sections, and exceptional photostability are the advantages of quantum dots. Multimodal imaging probes are developed by interfacing the unique optical properties of quantum dots with magnetic or radioactive materials. Besides, crystalline structure of quantum dots adds scope for high-contrast X-ray and TEM imaging. Yet another unique feature of a quantum dot is its spacious and flexible surface which is promising to integrate multiple ligands and antibodies and construct multi-functional probes for bioimaging. In this *critical review*, we will summarize recent advancements in the preparation of biocompatible quantum dots, bioconjugation of quantum dots, and applications of quantum dots and their bioconjugates for targeted and nonspecific imaging of extracellular and intracellular proteins, organelles and functions (181 references).

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Introduction

Nanomaterials have become fundamental building blocks for modern scientific and technological excellences. As the sizes of

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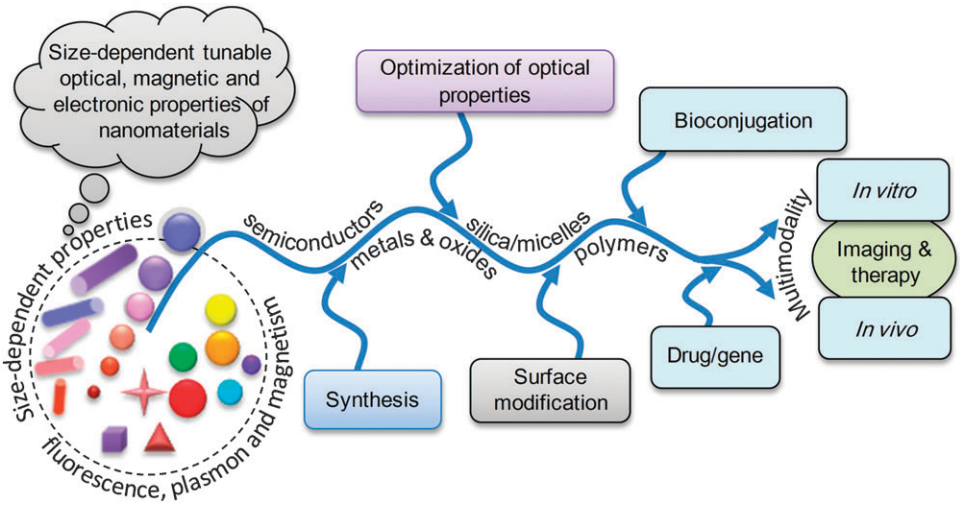

Fig. 1 Key steps involved in the interfacing of nanomaterials with biotechnology and biomedical fields.

inorganic and organic materials are decreased towards the nanometre scale, their optical and electronic properties largely vary from that in the bulk and become size- and shape-dependent. Such size- and shape-dependent properties of nanomaterials are the keys to the excellence of nanoscience and nanotechnology. It is not necessary to stress the basic concepts and emerging applications of semiconductor nanoparticles, also called quantum dots (QDs), as the key nanomaterials in this excellence. Size-dependent tunable absorption and emission in the visible and NIR regions, narrow emission and broad absorption bands, large one- and multi-photon absorption cross-sections, brightness, and exceptional photostability of QDs make them powerful sources of nanolight to advance device technology and biotechnology. In particular, bioconjugated QDs have become indispensable tools for extended imaging of cells and the structures and functions of subcellular organelles. Thus, synthesis, optimization of optical properties and bioconjugation of QDs have emerged into great research areas.

Nanobiotechnology is considerably advanced in recent years by the integration of nanomaterials with biotechnology. History of this advancement begins with the theoretical concept about unique electronic and optical properties of nanoparticles, and it extends towards biomedical imaging and nanomedicine. Nevertheless, there is an increasing demand for new materials and methodologies to breakdown the gap between nanobiotechnology and biomedical applications. Fig. 1 shows key steps in the integration of nanomaterials with biotechnology. As soon as colloidal synthesis of nanoparticles became straightforward, a broad spectrum of size- and shape-controlled nanomaterials from various chemical precursors were developed. In parallel, inorganic, polymer and hybrid nanomaterials for multimodal imaging and drug delivery were developed by utilizing the technology of shell growth and chemistry of bioconjugation. By the fine tuning of all these steps, it is now possible to formulate bioconjugated nanomaterials of our choice and challenge targeted multimodal imaging and therapeutic interventions of major diseases such as cancer. Among various nanomaterials, QDs attracted much attention in nanobiotechnology and biomedical fields due to the wide availability of precursors, straightforward synthesis, and the unique optical properties. In particular, biosensing, drug delivery, and *in vitro* and *in vivo* imaging are the most benefited areas from bioconjugated QDs. Here, we will summarize recent advances in cell, subcellular and biomolecular imaging using QDs by taking examples from our own research and cutting edge results reported by pioneers in the field. Preparation of bioconjugated QDs and various approaches for extracellular and intracellular labeling and imaging using bioconjugated QDs are summarized and correlated in this review (Fig. 2) with an intention to provide comprehensive information to newcomers in the field, which will be helpful for both extension of bioimaging applications of QDs and introduction of QD technology to other nanoparticles.

Systematic advancement in the science and technology of QDs was fuelled only after 1984, when Luis Brus derived

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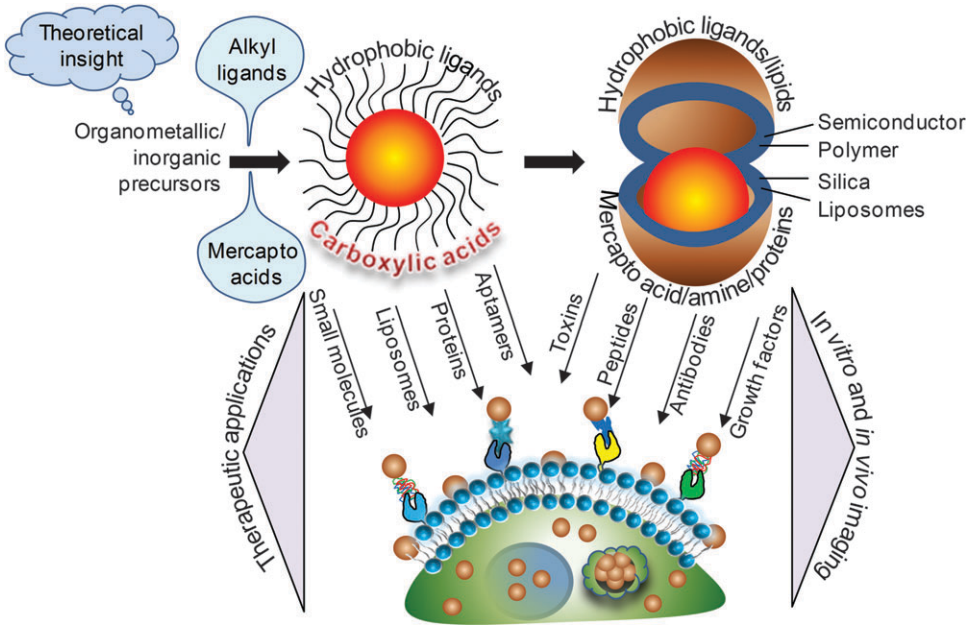

Fig. 2 Schematic representation of various methods for delivering QDs to cells.

a relation between size and band-gap for semiconductor nanoparticles by applying a particle in a sphere model approximation to the wave function for bulk semiconductors.[1,2] Subsequently, the quantum confinement of charge carriers in nanoparticles was verified by applying top-down and bottom-up approaches for obtaining nanoparticles. However, it took nearly a decade for a new drive in the nanoparticle research until Murray *et al.* accomplished the synthesis of colloidal CdX (*E* = S,Se,Te) QDs having size-tunable band-edge absorption and emission.[3] Through the systematic refinement of various parameters involved in the nanocrystal growth by a large number of researchers, synthesis of QDs has now become straightforward.[3–8] So far, CdX is the most investigated QDs due to the wide availability of precursors, well-defined technology of crystal growth, and size-tunable fluorescence in the UV-Vis-NIR regions.

Due to the wide-spread and rapidly growing applications of QDs, a comprehensive review from theory to technology is difficult and not attempted here; instead, we will provide an overview of the present status and prospects of QDs with special highlighting on bioconjugation of QDs, and applications of bioconjugated QDs for extracellular and intracellular labeling and imaging by taking specific examples. In particular, this review highlights on synthesis of biocompatible QDs, surface modification of QDs, conjugation of QDs with a variety of ligands and antibodies, potential targets of bioconjugated QDs in cells, and targeted and nonspecific extracellular labeling and intracellular delivery of QDs. Advancement in the science and technology of QDs from synthesis to bioimaging is summarized in Fig. 2. Specific examples given in this article does not mean that the outstanding contributions made by a large number of researchers are neglected. For further information on a particular topic, readers are requested to refer to key publications and review articles provided in each section.

1. Advantages of QDs in bioimaging

Fluorescent dye molecules are priceless entities to literally light up structures and functions of biomolecules and cells. However, despite the small size and widespread availability in various colors, organic dyes suffer from limitations such as narrow excitation band, small Stokes shift, broad fluorescence band, and photobleaching. Because of these limitations, dye molecules are less attractive labels for durable, sensitive, and multiplexed imaging. Semiconductor QDs on the other hand are exceptionally bright and photostable. Other promising optical properties of QDs for biological applications are size-dependent tunable absorption and emission in the visible and NIR regions, narrow emission and broad absorption bands, and large one- and multi-photon absorption cross-sections.[3,9–15] These unique optical properties originate from a combination of bulk semiconductor properties and quantum confinement effect. For most materials, the dimension that is important for quantum confinement is on the 2–10 nm scale. CdSe QDs having photoluminescence from near UV to NIR regions can be obtained by simply varying the size on the above scale. Fig. 3 shows size-tunable absorption and photoluminescence spectra of CdSe/ZnS QDs. The advantage of size-tunable photoluminescence is that common methods for surface modification or bioconjugation can be applied for obtaining multicolor probes. Broad absorption bands of QDs provide two advantages: (i) freedom to select any excitation wavelength below the band gap energy, and (ii) minimize background by increasing Stokes shift. Narrow photoluminescence bands of QDs are advantageous for minimizing bleed through during multiplexed imaging. Bright and stable photoluminescence of QDs permits durable and sensitive bioimaging even at single-molecule levels. Nevertheless, blinking photoluminescence[16–23] is a limitation in the advancement of QD technology towards single-molecule imaging. Nevertheless, synthesis of completely non-blinking ternary core/shell

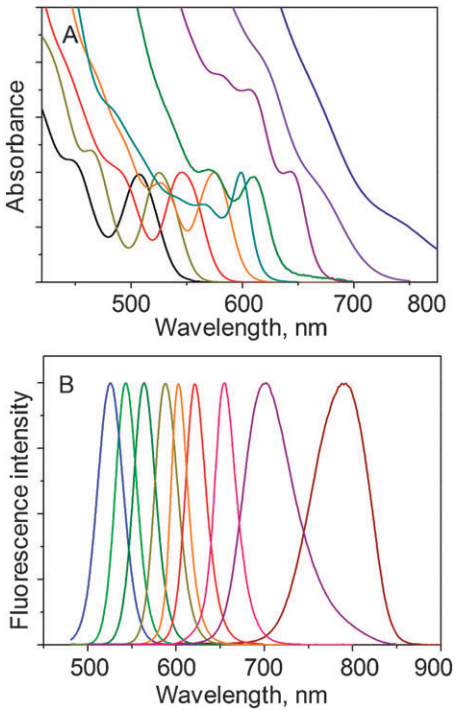

Fig. 3 (A) Absorption and (B) photoluminescence spectra of CdSe/ZnS QDs. From left to right: QD525, QD545, QD565, QD585, QD605, QD625, QD655, QD705, and QD800. Data was kindly provided by Invitrogen Corporation.

CdZnSe/ZnSe QD was achieved recently by radially alloying CdZnSe into ZnSe.[24] The large two-photon absorption cross-section [10^3–10^4 Goeppert-Mayer units][25] of QDs can be utilized for minimizing background by selecting NIR excitation of visible QDs. The advantage of NIR excitation is that it provides deeper tissue penetration, and is therefore preferable for applications such as *in vivo* imaging[9,11,25–32] and photodynamic therapy.[32–37] In addition to these gifted optical properties, the large surface area of QDs is promising for multiple bioconjugation and the preparation of multifunctional and multimodal probes. The unique optical and structural properties formulate QDs to be ideal alternatives for organic dyes, in particular, for multimodal and multiplexed imaging of single-molecules, cells, tissues and animals. Additional information on the optical properties of QDs that are relevant to bioimaging can be obtained from recent review articles.[9,11,25–32,38–41]

2. Synthesis of QDs

By introducing colloidal synthesis of cadmium chalcogenide (CdX, X = S/Se/Te) QDs by the pyrolysis of organometallic precursors of Cd and X, in 1993 Murray *et al.* brought radical changes to the basic research and applications of QDs.[3] In the key reaction, CdSe QD was synthesized by the pyrolysis of dimethyl cadmium ($CdMe_2$) and trioctylphosphine selenide (TOPSe) in a coordinating solvent mixture composed of TOP and its oxide (TOPO). The synthesis was carried out in an inert atmosphere by injecting the cadmium and selenium precursors dissolved in TOP into hot (300 °C) TOPO followed by growing the nanocrystals at ~230–260 °C. This synthesis provided a size-distribution of QDs and hence white photoluminescence. Narrow size-distributed QD samples having individual photoluminescence color were isolated by size-selective precipitation from a mixture of methanol and n-butanol. Also, CdS or CdTe QDs were prepared by this method by simply replacing TOPSe with bis(trimethylsilyl) sulfide (BTSS) or TOPTe. This classical synthesis of CdX QDs turned out to be the base of colloidal synthesis of various QDs. Katari *et al.* refined the above method for the synthesis of CdX QDs by selecting suitable injection temperature and nanocrystal growth temperature, and obtained size-selected QDs.[4] Although the use of pyrophoric and volatile $CdMe_2$ was pointed out as an occupational limitation, these two methods are widely accepted for the synthesis of CdX QDs even today. Peng *et al.*[5] and Talapin *et al.*[6] significantly improved the synthesis of CdX QDs by supplementing the solvent mixture (TOPO:TOP) with alkylphosphonic acids or alkyl amines. However, from time to time, $CdMe_2$ was substituted by nonvolatile cadmium precursors in the synthesis of high-quality and size-selected CdX QDs. Vossmeyer *et al.* accomplished the first alternative synthesis of colloidal QDs by replacing $CdMe_2$ with cadmium perchlorate ($CdClO_4$); more importantly, it is the first report on colloidal synthesis of QDs in aqueous phase.[7] Peng and coworkers significantly contributed to the synthesis of CdX QDs by introducing alternative cadmium precursors such as cadmium chloride ($CdCl_2$), cadmium oxide (CdO), cadmium acetate ($CdAc_2$), and cadmium carbonate ($CdCO_3$).[5,8,42] Synthesis of CdX QDs is now simplified and optimized by varying the precursors, temperature, and chelating ligands.[9,42–54] TEM images of CdSe QDs prepared from CdO, showing size- and shape-control as functions of time under reactions and precursor ratio is shown in Fig. 4A–G.[5,42] Also, a TEM image of uniform-size CdSe QDs prepared from $CdMe_2$ and TOPSe in the presence of TOPO, TOPO and hexadecyl amine (HDA) is shown in Fig. 4H and I.[6]

Among CdX QDs, CdSe and CdTe have attracted much attention in bioimaging due to their tunable and stable photoluminescence in the visible to NIR region. Gaponik *et al.* accomplished the synthesis of biocompatible CdTe QDs by passing H_2Te gas through an aqueous solution of cadmium perchlorate hexahydrate ($CdClO_4{\cdot}6H_2O$) and TGA kept at 100 °C and ~11.5 pH.[45] Here, H_2Te gas was generated by adding aluminium telluride into dilute sulfuric acid under inert atmosphere. InP QD is another promising candidate for bioimaging applications. Although optical properties of CdSe and CdTe QDs cannot be compromised with anything else, InP QD due to its non-toxic nature promise greatly for *in vivo* imaging and photodynamic therapy. InP QDs can be synthesized by modifying the high temperature synthesis procedure for CdX QDs, which is described in the previous paragraph. For example, Bharali *et al.* synthesized high-quality InP QDs by injecting tristrimethylsilyl phosphine (TTSP) into a dispersion of indium (III) myristate [$In(Mt)_3$] in 1-octadecene at ~280 °C.[48] Following the injection, the reaction mixture was diluted with 1-octadecene, and then QDs were grown at 180 °C. This synthesis provided hydrophobic-capped InP QDs in good yield. Detailed

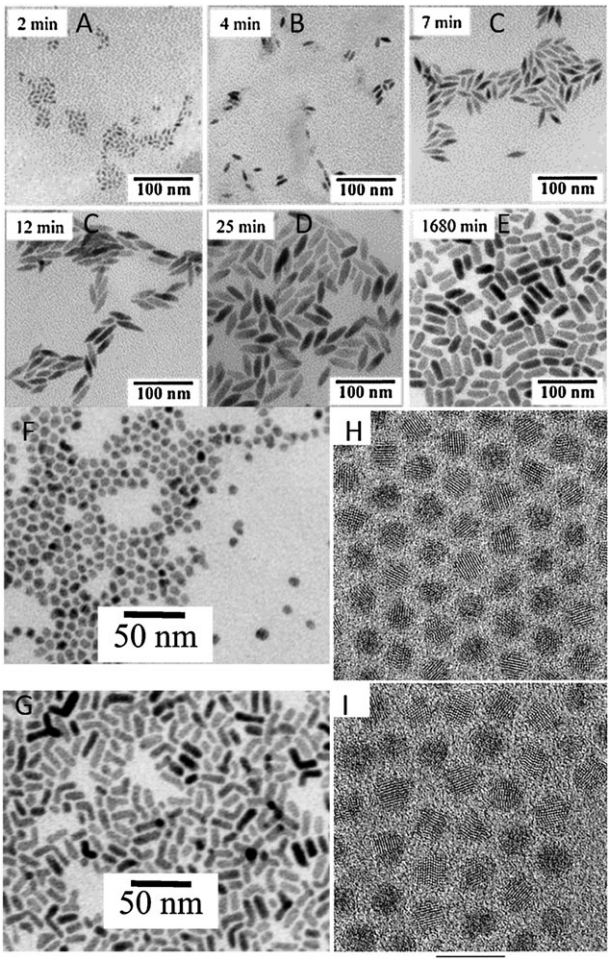

Fig. 4 (A–E) TEM images showing temporal evolution of CdSe nanocrystals from CdO, (F,G) TEM images of shape controlled CdTe nanocrystals synthesized from CdO, (H, I) high resolution TEM images of CdSe (H) and CdSe/ZnS core/shell (I) nanocrystals synthesized from $CdMe_2$. Reprinted with permission from ref. 42 (A–E), 5 (F,G), and 6; copyright (2001&2002), American Chemical Society 2001 and 2002.

procedures for the synthesis of various QDs that are suitable for biological applications are provided in recent review articles[9,43–46] and the references therein.

3. Surface modification of QDs for stability and biocompatibility

Core-only QDs are less attractive materials for bioconjugation and bioimaging because the core surface is less accessible for bioconjugate reactions and the optical properties of core-only QDs are unstable against chemical reactions. More importantly, the surfaces of most as-synthesized QDs are hydrophobically-capped, which makes them poorly compatible in the aqueous phase. Thus, surface modification of QDs has emerged into an active research area that can be divided into two: preparation of shells on core-only QDs, and surface modification of core and core/shell QDs with biocompatible molecules.

Shells on core QDs are of multipurpose. In general, shells protect the core against surface oxidation and leaching/dissolution in the form of toxic ions, and serve as platforms for ligand exchange and bioconjugate reactions. Also, shells from suitable semiconductor materials preserve and improve the optical properties of the core. For example, in type-I and type-II core/shell QDs such as CdS/ZnS, CdSe/ZnS, CdTe/ZnS, InP/ZnS, CdSe/CdS, CdTe/CdS, CdTe/CdSe, CdS/ZnSe and CdSe/ZnTe, shells not only serve as protecting layers but also offer improved photoluminescence quantum efficiency and red-shifted photoluminescence spectra. Preparation of semiconductor shells on core QDs was recently summarized by Reiss *et al.*[49] In particular, higher band-gap materials are preferred for shell preparation in order to minimize surface defects and improve optical properties of the core.[6,9,10,49–54] Likewise; thiols,[38,44–46,55–57] polymers[26,58–61] or silica[62–67] coatings on QDs serve as protecting layers and platforms for bioconjugation. Preparation of various core and core/shell QDs is summarized in Fig. 5.

3.1 Semiconductor capping

Core/shell QDs based on CdSe, CdTe or InP core and CdS, CdSe, ZnS or CdTe shell show stable photoluminescence in the visible to NIR regions. In particular, CdSe/ZnS, CdSe/CdS, CdSe/ZnCdS and CdTe/CdSe QDs are bright and robust materials for bioimaging. To-date, CdSe/ZnS is the most investigated core/shell QD in which the shell serves to be a platform form for the conjugation of thiols, polymers, liposomes, and biomolecules. Hines and Guyot Sionnest[50] and

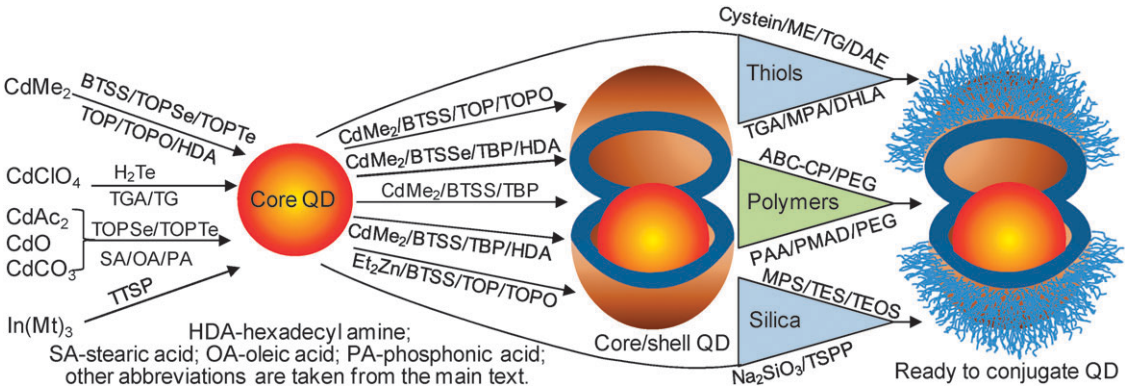

Fig. 5 Schematic presentation of the synthesis of core-QDs, preparation of shells on core-only QDs, and overlaying of core and core/shell QDs with additional layers for protection, biocompatibility and bioconjugation.

Dabbousi *et al.*[10] accomplished the preparation of core/shell QDs by the epitaxial growth of ZnS shells on CdSe QDs. Ideal precursors for ZnS shell are BTSS and diethyl zinc (Et_2Zn). Key steps involved in the preparation of ZnS shell are given below. First, a core QD solution was prepared by heating TOPO at 190 °C under vacuum, cooling to 60 °C and adding 0.1 equivalent TOP and 0.4 mmol CdSe QDs suspended in hexane. Then, hexane was distilled out, and a precursor solution for ZnS was added drop-wise into the core QD solution that was kept at 140 ~ 220 °C. The shell thickness, judged from the absorption spectrum, depends on the temperature and time under reaction. Once required thickness of ZnS shell was attained, the reaction was quenched by adding n-butanol, and the core/shell QDs were precipitated out by adding a mixture of n-butanol and methanol. Compared to CdSe QDs, CdSe/ZnS QDs show stable photoluminescence intensity and higher photoluminescence quantum efficiency. Peng *et al.* applied a strategy similar that described above and accomplished the synthesis of CdSe/CdS QDs by using a mixture of BTSS and $CdMe_2$ dissolved in tributyl phosphine (TBP) as the precursor solution for CdS.[51] These methods were further refined by Talapin *et al.* and accomplished the synthesis of CdSe/ZnS,[6] CdSe/CdS,[52] CdSe/CdS/ZnS[53] and CdSe/ZnSe/ZnS[53] QDs. Subsequently, Kim *et al.* accomplished the preparation of CdSe shells on CdTe core by using bis(trimethylsilyl) selenide (BTSSe) and $CdMe_2$ as the shell precursors.[54] Although semiconductor shells improve the optical properties of core QD and protect the core against chemical degradation, additional layers of silica, polymers or organic/bio-molecules are preferred on the shell for stability, biocompatibility, and bioconjugation.

3.2 Organic capping

As-synthesized high-quality core and core/shell QDs are usually covered by a layer of hydrophobic molecules. Thus, conversion of QDs from organic to aqueous phase has become necessary for biocompatibility, which is accomplished by coating or conjugating hydrophilic or amphiphilic molecules to the QD surface. For example, high-quality and water-soluble QDs can be prepared by conjugating QDs with mercapto acids such as thiogycolic acid (TGA) and dihydrolipoic acid (DHLA), hydrophilic dendrimers, silica-shells, mercaptopropyl silanes, amphiphilic block copolymers, micelles, proteins, peptides, and sugars. Recently, Medintz *et al.* compiled a valuable figure about surface modification of QDs.[38] In particular, the potentials of PEG, DHLA, TGA and silica layers for formulating water-soluble and biocompatible QDs are widely accepted.

Capping QD surface with thiols is a versatile approach for both exchanging hydrophobic QDs from organic to aqueous phase and introducing functional groups for bioconjugation. Coordination of thiol group to the surface metal atoms is the key to this exchange. Chan and Nie accomplished the conversion of CdSe/ZnS QDs from organic to aqueous phase by surface capping using TGA.[55] Subsequently, thiols were applied for direct synthesis of various water–soluble QDs. For example, Gopnik *et al.* synthesized water–soluble CdTe QDs by passing H_2Te gas into cadmium thiolates that were prepared by stabilizing $CdClO_4{\cdot}6H_2O$ in thiols such as 2-mercaptoethanol (ME), thioglycerol (TG), TGA, and 2-(dimethylamino) ethanethiol (DAE).[45] Various methods for the synthesis of thiol-capped CdTe QDs are recently summarized by Rogach *et al.*[46] Mattoussi *et al.* brought radical changes to the stability of phase-exchanged CdSe/ZnS QDs by substituting TOP/TOPO ligands on the surface of QDs with DHLA that acts as a bidentate ligand for surface atoms;[56] DHLA can be prepared by sodium borohydride reduction of freshly prepared thioacetic acid. Simply, TOP and TOPO ligands were exchanged by suspending size-selected QDs (100–300 mg) in DHLA (150–500 μL) and heating to ~60–70 °C for several hours. Then, the QD solution was diluted by adding dimethylformamide (DMF) and excess potassium *tert*-butoxide, which converts carboxylic acid groups into potassium carboxylates. In DMF, Potassium salt of DHLA-capped QD was precipitated out, which was separated by centrifugation and re-suspended in water. The potassium salt is stable in weak acids or weak bases and shows ~20–30% photoluminescence quantum efficiency. DHLA-capped CdSe/ZnS QDs are promising candidates for various bioconjugate reactions, detection of biomolecules by photoluminescence and Förster resonance energy transfer (FRET), and imaging of cells and tissues.[11,38,57] In short, any molecule containing one or more thiol group can be readily conjugated to QDs.

Coating or conjugation of polymers on QD surface has become an inevitable step for extended stability and biocompatibility of QDs. Polymer layers offer several advantages to QDs, such as protection against hydrolysis and biochemical reactions, improved biocompatibility, and adaptable surface for bioconjugation. Gao *et al.* successfully over-coated CdSe/ZnS QDs with a tri-block amphiphilic copolymer (ABC-CP), which protects QDs against hydrolysis and enzymatic degradation.[26] Further, they could improve biocompatibility and blood circulation of QDs by conjugating multiple polyethylene glycol (PEG) molecules on copolymer-coated QDs. The copolymer was composed of a polybutylacrylate segment, a polyethylacrylate segment, a polymethacrylic acid segment, and a hydrocarbon side chain. The hydrocarbon side chain facilitated binding of the polymer to TOP:TOPO-capped QDs through hydrophobic interactions. Also, the polymethacrylic acid segments rendered hydrophilicity and facilitated conjugation of PEG and biomolecules. Subsequently, Bentzen *et al.* accomplished the conjugation of amino-PEG molecules with varying molecular weight to amphiphilic poly(acrylic acid) (PAA)-coated CdSe/ZnS QDs.[58] More recently, Pellegrino *et al.* introduced a generalized approach for the preparation of water–soluble nanoparticles by using an amphiphilic and acid buffering polymer.[59] In this approach, nanoparticles were initially coated with poly(maleic anhydride *alt*-1-tetradecane) (PMAD) which contains roughly 25 monomers. Subsequently, the PMAD coating was changed into a stable polymer shell by cross-linking maleic anhydride groups with bis(6-aminohexyl) amine. Finally, water-solubility was introduced by the hydrolysis of several maleic anhydride groups left unreacted.

3.3 Silica capping

Several researchers have shown that encapsulation of QDs in silica shells can significantly improve the stability and compatibility of QDs in the aqueous phase. Preparation of silica shells on QDs was introduced by Correa-Duarte *et al.*[62] by first conjugating a layer of 3-mercaptopropyl trimethoxysilane (MPS) on the surface of citrate-stabilized CdS QDs, and then depositing silica layers from sodium silicate. The MPS layer facilitated both the deposition of silica layers and growth of thick silica shell. Thickness of the silica shell was increased by Stober synthesis, which involves the growth of additional layers of silica through base-catalyzed hydrolysis of tetraethoxysilane (TES). Subsequently, Rogach *et al.* refined this method and accomplished the synthesis of 'raisin bun' type silica-shelled CdSe, CdTe and CdSe/CdS QDs.[63] Briefly, CdTe QDs capped by TG or CdSe and CdSe/CdS QDs stabilized by citrate were coated first with MPS layers followed by seed layers of silica from sodium silicate solution. Subsequently, thick silica shells were grown on the seed layers by modified Stober synthesis. Gerion *et al.* introduced a slightly different, but more complex method for the preparation of high-quality silica shells on CdSe/ZnS QDs.[64] At first, they suspended QDs in methanol by adding MPS and tetramethylammonium hydroxide, and then silica shells were grown on the MPS layer by using (trihydroxysilyl)propyl methylphosphonate (TSPP). MPS offers a matching layer between the QD surface and silica shell. On the other hand, direct growth of silica shells on QDs causes mismatch between the QD surface and silica layers. The requirement of ideal double layers for the growth of perfect silica shells on QDs and metal nanoparticles was highlighted by Mulvaney *et al.*[65] With several modifications to the above mentioned methods, preparation of silica-coated QDs has become straightforward and well-controlled. For example, Nann and Mulvaney accomplished uniform encapsulation of single-QDs in silica shell.[66] Subsequently, Selvan *et al.* introduced a sol–gel process for the synthesis of silica-coated CdSe or CdSe/ZnS QDs which retained bright fluorescence.[67] They have shown that the number of QDs per silica shell and the thickness of the shell can be well-controlled by encapsulating QDs in reverse microemulsions followed by growing silica shells from tetraethyl orthosilicate (TEOS).

Whatever be the surface capping molecule/layer, the most important requirements for biological applications of QDs are that the surface cap should render intact or improved optical properties, mono-dispersion in aqueous phase, functional groups for bioconjugation, and protection against chemical/biochemical/physical damage and dissolution of toxic materials. In this regard, sequential introduction of a primary shell from a higher band-gap semiconductor, a silica shell and or a polymer shell, a PEG layer, and functional groups for bioconjugate reactions is preferred. However, the hydrodynamic size of QDs should be controlled for a better compromise among the size of QD, binding/labeling efficiency, and the functioning of targets after labeling. Preparation of various shells and capping layers on QDs are summarized in Fig. 5.

4. Bioconjugation

Recently, bioconjugated QDs have often become inevitable parts of biology and biotechnology for imaging of molecules, cells, tissues and animals.[9,11,25–32,38–41,68–71] Covalent or non-covalent conjugates of QDs with antibodies, proteins, peptides, aptamers, nucleic acids, small molecules, and liposomes can be considered as bioconjugated QDs, which are extensively used for direct and indirect labeling of extracellular proteins and subcellular organelles. Bioconjugated QDs are ideal substitutes for organic dyes when photostability or multiplexing is a requirement and excitation laser source is a limitation. Furthermore, QDs offer spacious and flexible surface for the conjugation of multiple tags. Advantages of QDs over organic dyes for bioimaging are discussed under Section 1. Also, additional information about QD-bioconjugates can be obtained from recent review articles.[9,11,32,38–41] Preparation of bioconjugated QDs is a prerequisite for bioimaging. Selection of a biomolecule on the surface QDs depends on a particular application such as biosensing, extracellular labeling, intracellular delivery, intracellular labeling, and *in vivo* imaging. To facilitate biolabeling, reactive functional group or molecule such as streptavidin, biotin, primary amine, thiol, maleimide, succinimide, or carboxylic acid is essential on the surface of core or core/shell QDs. When a specific functional group is absent, bioconjugated QDs can be prepared by non-covalent coating of peptides, proteins or liposomes on the QD surface as well. General methods for the conjugation of biomolecules to QDs are summarized in Fig. 6.

Labeling of cells using bioconjugated QDs can be classified into nonspecific and targeted. While nonspecific approaches have considerably contributed to an interface between biology and QDs; targeted delivery offers specific applications of QDs, such as imaging of the structures and functions of subcellular molecules and organelles. For example, targeted imaging of cells and tissues using QDs conjugated with anticancer antibodies and peptides has considerably improved the detection limit of cancer. *In vivo* applications of QDs are recently summarized by Michalet *et al.*[11] Walling *et al.*[70] and Smith *et al.*[71] Conjugation of QDs with specific tags enable imaging of extracellular receptors, intracellular cargo transport, gene and drug delivery, membrane dynamics, cancer cells, and embryonic development. We summarize these topics under four categories: (i) nonspecific extracellular labeling, (ii) nonspecific intracellular delivery, (iii) targeted extracellular labeling, and (iv) targeted intracellular delivery.

5. Nonspecific extracellular labeling

Nonspecific binding is hostile for high-contrast imaging of cells. Although nonspecific binding of QDs to cell membrane was initially exploited for extracellular labeling and intracellular delivery of QDs, it has become an undesirable property of QDs, which trims down the efficiencies of targeted extracellular labeling and intracellular delivery. Nonspecific binding of QDs to cell membrane takes place due to hydrophobic and electrostatic interactions between capping molecules on the surface of QDs and biomolecules in the cell membrane. Therefore, surface chemistry of QDs and the cell type play central roles in the nonspecific adsorption of QDs.[72,73] Jaiswal *et al.* identified considerable nonspecific adsorption of DHLA-capped CdSe/ZnS QDs to HeLa cells at 4 °C.[72] However, note that negative charge of DHLA does not

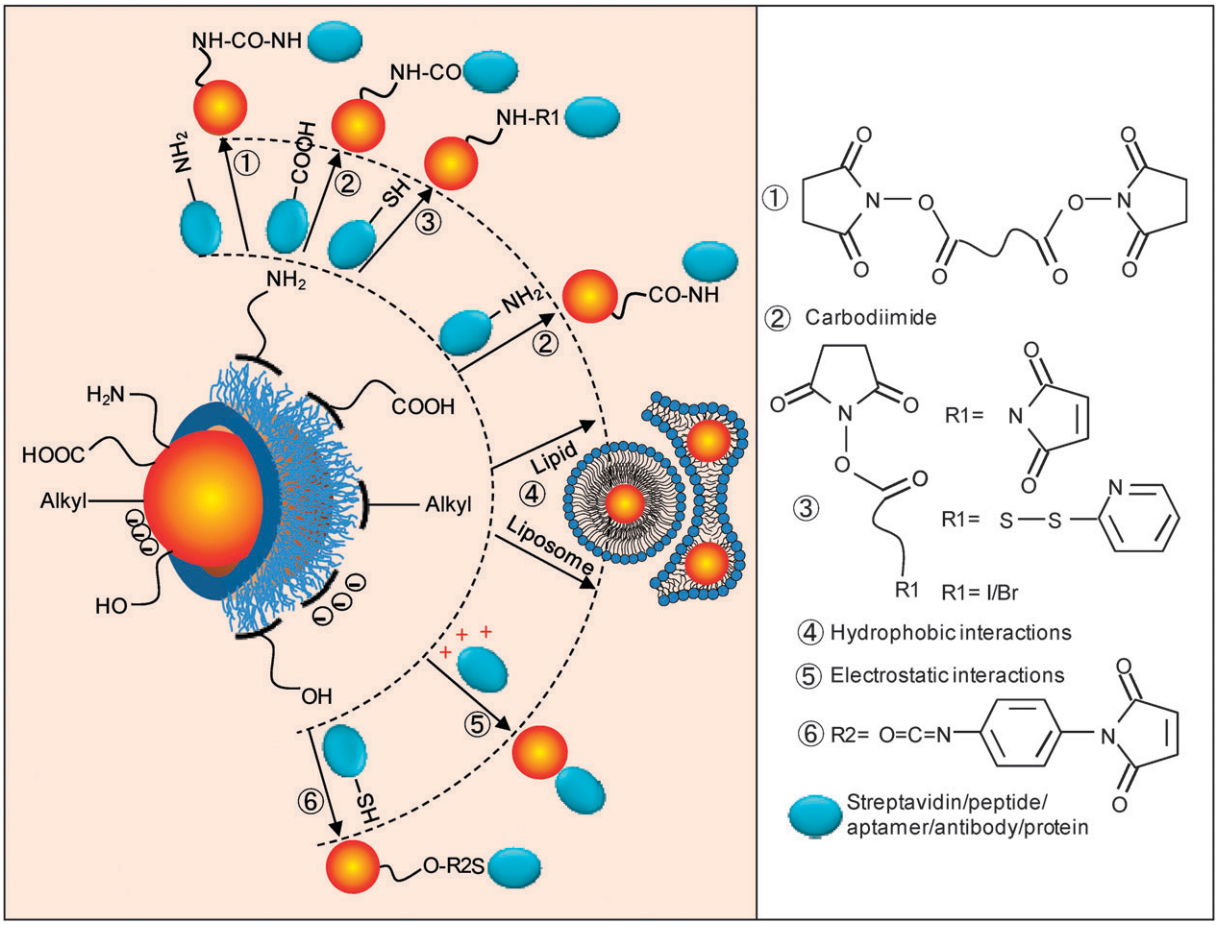

Fig. 6 Schematic presentation of various methods for the preparation of QD-bioconjugates. Bioconjugate reactions can be facilitated by selecting amino group, carboxyl group, or S–S de-protected sulfhydril group in biomolecules. ① bis(succinimide) derivatives, ③ maleimidosuccinimide/ succinimidylpyridyldithio/halosuccinimide derivatives, ⑥ N-(4-maleimido-phenyl) isocyanate.

support charge-based adsorption of QDs to negatively-charged cell membrane. Similarly, Gomez *et al.* detected strong adsorption of TGA-capped CdS QDs to neuroblastoma cells (SK–N-SH) under physiological pH.[73] The extent of QD adsorption was increased with increase in the particle size, which is large when synthesized at higher pH. A possible relation between size of QD and nonspecific adsorption to cell membrane is that larger QDs carry more ligands, which induce electrostatic interactions with cell membrane, in particular, under acidic conditions. Still, binding of DHLA- and TGA-capped QDs to cell membrane at physiological pH remains unanswered. Analogous to these observations is the nonspecific binding of zwitterionic molecules such as D-penicillamine to human monocytic cells.[74] Interestingly, Gomez *et al.* found that compared to SK–N-SH cells, the degree of nonspecific adsorption of CdS QDs was relatively low for rat adrenal pheochromocytoma cells (PC 12) and neonatal cortical cells.[73] The difference in the degree of nonspecific adsorption could be due to different cell culture medium, cell membrane composition and excreted extracellular matrix components. Subsequently, Benzen *et al.* found that CdSe/ZnS QDs coated with amphiphilic poly(acrylic acid) (AMP) nonspecifically bind to human epithelial kidney (HEK) cells at a greater extent than to mouse fibroblast cells (NIH3T3).[58] Like polymer capped QDs, QDs coated with cationic lipids result in their electrostatic binding to cell membrane. Murcia *et al.* exploited nonspecific binding of phospholipid-conjugated CdSe/ZnS QDs as a method for single-molecule imaging and tracking of QDs in solid supported phospholipids bilayers, or in the membranes of normal rat kidney (NRK) fibroblasts, African Green Monkey SV40-transfected kidney fibroblasts (COS 7), HEK 293 or embryonic mouse fibroblasts (Swiss 3T3) cells.[75] Also, QDs conjugated with various other molecules such as silanes, amines, cationic peptides, carboxylic acids, proteins, polymers and surfactants bind to cell membrane, indicating that multiple modes of interactions are pertinent in the nonspecific adsorption of QDs to cells. A few examples for nonspecific interactions between QDs and cell membrane are shown in Fig. 7.

Nonspecific extracellular binding of QDs is advantageous in the sense that cells can be labeled without extensive and tedious bioconjugation steps. However, the overall strength of nonspecific binding is mostly unknown, leaving a possibility that QDs can be dissociated with changes in pH, temperature, or chemical/biochemical environments. Because surface chemistry of QDs and bioconjugate chemistry have well advanced, selective labeling of extracellular molecules and subcellular organelles is not a concern anymore. In other words, as an

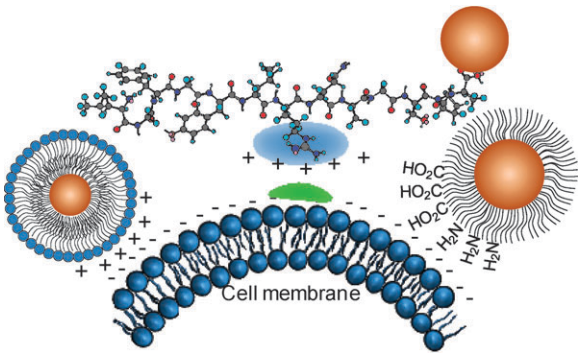

Fig. 7 Schematic presentation of nonspecific interaction and adsorption of peptides/lipids/liposomes/amines/carboxylic acids-coated QDs to cell membrane.

interface among QDs, bioconjugate chemistry, information about target biomolecules in cells, and cells biology has rapidly advanced, nonspecific adsorption of QDs to cells has became an undesirable property. For example, Ryman-Rasmussen *et al.*[76] and Mortensen *et al.*[77] have shown that dermal absorption and subsequent uptake of QDs, the extent of which depends on the size and surface chemistry of QDs, are important matters to be considered for occupational safety. Several researchers recognized that coating or conjugation of PEG molecules on the surface of QDs is unique approach for minimizing or preventing nonspecific interactions of QDs with biomolecules, cells, and tissues. For example, Bentzen *et al.* found that nonspecific binding of AMP-coated CdSe/ZnS QDs to HEK and NIH3T3 cells can be suppressed by 18% and 90%, respectively, by overlaying QD-AMP with PEG.[58] Also, they found that nonspecific binding of QD-AMP to cells can be suppressed by increasing the amount of PEG. However, on the other hand, if the construction of QD-PEG conjugate is not perfect, PEG layer can act as a barrier for intracellular delivery, while other molecules such as cationic lipids can hold QDs in the cell membrane. For example, Gopalakrishnan *et al.* found an interplay between membrane labeling and intracellular delivery of CdSe QDs coated with two lipid constructs.[78] In construct I, QDs were encompassed in vesicles composed of 1,2-dimyristoyl-*sn-glycero*-3-phosphatidylcholine (DMPC) and 1,2-dioleoyl-3-trimethylammonium propane chloride salt (DOTAP). In construct II, QDs were encompassed in vesicles composed of DMPC, DOTAP, and 1,2-distearoyl-*sn-glycero*-3-phosphatidyl ethanolamine-*N*-(biotinyl-PEG2000) (DPPE-PEG2000). Although both the constructs, due to the presence of 25% cationic DOTAP, were expected to deliver QDs inside the cytosol; construct II was stuck at the cell membrane because of a barrier created by PEG molecules for direct interaction between liposome and cell membrane. Nonetheless, systematic coating of QDs with PEG has been well established, and the potentials of PEG to prevent nonspecific adsorption of QDs to proteins, cells and tissues is widely appreciated.[26,58,78–84]

6. Nonspecific intracellular delivery

Nonspecific intracellular delivery of QDs can be classified into methods applied by man and methods adopted by cells. Applied method means forceful delivery of QDs into the cytosol or the nucleus by transiently destabilizing the cell/nuclear membrane. Applied methods include micro/nanoinjection, electroporation, and osmotic lysis. Adopted methods follow the general routes of endocytosis such as phagocytosis, macropinocytosis, clathrin-dependent endocytosis, caveolae-dependent endocytosis, and routes other than clathrin- or caveolae-dependent endocytosis.[85] Phagocytosis is the process of engulfing large foreign particles such as pathogens, minerals or cell debris by specialized cells such as phagocytes and protists. Thus, phagocytosis is less important in the intracellular delivery of nanoparticles such as QDs. In macropinocytosis, membrane protrusions fuse into macropinosomes by engulfing foreign materials along with surrounding fluid. Macropinocytosis is common mechanism for most cells in the nonspecific uptake of small particles. In clathrin-mediated endocytosis, foreign materials such as biomolecules and nanoparticles first attach to the membrane receptors/proteins, and then get trapped along with clustered receptors into ~100 nm size clathrin-coated pits. These pits pinch off the membrane in the form of clathrin-coated vesicles (CCV). The regulatory enzyme phosphoinositide 3-kinase (PI3K) assists the formation of clathrin-coated pits, and the hydrolase enzyme dynamin supports the detachment of CCV. Clathrin-mediated endocytosis is common for all cell types. In caveolae-dependent endocytosis, nanoparticles or biomolecules are trapped into relatively small (~50 nm size) caves formed by lipid-raft invaginations in the cell membrane. The transmembrane protein caveolin assists the formation of caveolae that are enriched by proteins, cholesterol and sphingolipids. Caveolae-dependent endocytosis is common for smooth muscle cells, pneumocytes, fibroblasts, adipocytes, and endothelial cells. In short, phagocytosis and macropinocytosis are completely nonspecific endocytosis; whereas clathrin- and caveolae-dependent endocytosis can be either nonspecific or receptor-mediated pathways.

As nonspecific endocytosis is not mediated by any target molecule, it will neither limit the intracellular delivery of QDs in a particular type of cell nor facilitate targeting of a particular organelle/molecule in cells. Thus, nonspecific endocytosis of QDs is important in the extended imaging of cell samples and investigation of endocytosis mechanism. Nonspecific intracellular delivery of QDs cannot be completely estranged from nonspecific extracellular labeling. Indeed, in a cell sample, the fate of QDs coated with liposomes, certain peptides, surfactants, carboxylic acids, silanes, polymers, amines, and certain proteins depends mostly on temperature. At 4 °C, such QDs stuck at the cell membrane; whereas at 37 °C, endocytosis will be operative. In the following sections, we will first summarize applied methods, and then move into the classification of adopted methods according to the surface chemistry of QDs.

6.1 Applied methods

6.1.1 Micro/nanoinjection. In microinjection or nanoinjection, a small amount of QD is introduced into the cytosol or nucleus of a cell using a fine capillary or a nanotube. Microinjection of QDs was first tested by Dubertret *et al.*

for extended *in vivo* imaging of Xenopus embryonic cells.[27] Simply, a solution of CdSe/ZnS QDs encapsulated in phospholipid micelles that are composed of PEG phosphatidylethanolamine (PEG-PE) and phosphatidylcholine (PC) was microinjected into individual embryonic cells. Interestingly, QDs were confined to the injected cell and its progeny. The exceptional photostability and brightness of QDs were helpful to follow embryogenesis up to the tadpole stage. Similarly, Slotkin *et al*. applied microinjection of QDs for detecting compartmentalized division of mouse embryonic cells *in vitro*.[86] They injected green and red QDs separately into the embryo at two-cell stage. The microinjected QDs were uniformly distributed in the cytoplasm without affecting cell division. Besides, microinjection can be utilized for intracellular labeling if QDs are tagged with certain molecules that target subcellular organelles. For example, Derfus *et al*. accomplished selective labeling of the nucleus (Fig. 8A) and mitochondria (Fig. 8B) in 3T3 cells by microinjecting QDs that are conjugated with either a nuclear localization signal (NLS) peptide derived from the Simian Vacuolating Virus 40 large T antigen (SV40 TAg) or a mitochondrial localization signal (MLS) peptide.[87] More recently, Ishihama and Funatsu investigated channeled single-molecule mRNA diffusion in interchromatin regions by microinjecting an intron-less mRNA containing A_{40} and QD655-U_{22} into the nucleus of COS 7 cell.[88] Similarly, Roberti *et al*. microinjected QD605-α-synuclein (AS) conjugate into HeLa cells and detected AS amyloid fibril deposition, which is causative for neurodegenerative diseases such as Parkinson's disease.[89] More recently, Yum *et al*. extended microinjection into unique nanoneedle-based reductive-release nanoinjection technology for solvent-free delivery of a small amount of QDs into the cytosol or nucleus.[90] They have utilized ~50 nm diameter gold-coated boron nitride nanotube as the nanoneedle, which was decorated with QDs through QD-streptavidin-biotin-S–S–Au linkage. The nanotube was connected to a microscopic needle and injected into the cytoplasm or nucleus of HeLa cells. Interestingly, QDs were spontaneously released from the nanotube by cleaving the disulfide bond under the reducing equilibrium in the cytoplasm and nucleus.

The main advantages of micro/nanoinjection are high-efficiency and selectivity. Also, microinjection enables uniform distribution of QDs in the cytosol or nucleus by bypassing endosomal arrest. Nevertheless, microinjection is a low throughput, tedious and time-consuming approach, making it less promising for labeling a large number of cells.

6.1.2 Electroporation. Electroporation is the process of transiently creating small hydrophilic pores in the cell membrane by applying a large voltage for a short period. The applied voltage charges the cell membrane, resulting in the rearrangement of lipid molecules and the formation of pores through which foreign materials and extracellular fluid enter the cytosol. Electroporation is an attractive method for intracellular delivery as it can deliver a large amount of QDs in the cytosol within a short period. Derfus *et al*. accomplished intracellular delivery of QDs in HeLa cells by electroporation. However, irrespective of the surface-coating of QDs, electroporated QDs were aggregated in the cytosol.[87] Similarly, Chen and Gerion detected large aggregates of CdSe/ZnS QDs, which were conjugated with NLS SV40 TAg peptide or a random peptide (RP) and electroporated into the cytosol of HeLa cells.[79] Subsequently, the QD-NLS conjugate was transported to the perinuclear region and delivered in the nucleus, indicating that the aggregates can gradually dissociate into more uniform particles. This result shows that electroporation

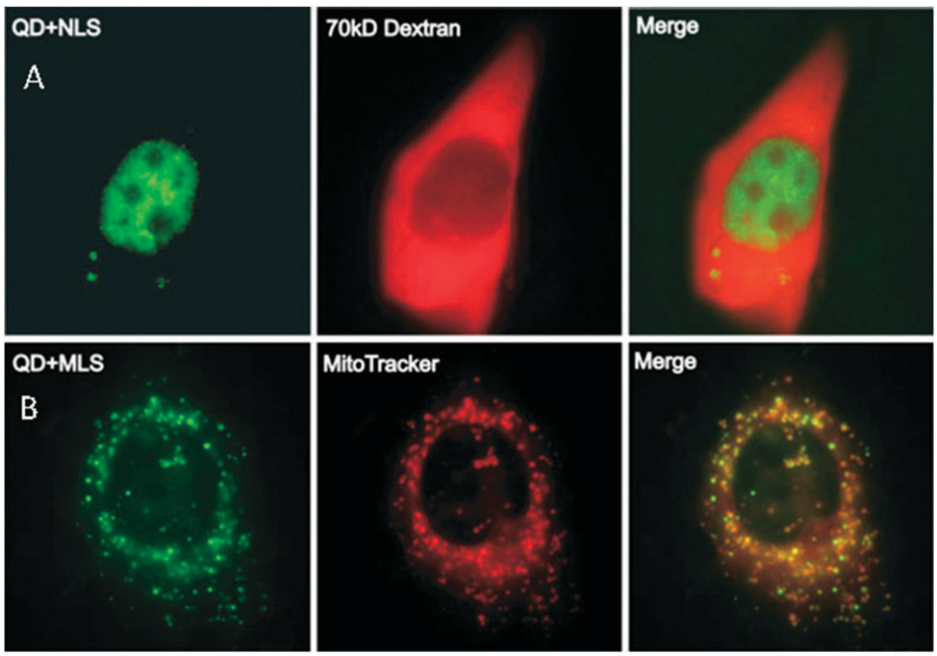

Fig. 8 Photoluminescence images of HeLa cells (A) co-microinjected with QD-NLS and 70 kDa rhodamine dextran, and (B) microinjected with QD-MLS conjugate followed by colocalization with MitoTracker Red. Reprinted with permission from ref. 87. Copyright (2004), Wiley-VCH Verlag GmbH & Co.

can be utilized for intracellular targeting if QDs are properly tagged. More recently, Slotkin *et al*. applied electroporation for *in vivo* delivery of QDs into the neural stem and progenitor cells in mouse embryo.[86] At first, they have intracerebrally injected a mixture of carboxylic acid functionalized CdSe/ZnS QDs and eGFP-F plasmid, and then electroporated the QDs into the ventricular neuroepithelium of mouse embryo by applying 33 V short pulses using an anode placed outside the uterine muscle. This *in vivo* approach and the high efficiency of intracellular delivery show that electroporation is superior to microinjection.

6.1.3 Osmotic lysis. Osmotic lysis is a promising technique for the intracellular delivery of a large number of QDs into the cytosol. In osmotic lysis, desired molecule or material for intracellular delivery is encompassed in pinocytic vesicles and applied to a cell sample. When a hypotonic solution is added into the sample, the pinocytes break as well as endosmosis takes place. By this method, Courty *et al*. successfully delivered QD-drosophila kinesin conjugates in HeLa cells,[91] and detected the intracellular movement and processivity of single-molecule motors. More recently, Nelson *et al*. exploited osmotic lysis for the intracellular delivery of QD-myosin Va conjugate in COS 7 cells.[92] The internalized conjugate was used for estimating the step size at 75 nm and speed at 604 nm s^{-1} for cargo movement by single-molecule myosin Va along actin filaments.

6.2 Nonspecific endocytosis adopted by cells

Surface charge and hydrophobicity are two factors important in the nonspecific endocytosis of QDs coated with cationic lipids/liposomes, amines/polyamines, peptides, certain proteins and carbohydrates. Such QDs are taken up by cells through one or multiple endocytic pathways discussed above. We will summarize nonspecific endocytosis of QDs in terms of the surface functionality of QDs.

6.2.1 Lipids/liposomes. Cationic lipids/liposomes are extensively used for *in vitro* and *in vivo* delivery of a wide spectrum of drugs, genes, and nanomaterials. There are several advantages of cationic lipids/liposomes, such as high-efficiency for intracellular delivery, wide availability, cost effectiveness, chemical and physical stability and biocompatibility. Although there are controversial reports on the mechanism underlying the intracellular delivery of cargos by cationic liposomes;[93] the general concept is that lipid molecules electrostatically attach to the cell membrane, then fuse with the membrane, and finally release the contents into the cytoplasm.[94] Indeed, the efficiency of liposome-mediated delivery of cargos varies with cell type.

Derfus *et al*. accomplished efficient intracellular delivery of PEG-conjugated CdSe/ZnS QDs in HeLa cells by mixing the QDs with transfection reagents such as lipofectamine 2000, activated dendrimers, or translocation peptides.[87] Among these reagents, cationic liposomes derived from lipofectamine 2000 provided highest efficiency for intracellular delivery. Endocytosis of QDs encapsulated in liposomes was evidenced by both the presence of large aggregates in the cytosol and colocalization of QDs with fluorescent labeled-epidermal growth factor (EGF), which is a known endosome marker due its receptor (EGFR)-mediated endocytosis. Similarly, Hsieh *et al*. and Lagerholm *et al*. accomplished high-efficiency for the intracellular delivery of QDs encapsulated in lipofectamine PLUS liposomes[95] or QDs coated with cationic surfactants such as di-dodecyl/di-hexadecyl dimethylammonium bromide.[96] Despite the high efficiency for the intracellular delivery of QDs by lipids/liposomes; endosomal arrest, endolysosomal degradation and exocytosis of QDs are not yet completely addressed.

6.2.2 Polymers. Nonspecific endocytosis of QDs coated with polymers is a matter of great concern for both occupational safety[76,77,97] and targeted intracellular delivery. Skin and respiratory track are the two potential routes of occupational exposure. Thus, nonspecific intracellular delivery and cytotoxicity of QDs were investigated in human skin and lung fibroblast cells. Hsieh *et al*. investigated gene expression and cytotoxic effects by silica-shelled and PEG-coated CdSe/ZnS QDs as a function of nonspecific uptake in human skin (HSF 42) and lung epithelial (IMR 90) cells.[95] Whilst IMR 90 cells were not affected detectably, genes related to cell cycle progression were slightly down-regulated in HSF 42 cells. In another study among QDs conjugated with PEG, $-NH_2$ and –COOH functionalities, Ryman-Rasmussen *et al*. found curiously fast uptake of QD–COOH by normal human epidermal keratinocytes (NHEK).[76] Nonspecific endocytosis was also detected for QDs coated with polymers such as PEG-grafted-polyethyleneimine (PEG-*g*-PEI),[98] poly(L-lysine) (PLL)-PEG-citraconic amide,[99] poly(maleic anhydride/acid–*alt*-1-tetradecane) (PMAD),[100] thiolated polyacrylamide,[101] and poly(D,L-lactide-*co*-glycolide) (QDNC).[102] These reports advise that QD-polymer conjugates can be endocytosed by various cell types.

One of the promising aspects about nonspecific endocytosis of QDs conjugated with certain engineered polymers is the endosome disrupting property of the polymer and uniform distribution of QDs in the cytosol. For example, Duan and Nie reconstructed an endosome-disrupting PEG-*g*-PEI copolymer and conjugated it to CdSe/CdS/ZnS QDs.[98] The PEI segment is known to provide a proton sponge effect with which the cargos escape from the endolysosomal compartments. The QD-PEG-*g*-PEI conjugate was strongly bound to HeLa cells by electrostatic interactions and subsequently delivered in the cytosol by nonspecific endocytosis or macropinocytosis. The fate of QDs in the cytosol depends on the ratio between PEG and PEI. For example, QDs conjugated with PEI-*g*-PEG_4 were preferentially trapped in endosomes (Fig. 9A); whereas, QDs conjugated with PEI-*g*-PEG_2 successfully were escaped from endosomes and uniformly distributed in the cytoplasm (Fig. 9B). Proton sponge effect facilitates enhanced delivery of contents trapped in endosomes by buffering H^+ and subsequent accumulation of Cl^-. Molecules such as PEI having multiple amino groups can efficiently buffer H^+. As a result of an increase in the ionic concentration, the endosome swells by osmosis and subsequently breaks. Proton sponge effect of PEI and its endosomal escape are schematically presented in Fig. 10. Yezhelyev *et al*. extended the proton sponge effect towards pH-dependent siRNA

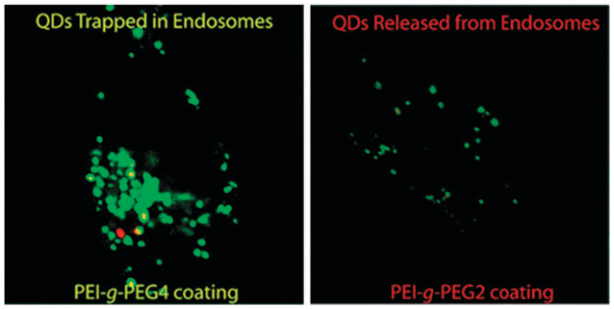

Fig. 9 Photoluminescence images of HeLa cells treated with QD-PEI-*g*-PEG_4 (left) and QD-PEI-*g*-PEG_2 conjugates. Reprinted with permission from ref. 98; copyright (2007), American Chemical Society.

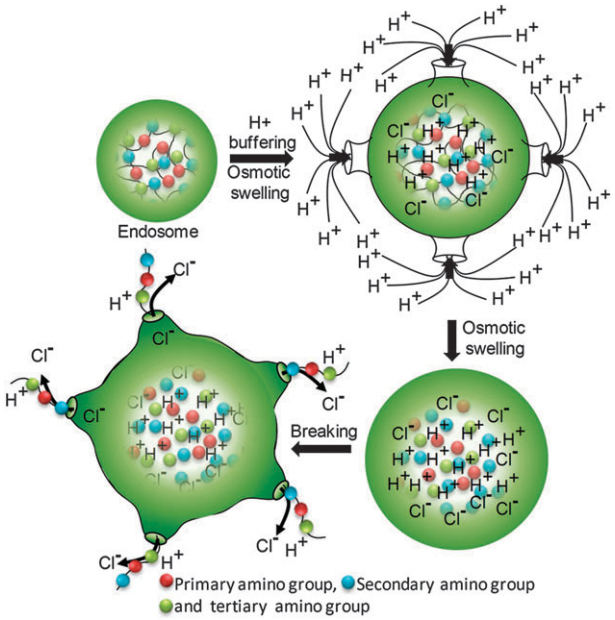

Fig. 10 Schematic presentation of proton sponge effect and endosomal escape of polyamines.

delivery by CdSe/ZnS QDs coated with PMAD.[100] Similarly, Qi and Gao accomplished the intracellular delivery and endosomal escape of siRNA by utilizing the proton sponge effect offered by maleic acid groups in an amphiphol.[103] More recently, Kim *et al.* took a step ahead in this direction by successfully targeting intracellular actin filaments using QDs coated by an endosome disrupting lactide-*co*-glycolide polymer (QDNC).[102]

6.2.3 Peptides. Peptides are common ligands for the intracellular delivery of QDs. The advantage of peptides, in particular cationic peptides, is that electrostatic interactions between peptides and cell membrane facilitate initial attachment of QDs to cells. Subsequently, QD-peptide conjugates are translocated into the cytosol by macropinocytosis, direct cell-penetration, or clathrin- or caveolae-dependent endocytosis. The efficiency and the mechanism of intracellular delivery of QD-peptide conjugates depend on various factors such as charge and amino acid sequence of peptides, hydrodynamic size of the conjugates, and the cell type.

6.2.3.1 Arginine-rich peptides. To facilitate high-efficiency transfection, researchers have developed non-viral vectors based on peptide containing polyarginine sequences or multiple arginine units. The concept of arginine-rich peptides in the intracellular delivery of QDs and other cargos originated from the uptake of *trans*-activating transcriptional activator (Tat) by numerous cell types. Simply, peptides containing multiple arginine units carry net positive charge, attach to the cell membrane through electrostatic interactions, and translocate into the cytosol. Thus, arginine-rich peptides[96,104–107] and Tat peptides[108–111] are extensively utilized for the intracellular delivery of QDs. Lagerholm *et al.* accomplished efficient intracellular delivery of poly-L-arginine (L-Arg_9)-conjugated QD565, QD605 and QD655 in Swiss 3T3 cells, HeLa cells and MG63 cells.[96] The efficiency of intracellular delivery of QD-L-Arg_9 conjugate is equal to that of QDs coated with cationic surfactants. However, intracellular aggregation of QD-polyarginine conjugates in endolysosomal compartments, evidenced by colocalization experiments using fluorescent-labeled antibody to lysobisphosphatidic acid or LysoTracker, is a limitation.[104] Delahanty *et al.* accomplished the intracellular delivery of QDs by coating a bi-functional octaarginine-*co*-octahistidine peptide on the QD surface.[105] In this approach, the octahistidine residue due to its affinity for metal atoms facilitated noncovalent conjugation to CdSe/ZnS QDs, and the octaarginine residue due to its positive charge facilitated endocytosis in HEK 293T/17 and COS-1 cells. Subsequently, Medintz *et al.* extended this approach towards the intracellular delivery of small and large protein cargos such as monomeric yellow fluorescent protein (YFP) and streptavidin-tagged β-phycoerythrin (β-PE).[107] The key mechanism underlying the intracellular delivery of QD-polyarginine conjugates is endocytosis, which was evidenced by colocalization of QDs with AlexaFluor-transferrin conjugate;[105] transferrin is well known for transferrin receptor-mediated endocytosis. Later, Zhang *et al.* validated the role of arginine in the intracellular delivery of QDs by analyzing matrix metalloprotease-2 (MMP-2)- and MM-7-modulated deprotection of arginine segments followed by the uptake of QDs by human fibrosarcoma cells (HT 1080).[106] In this method, an oligoarginine unit in a QD-peptide conjugate was first blocked with the substrate PLGVR for MMP-2 or RPLALWRS for MMP-7, and then investigated the intracellular delivery of QDs before and after deprotecting the substrates. The intracellular delivery of the QD-peptide conjugates was facilitated only after de-protecting the arginine residue by MM-2/MM-7-assisted cleavage of the substrates.

Ruan *et al.* evaluated the mode of intracellular delivery of QDs by Tat.[108] They found that QDs conjugated with multiple Tat peptides (QD:Tat = 1 : 20) can efficiently bind to the cell membrane, and subsequently taken up by HeLa cells (Fig. 11A and B). The mechanism of Tat-mediated intracellular delivery of QDs was investigated by treating the cells with QD-Tat conjugates at 4 °C (Fig. 11B) or after disrupting the cytoskeleton. Intracellular delivery of QDs was blocked in both cases, indicating that the conjugates were taken up by macropinocytosis. The QD-Tat conjugates were eventually transported along the microtubule tracks to the microtubule organizing center in the perinuclear region (Fig. 11C).

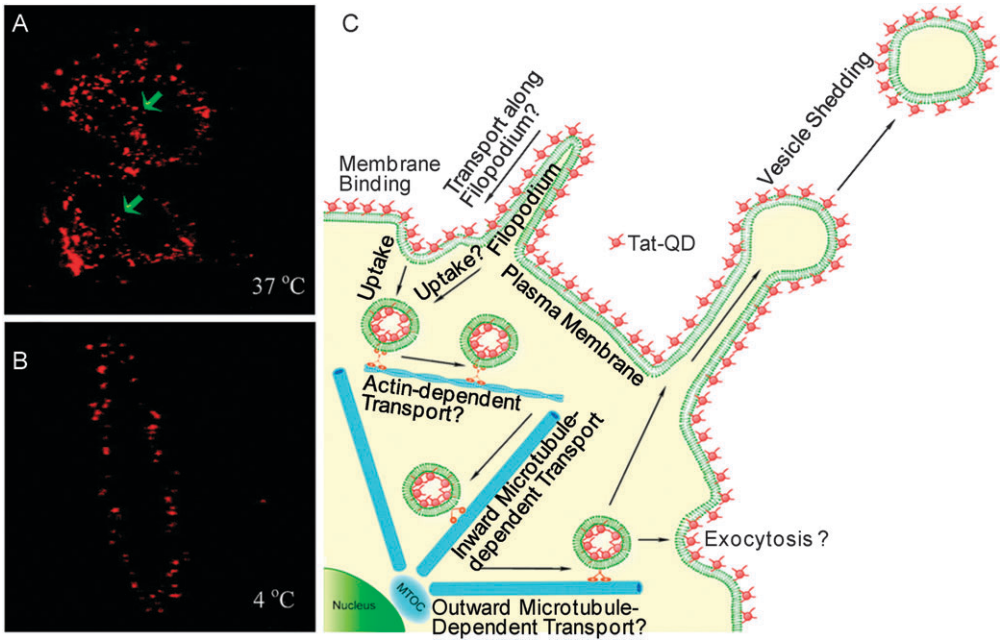

Fig. 11 Fluorescence images of HeLa cells treated with QD-Tat conjugate at 37 °C (A) and 4 °C (B). (C) Diagram illustrating key steps involved in the uptake and intracellular transport of QD-Tat conjugates. Reproduced with permission from ref. 108; copyright (2007), American Chemical Society.

Subsequently, Lei *et al.* sophisticated Tat-mediated intracellular delivery of QDs by first encompassing CdSe/ZnS QDs in lipid micelles followed by conjugating Tat peptide to the lipid coat.[109] The QD-micelle-Tat assembly was efficiently endocytosed by mesenchymal stem cells. More recently, Chen *et al.* investigated the intracellular delivery of QDs in human alveolar basal epithelial carcinoma cells (A549) as a function of QD:Tat ratio.[111] They prepared the QD-Tat conjugate by tethering biotinylated Tat to streptavidin-coated CdSe/ZnS QDs up to 1: 40 QD:Tat ratio. Interestingly, the efficiency of intracellular delivery was increased with increase in the ratio of Tat. Endocytosis of the QD-Tat conjugate was evidenced by a considerable suppression in its intracellular delivery either at 4 °C or when ATP was inhibited by treating the cells with NaN3/2-deoxy-D-glucose. The contribution of clathrin-mediated endocytosis to the intracellular delivery of QD-Tat conjugate was investigated by either depleting K^+ in the cell culture medium or treating the cells with hypertonic sucrose. These treatments result in the inhibition of intracellular delivery of transferrin but QD-Tat. On the other hand, interestingly, the intracellular delivery of the conjugate was arrested when lipid-rafts associated with the membrane cholesterol was depleted, indicating that caveolae dependent endocytosis can be the most important endocytic pathway for QD-Tat. All the above examples show that efficient intracellular delivery of QDs can be accomplished by conjugating QDs with arginine-rich peptides. However, such QD-peptide conjugates are mostly trapped in endolysosomal vesicles. Thus, a combination of arginine-rich peptides and endosome disrupting coating is necessary to advance the intracellular applications of QDs.

6.2.3.2 Nuclear localization signals (NLS). NLS are peptide ligands which deliver foreign materials into the cytosol and nucleus. As the name states, the main function of NLS is intranuclear signaling through interactions with importin in the nuclear pore complex. Due to the positive charge of NLS, it alone or in combination with other peptides can be utilized for the intracellular and intranuclear delivery of QDs. Chen and Gerion accomplished the intracellular and intranuclear delivery of CdSe/ZnS QDs conjugated with NLS derived from SV40 TAg.[79] The QD-NLS conjugate was prepared by mixing biotinylated NLS with QD-streptavidin conjugate. On the other hand, only intracellular but intranuclear delivery was detected for QDs conjugated with a random peptide sequence. Similarly, Rozenzhak *et al.* utilized a carrier peptide (Pep-1) for the intracellular delivery of QD-streptavidin, QD-NLS, and QD-GH3 domain sequence (derived from the Grim protein) conjugates.[112] They found that Pep-1 serves only as a transmembrane carrier of QDs. Interestingly, the complexes between bioconjugated QDs and Pep-1 dissociated in the cytoplasm and resulted in the release of QD-streptavidin, QD-NLS, and QD-GH3. Thus, QD-NLS and QD-GH3 conjugates delivered in the cytoplasm could further target the nucleus and mitochondria, respectively. More recently, Hoshino *et al.* utilized NLS for the intracellular delivery CdSe/ZnS QD-eGFP conjugate in HEK293T cells.[113] They could successfully validate the intracellular delivery of QD-eGFP conjugate based on the intracellular expression of GFP.

6.2.3.3 Insect neuropeptide. Recently, the potentials of natural peptides other than Tat-based peptides have been investigated for intracellular delivery of QDs and other nanoparticles. We have shown that allatostatin I (AST1, APSGAQRLYG FGL-NH_2), a neuropeptide present in insects and crustaceans, can be a promising candidate for the intracellular delivery of QDs in living mammalian cells such as human epidermoid ovarian carcinoma cells (A431) and

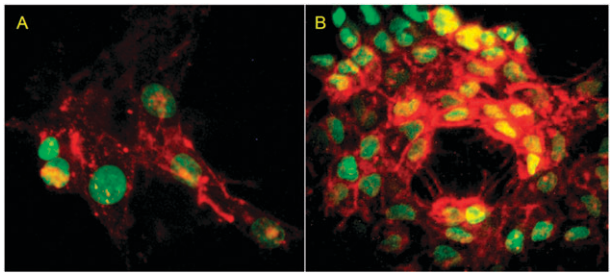

Fig. 12 Fluorescence images of (A) 3T3 and (B) A431 cells incubated with a 5 μM solution of Syto16 dye for 10 min followed by a 1 nM solution of QD605-AST1 for 30 min. Cell nucleus is preferentially stained green by Syto16 due to its cell-permeability and ability for intercalation with DNA. The yellow-orange color indicates colocalized QD-AST1 and Syto16. Reprinted with permission from ref. 115; copyright (2009), American Chemical Society.

NIH3T3 cells.[110,111] By selecting AST1 as the carrier peptide of QDs, we could validate multiple pathways in the peptide-mediated intracellular delivery of QDs. First, AST1 or its mutant (MAST1) which lacks an arginine unit was biotinylated using biotin-NHS ester and subsequently conjugated to streptavidin functionalized QD605. These conjugates were delivered efficiently in the cytosol and sparingly in the nucleus of NIH3T3 (Fig. 12A) and A431 cells (Fig. 12B). Apparently, the QD-AST1 conjugate was first attached to the cell membrane, and successively transported into the cytosol and nucleus. In the cytosol, QDs were mostly present in the form of aggregates, indicating endosomal arrest. Although details about the endosomal escape of QD-AST1 conjugate is poorly understood, the presence of QDs in the nucleus indicates that the aggregates of QDs could partly dissociate and successively cross the nuclear pore complex.

To obtain a general picture about the intracellular delivery of QDs by peptides, we have combined flow cytometry and fluorescence microscopy and investigated various pathways such as clathrin-mediated endocytosis, galanin receptor-mediated endocytosis, charge-based cell penetration, and temperature-dependent endocytosis involved in the intracellular delivery. Clathrin-mediated endocytosis can be receptor-mediated or nonspecific. We have shown that the intracellular delivery of QD-AST1 can be considerably suppressed by inhibiting the regulatory enzyme PI3K with wortmannin and blocking the formation of clathrin-coated vesicles. In this inhibition assay, cells were pre-incubated with 50 nM wortmannin and copiously washed before incubating with QD-AST1. Wortmannin is a cell-permeable steroidal furanoid which irreversibly inhibits PI3K by blocking its ATP binding pocket and modifying the lysine side chain. In parallel, clathrin-mediated endocytosis was validated on the basis of colocalization of QD605-AST1 with *anti*-clathrin antibody (CAb) that was labeled with QD565 (Fig. 13A,B). Clathrin-mediated endocytosis and its inhibition by wortmannin are schematically presented in Fig. 13C. Next, we chose to investigate receptor-mediated endocytosis because the target receptors for AST1 in crustaceans and galanin in mammals are analogous. However, the intracellular delivery of QD-AST1 was not considerably affected in the presence of galanin antagonist. Indeed, the level of galanin-receptor is low in A431 and 3T3 cells, indicating

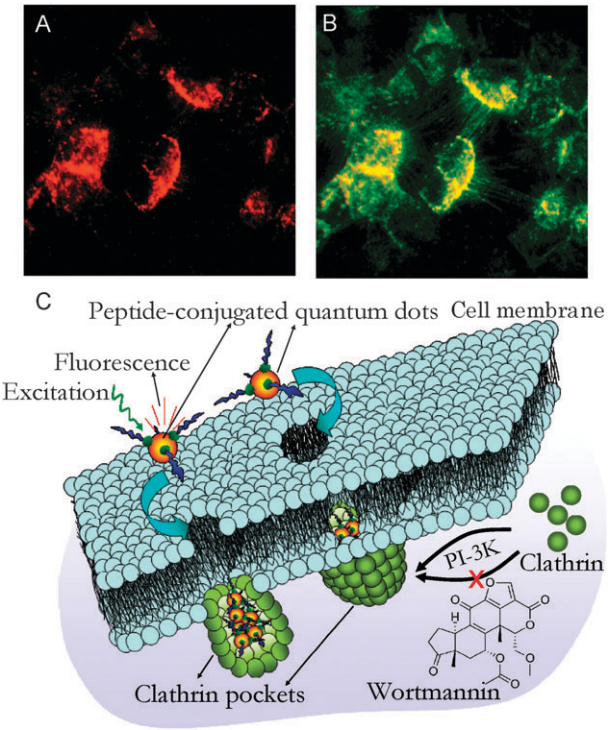

Fig. 13 (A, B) Fluorescence images of A431 cells incubated with QD605-AST1 conjugate for 30 min, permeabilized using methanol, and subsequently incubated with QD565-CAb conjugate: (A) image acquired through a filter for QD605 and (B) image acquired through a filter for QD565 and overlaid with A. (C) Schematic presentation of clathrin-mediated endocytosis of QD-AST1. Reprinted with permission from ref. 115; copyright (2009), American Chemical Society.

that galanin receptor-mediated endocytosis of QD-AST1 is negligible in these cell lines. Next, we chose to investigate charge-based cell penetration of QD-AST1 because AST1 contains an arginine moiety, which carries a net positive charge. Interestingly, when the arginine unit was replaced with an alanine unit (MAST1), the efficiency of intracellular delivery of QD was lowered by ~13% for A431 and ~27% for 3T3 cells from the efficiency for QD-AST1. Similarly, when the negative charge of cell membrane due to heparan sulfate was suppressed by pre-incubating the cells with heparinase enzyme, the efficiency of intracellular delivery of QD-AST1 was decreased by ~18% for A431 and ~30% for 3T3 cells, indicating the contributions by charge-based intracellular delivery. However, direct comparison between the efficiency of intracellular delivery with charge need not be accurate because electrostatic attachment of QD-AST1 to the cell membrane cannot be underestimated in different endocytic pathways. Direct cell penetration but endocytosis is an energy independent process unaffected at low temperatures. Thus, we have shown the difference between cell penetration and endocytosis by incubating the cells at 4 °C. By considering that the intracellular delivery of QD-AST1 at 37 °C is 100%, the efficiencies at 4 °C were ~87% for A431 and ~55% for 3T3 cells, suggesting that direct cell penetration (~13% for A431 and ~45% for 3T3) is operative in the intracellular delivery of QD-AST1. Based on our own investigations[114,115]

and investigations by Ruan *et al.*[108] and Chen *et al.*,[111] apparently multiple pathways are pertinent in the intracellular delivery of QD-peptide conjugates.

Versatility of peptide-mediated intracellular delivery of QDs was tested for several other natural and synthetic peptides. For example, Walther *et al.* employed calcitonin, a linear polypeptide hormone found in most animals and man, as a carrier peptide for QDs and RNA in HeLa and HEK-293 cells.[116] QD-calcitonin conjugate was endocytosed mainly by lipid-raft-dependent pathways, but the contributions of clathrin-mediated endocytosis and macropinocytosis were minor. However, calcitonin is the ligand for the G-protein coupled calcitonin receptor in osteoclasts and thus, receptor-mediated endocytosis will be operative for QD-calcitonin conjugate in osteoclasts. Maurocalcine, a 33 amino acid peptide toxin found in scorpion venom, is another natural peptide that was investigated for the intracellular delivery of QDs. Jayagopal *et al.* accomplished biotinylation of this peptide, conjugation of the biotinylated peptide to streptavidin-coated QD585 and QD655, and efficient intracellular delivery of QD-maurocalcine conjugate in immunomagnetically isolated monocytes and T-lymphocytes.[117] The above examples show that the extent and the mode of intracellular delivery of QD-peptide conjugates depend on various factors such as the amino acid sequence of the peptide, charge of the peptide, and the cell type.

6.3 Proteins

Proteins such as collagen, serum albumin and virus capsid proteins are found to be cost-effective agents for nonspecific intracellular delivery of QDs and other cargos. Parak *et al.* utilized collagen-coated QDs for evaluating the motility of cancer cells.[118] They seeded human mammary epithelial tumor cells (MDA-MB-231) and non-tumor cells (MCF-10A) on plates coated with collagen and silica-shelled CdSe/ZnS QDs. Interestingly, the QD-collagen complex was initially adhered to the cell surface glycoproteins and glycolipids and subsequently taken up by pino/endo/phagocytosis. Although both cancer cells and normal cells engulfed the QD-collagen complex, the uptake was prominent in the case of cancer cells due to their motility. Thus, the motility of cancer cells could be determined from non-fluorescent tracks made by engulfing QD-collagen complexes. Serum albumin is another example for cost-effective and widely available protein for the intracellular delivery of QDs. The efficiency of serum albumin to deliver QDs into the cytosol was first examined by Hanaki *et al.* by mixing a CdSe/ZnS QD solution with serum from sheep, bovine, human, rabbit, pig, mouse or canine.[119] They found that QD-serum mixture can be efficiently endocytosed by Vero cells. One of the advantages of QD-serum complex over other QD-conjugates is that serum albumin renders QDs non-toxic.[120] Proteins derived from virus capsids are promising alternatives to deliver QDs into cells through caveolae-dependent pathway, which can avoid endolysosomal trapping and degradation of QDs. Recently, Li *et al.* accomplished efficient intracellular delivery of CdSe/ZnS QDs in Vero cells by complexing QDs with capsid protein from SV40 virus.[121] They have constructed a chimeric virus-like nanostructure by encapsulating TGA-capped QDs in SV40 virus protein pentamers. Resembling the endocytosis of virus particles, the QD-protein assembly was delivered through caveolar endocytosis, which was confirmed by colocalizing QDs with caveolin-1 that was fused with cyanine fluorescence protein (CFP). On the other hand, clathrin-mediated endocytosis was ruled out as transferrin was not colocalized with QDs. The endocytosed nanoparticles were transported in the cytosol along microtubules and accumulated in the endoplasmic reticulum, a process that mimics the early stage of viral infection.

6.4 Small molecules

Nonspecific endocytosis of QDs by small molecules such as TGA,[12,72] MPA,[73,122] DHLA[72] and trimethoxysilylpropyl urea[123] was relevant only until bioconjugate chemistry of QD was established. Recently, QDs conjugated with such small molecules are utilized in control experiments or experiments related to occupational safety. Bruchez *et al.* tested the first biological application of QDs by delivering silica-shelled and trimethoxysilylpropyl urea-conjugated CdSe/CdS QD in 3T3 cells.[123] They found that the internalized QDs can be utilized for staining the nucleus. Also, they could successfully target F-actin filaments in 3T3 cells by treating the cells successively with biotinylated phalloidin, streptavidin, and QD-biotin. Subsequently, Jaiswal *et al.* utilized DHLA-capped CdSe/ZnS QDs for intracellular labeling of HeLa cells.[72] They have evaluated the endocytosis of QD-DHLA conjugate by colocalizing QDs with an endosome specific protein to which CFP was fused. Sun *et al.* successfully detected nonspecific uptake of TGA or MPA-conjugated CdSe/ZnSe/ZnS QDs by human ovarian cancer cells (SKOV-3), multiple myeloma cells (RPMI 8226) and pancreatic cancer cells (AsPc-1), but NIH3T3 cells.[122] More recently, Xu *et al.* found that a combination of UV light and the membrane permeable dye 4′,6-diamino-2-phenylindole (DAPI) can facilitate intracellular and intranuclear delivery of QDs.[124] These reports indicate that nonspecific endocytosis of QDs depends on both the molecules on the surface of QDs and the cell type.

6.5 Carbohydrates

Coating or conjugation of carbohydrates to QDs offers water-solubility and biocompatibility to QDs. Also, complexes between QDs and carbohydrates have shown substantial intracellular delivery in a variety of cell types. For example, Hasegawa *et al.* successfully delivered QDs-coated with a thick nanogel-shell composed of cholesterol and primary amine functionalized pollulan ($CHPNH_2$), a polysaccharide.[125] They assembled the QD-nanogel complex by coating positively-charged $CHPNH_2$ to negatively-charged QDs (Fig. 14A). Interestingly, the efficiency of intracellular delivery of the QD-nanogel complex depended on the number of amino groups in pollulan. For example, QD-nanogel containing 9 amino groups per 100 glucose units provided intracellular delivery efficiency equal to that of QDs encompassed in lipofectamine. As the number of amino groups per 100 glucose units was increased to 15, the efficiency of intracellular delivery was increased by a factor of 3.4 (Fig. 14B–D).

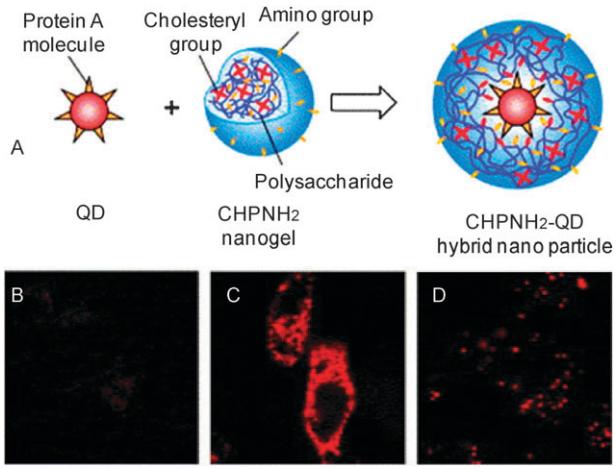

Fig. 14 (A) Preparation of $CHPNH_2$–QD hybrid nanoparticles, (B–D) confocal fluorescence images of HeLa cells labeled with (B) QD, (C) QD-conjugated to $CHPNH_2$ having 15 amino groups per 100 glucose units, and (D) QD-liposome. Reprinted from ref. 125; copyright (2005), with permission from Elsevier.

These results indicate that positive charge of the nanogel due to amino group has considerably contributed to the intracellular delivery of QDs. Similarly, Xie *et al.* and de Farias *et al.* successfully delivered QDs-coated with chitosan[126] or glucose[127] in yeast cells. Simply, a solution of TOPO-capped QDs in hexane was minced with chitosan or carboxymethyl chitosan, re-suspended in water, and applied to yeast cells.[126] Although exact mechanism underlying the intracellular delivery of QD-carbohydrate system is poorly understood, the uptake of QD-glucose conjugate by yeast cells can be attributed to an active transport by transmembrane proteins.

7. Targeted extracellular labeling

Selective labeling of cell membrane using QDs can be accomplished by targeting membrane proteins/receptors with QD-antibody or QD-ligand conjugates. In this section we will summarize targeted extracellular labeling in line with the type of antibody or ligand that is conjugated to QDs and the type of target protein/receptor in the cell membrane. It may be noted that targeted extracellular labeling cannot be completely estranged from targeted intracellular delivery because most membrane proteins/receptors are taken up and recycled by cells. Therefore, extracellular labeling should be considered as an early event in the targeted intracellular delivery of bioconjugated QDs.

7.1 Antibodies and secondary antibodies

Site-specific extracellular labeling is essential for unraveling the functioning of cells and membrane proteins for which antibodies are ultimate candidates due to the high-specificity for antigen-antibody interactions. Membrane proteins in living cells can be labeled either directly with QD-antibody conjugates or indirectly with QD-secondary antibody conjugates. Indeed, labeling of cells with QD-antibody/secondary antibody conjugates means taking advantages of the unique optical properties of QDs by replacing fluorescent dyes in standard immunofluorescence methods. In this line, Chan and Nie tested the selectivity and effectiveness of QD-antibody conjugates for antigen detection by binding CdSe/ZnS QD-human immunoglobulin G (IgG) conjugate to a polychlonal antibody, but without any cell.[55] Applications of QD-antibody conjugate for cell labeling and imaging were first investigated by Winter *et al.* by targeting α_v portion of the $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins in human neurons (SK–N-SH).[128] At first, they labeled the α_v portion of the integrins with a primary antibody (*anti*-CD51), and subsequently labeled the primary antibody with CdS-IgG conjugate. Recently, antibody/secondary antibody-based labeling of cells using QDs has been widely appreciated for cell imaging applications such as simple imaging of cells,[72,129] selective detection and extended imaging of over-expressed receptors in cancer cells,[12,26,102,130–136] discriminating cancer cells from each other,[26,130,132] investigating the spatiotemporal distributions of receptors at ensemble and single-molecule levels,[136–140] and analyzing the growth and differentiation of cells.[72,141] Extracellular labeling using antibodies should fall within one of the following four categories (Fig. 15): (i) direct labeling of a target protein with a QD-primary antibody conjugate, (ii) labeling of a target protein first with a biotinylated primary antibody followed by a QD-streptavidin conjugate, (iii) labeling of a target protein first with a primary antibody followed by a QD-secondary antibody conjugate, or (iv) sequential labeling of a target protein with a primary antibody, biotinylated secondary antibody, and QD-streptavidin conjugate.

The usefulness of primary antibody for extracellular labeling using QDs was shown by Jaiswal *et al.* by labeling of the extracellular epitope of the multidrug transporter P-glycoprotein (Pgp) in HeLa cells.[72] In this work, cells were incubated either directly with a QD-primary antibody (clone 4E3 antibody for Pgp) conjugate, or first with biotinylated primary antibody, and then with avidin-coated QD. They prepared QD-avidin assembly by electrostatically coating avidin on DHLA-capped CdSe/ZnS QDs, and Pgp was expressed in HeLa cells by cotransfecting with a Pgp-eGFP construct. Jayagopal *et al.* have shown that QDs conjugated with monoclonal antibodies for cell adhesion molecules (CAM) such as platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are ideal candidates for extracellular labeling as well as fluorescence imaging of cells.[82] The QD-antibody

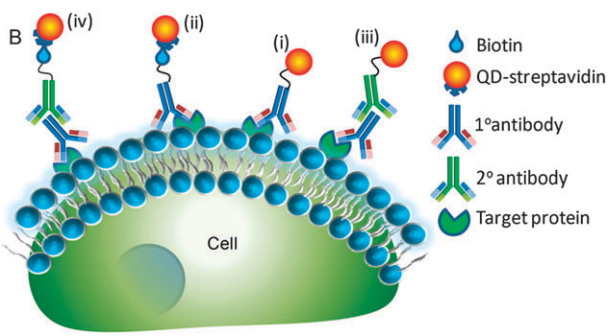

Fig. 15 Schematic presentation of antibody-based labeling of cells with QDs.

conjugates could be selectively attached to CAMs on TNF-α stimulated rat endothelial cells, leukocytes, or retinal endothelium. In contrast to these generalized approaches for cell labeling, QDs conjugated with antibodies to over-expressed Her2 receptor in many human breast cancer cell lines such as SK-BR-3,[12,102,130] MCF 7,[130,131,133] BT 474[131] and MDA-MB-231[131] have been extensively utilized for selective extracellular labeling. For example, Wu *et al.* accomplished selective labeling of Her2 receptor in SK-BR-3 cells by treating the cells with trastuzumab (herceptin), an *anti*-Her2 antibody, followed by QD-IgG conjugate.[12] Alternatively, the cells were incubated sequentially with humanized *anti*-Her2 antibody, biotinylated *anti*-human IgG, and QD-streptavidin conjugate. Conjugates of QDs and antibody were also tested for labeling prostate specific membrane antigen (PSMA), prostate stem cell antigen (PSCA) and Her2 receptor, which are over-expressed in human prostate cancer cells such as C4-2 and LNCaP.[26,133] Gao *et al.* accomplished selective extracellular labeling of PSMA-positive C4-2 cells by using QDs conjugated with the monoclonal *anti*-PSMA antibody; whereas, neither the conjugate recognized PSMA-negative human prostate adenocarcinoma cells (PC 3) nor QD alone could label C4-2 or PC 3 cells.[26] More recently, Barat *et al.* successfully labeled LNCaP cells by simultaneously targeting PSCA and Her2 receptor using NIR QDs conjugated with PSCA antibody and an *anti*-Her2 antibody fragment (*anti*-Her2 cys-diabody).[133] This report suggests that both small fragments of antibody and multiple targets can be exploited for effective detection of cancer cells. Similarly, Yezhelyev *et al.*[131] and Kawashima *et al.*[136] accomplished extracellular labeling of cancer cells such as MCF 7, BT 474, MDA-MB-231 and A431 by using a combination of QDs and *anti*-EGFR antibody. Other examples for extracellular labeling of cancer cells using QDs-antibody combinations are targeting of CD56 receptor in natural killer cell (NK92MI) using QD-*anti*-CD56 antibody conjugate,[134] and discriminating the levels of EGFR and *E*-cadherin (*E*-cad) in cancer cells derived from non-small cell lung carcinoma (NSCLC) and non-squamous cell carcinoma of the head and neck (SCCHN) by labeling the cells with *anti*-EGFR or *anti*-*E*-cad antibody followed by QD605-IgG conjugates.[132] More recently, Zhang *et al.* realized that CdTe QDs conjugated with an antibody (AVE-1642) to type 1 insulin-like growth factor receptor (IGF1R) is an ideal candidate for selective detection of over-expressed IGF1R in MCF 7 cells.[135]

Besides the above reports on labeling and imaging of cells, QD-antibody conjugates are extensively utilized for investigating the functioning of cells and biomolecules as well. For example, Dahan *et al.* have shown that QD-antibody conjugates can be utilized for understanding the diffusion dynamics of glycine receptors (GlyRs) in neurons at single-molecule resolution.[137] At first, they conjugated streptavidin-coated QDs with anti-mouse Fab fragments, which are primary antibodies to glycine receptor α1 subunits, and then accomplished the labeling of GlyRs in cultured spinal neurons. Blinking fluorescence of QDs was adopted as a marker for single QD-GlyR conjugates. Owing to the exceptional photostability of QDs, lateral diffusion of GlyRs between synaptic and extrasynaptic domains was realized by extended imaging at single-molecule level. Similarly, Chen *et al.* have shown that photostability of QDs is promising for extended imaging and analysis of integrin dynamics during osteogenic differentiation of human bone marrow derived progenitor cells (BMPC).[141] First, integrin receptors in cells were labeled using QDs by treating the cells with *anti*-integrin antibody followed by QD655-secondary antibody conjugate, and then osteogenic differentiation was induced. More recently, Bouzigues *et al.* utilized QD-antibody conjugates for real-time single-molecule imaging of GABA (A) receptors in rat spinal neuron membrane cells.[138] The γ2 subunit of the GABA receptor was labeled using QDs by treating neurons sequentially with guinea pig *anti*-γ2 antibody, biotinylated *anti*-guinea pig antibody, and QD605-streptavidin conjugate. Photostability of QDs facilitated the detection of transient interactions between receptors and microtubules as well as asymmetric receptor redistribution across growth cones. Although antibodies offer efficient labeling of extracellular proteins/receptors, cells gradually uptake QD-antibody-receptor complexes by receptor-mediated endocytosis. Thus, many reports related to labeling of cells using QDs and antibody are further discussed under targeted intracellular delivery of QD-antibody conjugates.

7.2 Ligands for fusion proteins

Despite the selectivity of QD-antibody conjugates for site-specific labeling of cells, antibodies are expensive. Furthermore, antibodies-conjugated to large nanoparticles such as QDs need not be equally active as in their native state. Thus, alternative methods for targeted extracellular labeling were sought after. A valuable alternative approach is the fusion of a 15-amino acid acceptor peptide (AP) to any target protein followed by selective biotinylation of the lysine side chain in AP by using the Escherichia Coli enzyme biotin ligase (BirA). Then, biotinylated AP can be labeled simply with QD-streptavidin conjugate (Fig. 16A). Thus, AP-BirA combination has received wide acceptance for selective labeling of membrane proteins. Howarth *et al.* introduced this approach to the QD field by fusing AP with CFP or EGFR in HeLa cells.[142] Also, they validated the versatility of this method by labeling the tetrameric α-amino-3-hydroxy-5-methyl-4-isoxasolepropionate (AMPA) glutamate receptor (GluR2) in hippocampal neurons at ensemble and single-molecule levels. For this labeling, the neurons were first cotransfected with AP-GluR2, and the synaptic marker postsynaptic density (PSD)-95 was fused with YFP. Then, AP was biotinylated using BirA and successively labeled with QD-streptavidin (Fig. 16B). Subsequently, AP-BirA combination was utilized for labeling QDs to various membrane proteins such as glutamate receptor, neuroligin and EGFR in HeLa cells,[143] and type I interferon receptor in COS 7 cells.[144]

As in the case of the AP-BirA pair, selective labeling of fusion proteins such as cutinase, polyhistidine, and HaloTag by using corresponding ligands is an alternative method for antibody-based labeling. Bonasio *et al.* accomplished the fusion of cutinase to integrin lymphocyte function-associated antigen 1 (LFA-1) in K562 cells, and then targeted cutinase by using QDs conjugated with p-nitrophenyl phosphonate (pNPP).[145] Here, selective binding of pNPP to LFA-1 was

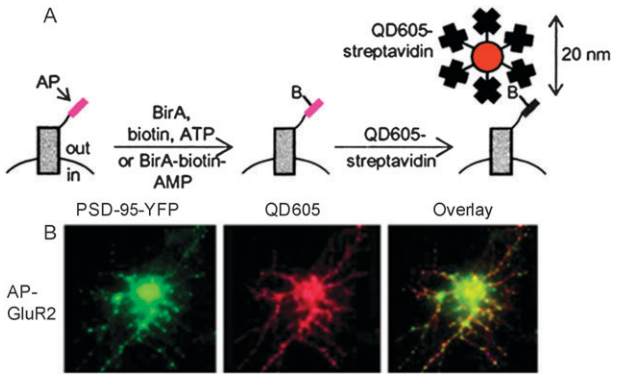

Fig. 16 (A) General scheme for targeting cell surface proteins with the AP-BirA combination, and (B) fluorescence images of a neuron expressing AP-GluR2 and the synaptic marker PSD-95-YFP. Here, AP was biotinylated using BirA and subsequently labeled with QD-streptavidin. Taken from ref. 142.

used for real-time imaging of LFA-1-assisted cell migration. Similarly, Kim *et al.* accomplished selective labeling of a hexahistidine sequence that was genetically fused to the extracellular domain of 5HT2C serotonin receptor in HEK-293 cells.[146] In this approach, they used QD-Ni-nitrilotriacetic acid (QD-Ni-NTA) conjugate as the ligand for hexahistidine. The key in the Ni-NTA-based targeting is that Ni ion can complex with polyhistidine. Recently, Roullier *et al.* and Dif *et al.* applied the Ni-NTA-based targeting method for labeling a decahistidine unit fused with CFP in HeLa cells[144] or polyhistidine-tagged (histag) proteins inherent to HeLa cells.[147] In an analogous approach, So *et al.* accomplished extracellular labeling of living COS 7 cells with QDs by expressing an engineered bacterial enzyme, haloalkane dehalogenase, also called HaloTag protein (HTP), followed by incubating the cells with QD-HaloTag conjugate.[148]

7.3 Peptides for membrane proteins

Peptides are cost-effective and stable ligands for selective and efficient labeling of cell; however, specific peptides and targets are limited for such labeling purposes. Peptides containing arginine-glycine-aspartic acid (RGD) sequence offer selective labeling of $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins which are over-expressed in angiogenetic endothelial cells and many cancer cells including metastatic cancer cells. Winter *et al.*[128] and Mulder *et al.*[149] utilized QD-RGD conjugates for selective labeling of $\alpha_v\beta_3$ integrins in SK–N-SH cells and human umbilical vein endothelial cells (HUVEC). In this labeling, RGD peptides were found to be equally good as a combination of *anti*-CD51 antibody and QD-IgG conjugate.[128] Subsequently, Cai *et al.* demonstrated selective detection of cancer cells using CdTe/ZnS QD-RGD conjugates by targeting and imaging over-expressed $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins in human breast cancer cells (MDA-MB-435) and human glioblastoma cells (U87MG), but MCF 7 cell which does not express $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrin.[28] Akerman *et al.* accomplished extracellular labeling using QDs-conjugated with peptides such as CGFECVRQCPERC (GFE) that targets the membrane dipeptidase in endothelial cells, KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK (F3) that targets blood vessels and tumor cells, and CGNKRTRGC (LyP-1) that targets lymphatic vessels and tumor cells.[29] With the QD-GFE conjugate, they could selectively label lung endothelial cells. On the other hand, neither QD-GFE conjugate could label brain endothelial cells nor QD-LyP-1 conjugate could label lung endothelial cells. Similarly, by using QD-F3 and QD-LyP-1 conjugates, but QD-GFE, they could label MDA-MB-435 cells. In another example for peptide-based labeling, Pinaud *et al.* have shown that QDs conjugated with a series of synthetic peptides related to phytochelatin can target certain extracellular proteins.[150] First, they attached hydrophobically-modified C-terminus of the peptides to hydrophobic QDs, and then biotinylated N-terminus of the peptide was used for labeling glycosylphosphatidylinositol (GPI)-anchored avidin-CD14 chimeric proteins expressed in HeLa cells. Thus, although these synthetic peptides could bridge between QD and cell surface, this method is equivalent to biotin-avidin based labeling. While the potentials of various peptides for intracellular delivery of QDs and other nanoparticles are being investigated more and more, QD-RGD conjugates rank top in their labeling efficiency.

7.4 Ligands for membrane proteins

Ligands such as growth hormones are natural entities for specific labeling of extracellular proteins/receptors. Thus, QD-ligand conjugates have been extensively employed for labeling and imaging of cells, and investigating the functions of membrane proteins at ensemble and single-molecule levels. Examples of ligands conjugated with QDs are EGF for EGFR,[83,136,151,152] nerve growth factor (NGF) for the receptor tyrosine kinase A (TrkA),[153–155] integrin for integrin receptor,[141] neuroligin-1β(Nrx1β) for neurolignin-1 (Nlg1),[156] hyaluronic acid (HA) for CD44/lymphatic vessel endothelial receptor (LYVE-1),[157] single-stranded GBI-10 aptamer for extracellular matrix protein tenascin-C in glioma cells,[158] *anti*-PSMA aptamers for PSMA positive prostate cancer cells,[159] MHC-I viral pepticle for the T-cell receptor phycoerythrin,[160] the antagonist muscimol for rho 1 GABAC receptor,[81] and toxins such as cholera toxin (CTX) for MMP-2 and dendrotoxin-1 (DTX-1) for shaker related voltage gated potassium channel (Kv1.1).[161] A few examples for extracellular labeling using QD-ligand conjugates and potential applications of such conjugates are summarized below.

7.4.1 EGF. Specific binding of EGF to EGFR has been utilized for labeling cell membrane with fluorescent dyes or QDs and analyze the activation dynamics of EGFR. Lidke *et al.* utilized QD-EGF conjugates for targeting erb1-eGFP or erb3-mCitrine in Chinese Hamster Ovary (CHO) cells or A431 cells.[151] The QD-EGF conjugate, which was prepared by mixing QD-streptavidin conjugate with biotinylated-EGF, is an ideal candidate for the detection of heterodimerization between erb1 and erb2 (Fig. 17). Also, owing to the exceptional photostability of QDs, the QD-EGF conjugate could be used for detecting the courses of EGF-QD to erb1 binding and retrograde transport of QD-EGF-erb complexes from filopodia to the cell body, both at single-molecule level. Subsequently, Liu *et al.* and Diagaradjane *et al.* utilized QDs for real-time

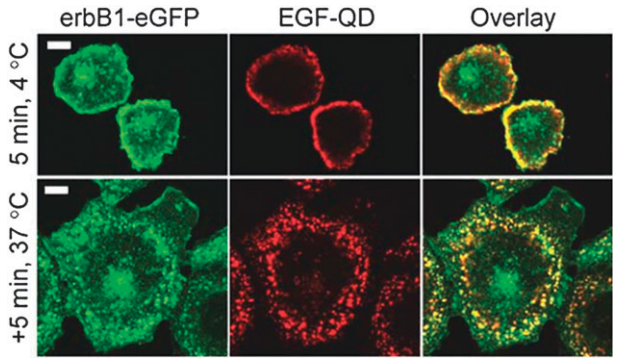

Fig. 17 Fluorescence images of CHO cells expressing erbB1-eGFP. The cells were exposed to 7 nM solution of biotin-EGF for 10 min at 4 °C and subsequently labeled with QDs. Lower panel, images after 5 min at 37 °C. Scale bars, 20 μm. Reprinted by permission from McMillan Publishers Ltd: [Nature Biotechnology], ref. 151, copyright 2004.

tracking of EGFR-EGF complex in HeLa cells,[83] human colon cancer cells,[152] and Xenograft tumors in mice.[152]

Recently, by extended single-molecule fluorescence imaging and FRET analysis using QD605-labeled EGF and Cy5-labeled *anti*-EGFR antibody (Ab11), we have shown for the first time that the lateral propagation of EGFR activation takes place through reversible association of a heterodimer [EGF-$(EGFR)_2$] with predimers [$(EGFR)_2$].[136] On the other hand, without extended single-molecule imaging using photostable QDs, reversible receptor dimerization in the lateral activation of EGFR remained obscured in previous investigations at single-molecule and ensemble levels. At first, we characterized the activation of EGFR by colocalizing Cy5-Ab11 and QD-EGF in A431 cells. We found large aggregates of EGFR in cells that are activated with >2 nM QD-EGF (Fig. 18A); whereas, heterodimers and signaling dimers [$(EGF\text{-}EGFR)_2$] were uniformly distributed in cells activated with sub-nanomolar QD-EGF solutions (Fig. 18B). Interestingly, EGFR single-molecules traced both linear and random trajectories which extended up to 3 μm, validating the presence of large micro-domains for EGFR activation. The linear trajectories traced by QD-EGF-EGFR complexes support previous reports on the binding of predimers to cytoskeleton. We could detect long-range and reversible propagation of EGFR activation, owing to a combination of the exceptional photostability of QDs and time- and distance-correlated single-molecule imaging.

Conversely, in previous reports, photobleaching of organic dyes and fluorescent proteins was a limitation in the detection and analysis of long-range and reversible activation of EGFR. Also, we could detect transient increases and decreases (Fig. 18C) in the fluorescence intensities of single-molecules, indicating that two heterodimers have associated into a signaling dimer, which was subsequently dissociated into two heterodimers. Reversible association of heterodimers was further characterized using FRET from QD-EGF-EGFR to Cy5-Ab11-EGFR. Intermolecular interactions among heterodimers, predimers and signaling dimers are shown in Fig. 18D. In short, by analyzing fluorescence images, intensity trajectories and FRET of single-molecule we found that reversible association of heterodimers into homodimers stimulates multiple signaling in cells. The reversible dimerization of EGFR revealed by single-molecule

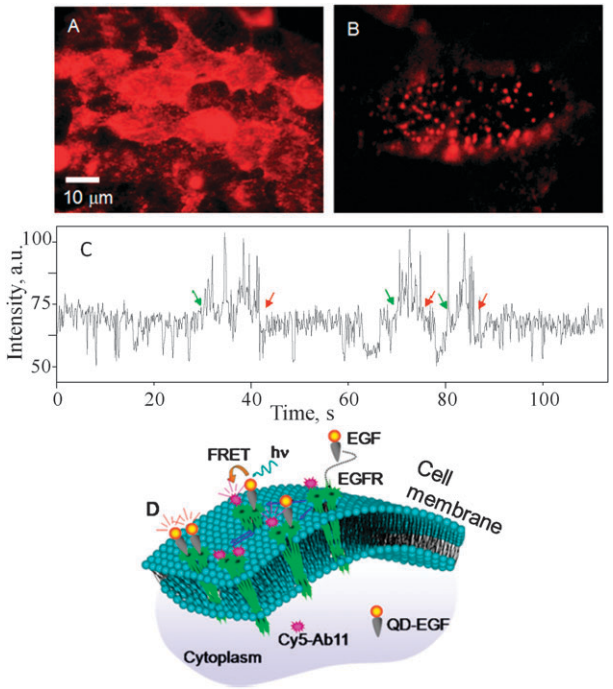

Fig. 18 (A,B) Fluorescence images of A431 cells incubated with (A) 2 nM and (B) 0.5 nM QD-EGF conjugate solutions, (C) single-molecule fluorescence intensity trajectory of a heterodimer under reversible association with another heterodimer. The green arrows in 'C' indicate association of two heterodimers and the red arrows indicate dissociation of a signaling dimer, (D) schematic presentation of intermolecular interactions among predimers, heterodimers and signaling dimers in A431 cell. Reprinted with permission from ref. 136; copyright (2010), Wiley-VCH Verlag GmbH & Co.

imaging using quantum dots, which we first submitted to Angewandte Chemie International Edition in January 2009, is further supported by a similar single-molecule study that was recently reported by Schlessinger and co-workers.[181]

7.4.2 NGF. Analogous to the binding of QD-EGF to EGFR, QD-NGF conjugates can selectively bind to TrkA and label neurons. For example, Vu *et al.* successfully detected downstream signaling and neuronal differentiation in PC 12 cells by activating TrkA with QDs conjugated to the β subunit of NGF (βNGF).[153] Here, functional bioassays of neurite growth indicated that the level of cell signaling by QD-βNGF is lower than that by βNGF. Similarly, by activating TrkA or p75 NGF receptor in PC 12 cells with QD-NGF or QD-ricin toxin A (RTA) conjugate, Rajan *et al.* evaluated endocytosis, cytoplasmic redistribution, and shuttling of QD-NGF-receptor complexes at ensemble and single-molecule levels.[154,155] In particular, they found that extended imaging of QD-NGF-TrkA single-molecule complexes can stage distinct endocytic phases and cytoplasmic transport of TrkA with high spatial and temporal resolutions.[155]

7.4.3 Aptamers. Aptamers are oligonucleic acids which selectively bind with certain target molecules in cells. Aptamers show targeting efficiencies equivalent to that of antibodies, and are stable and cost-effective substitutes for certain antibodies.

Thus, QD-aptamer conjugates have been used for labeling particular membrane proteins.[158,159,162] For example, Chu *et al*. and Bagalkot *et al*. utilized CdSe/ZnS or CdTe QDs conjugated with an A9[159] or an A10[162] aptamer for extracellular labeling of PSMA positive LNCaP cells. On the other hand, PSMA-negative PC 3 cells were not labeled by the conjugates. For this labeling, the A9 aptamer was first biotinylated using biotin hydrazide, and then either conjugated to streptavidin-coated CdSe/ZnS QD or linked to biotinylated CdTe QD through streptavidin bridge. The A10 aptamer was cross-linked to carboxylic acid functionalized CdSe/ZnS QD by carbodiimide coupling (Fig. 6). The QD-aptamer conjugates were found to be equally efficient as QD-PSMA antibody conjugate for the selective labeling of PSMA-positive prostate cancer cells. With time under incubation, the QD-aptamer conjugates were delivered in the cytosol by receptor-mediated endocytosis,[162] which was examined by FRET analysis. For FRET analysis, fluorescence of the QD-aptamer conjugate was initially quenched by attaching doxorubicin (Dox) to QD; however, in the cytosol, Dox was released from QD within 90 min after incubation, which prevented FRET and resulted in the recovery of emission from QD.

7.4.4 Annexins. Annexins are a family of cellular proteins involved in various vital cell functions such as exocytosis, membrane organization, trafficking, apoptosis, inflammation, coagulation, and fibrinolysis. Although more than a dozen annexin proteins have been identified, investigations of QD-annexin conjugates are limited to annexin V/A5, which is an important marker for apoptosis.[163–165] Thus, QD-annexin conjugates are used for labeling apoptotic cells. For example, van Tilborg *et al*. detected apoptosis in Jurkat T-lymphoma cells by using a multimodal QD-annexin A5 conjugate.[163] Apoptosis was either inherent to the cells or induced by treating the cells with *anti*-Fas (CD95). Selective labeling of apoptotic cells by QD-annexin A5 conjugate was detected by fluorescence as well as magnetic imaging. The key in this detection is that in the presence of Ca^{2+} annexin A5 selectively binds to phosphatidylserine, which is located in the outer membrane of apoptotic cells. In normal cells, phosphatidylserine is located in the inner membrane and it will be externalized during apoptosis. Similarly, by using QD-annexin V conjugate, Le Gac *et al*. detected apoptotic human promyelocytic leukemia cells (HL 60) in which apoptosis was induced by either irradiating with UV light or treating with apoptosis drugs such as etoposide, camptothecin, TNF-α or cycloheximide.[164]

8. Targeted intracellular delivery

Targeted intracellular delivery of QDs is essential for extended imaging of the structures of subcellular organelles and the functions of intracellular molecules. Receptor-mediated endocytosis is the main strategy for targeted intracellular delivery of QDs. Membrane receptors tagged by QD-antibody or QD-ligand conjugates will be rapidly endocytosed by the activation of receptors. In such cases, QD-antibody/QD-ligand conjugates are taken up by receptor-mediated endocytosis. QDs trapped in endosomes will be transported by microtubules and actin filaments to the lysosome organization center, and degraded under enzymatic reactions or acidic conditions in the endolysosomal compartments. Thus, endosome-disrupting coating will be necessary for intracellular targeting. Here we will summarize various antibodies and ligands used for targeted intracellular delivery of QDs.

8.1 Antibodies

Antibodies to membrane receptors or subcellular organelles have been extensively utilized for both intracellular delivery of QDs and subcellular labeling. While membrane permeability is indispensable for the intracellular delivery of QDs conjugated with antibodies to intracellular proteins, membrane receptors labeled with QD-antibody conjugates can be taken up by live cells by way of receptor-mediated endocytosis. Wu *et al*. elegantly demonstrated the intracellular and intranuclear delivery of bioconjugated multicolor CdSe/ZnS QDs in human epithelial cells or 3T3 cells by sequentially incubating fixed cells with primary antibody, biotinylated secondary antibody, and QD-streptavidin conjugate.[12] Bioconjugated-QDs were prepared by successively coating CdSe/ZnS QDs with poly(acrylic acid) (PAA), cross-linking PAA using lysine or PEG-lysine, and conjugating lysine to streptavidin. Labeling of microtubules in 3T3 cells was accomplished by sequentially incubating the cells with monoclonal *anti*-α-tubulin antibody, biotinylated anti-mouse IgG, and streptavidin-QD conjugate (Fig. 19A). Similarly, labeling of nuclear antigen in human

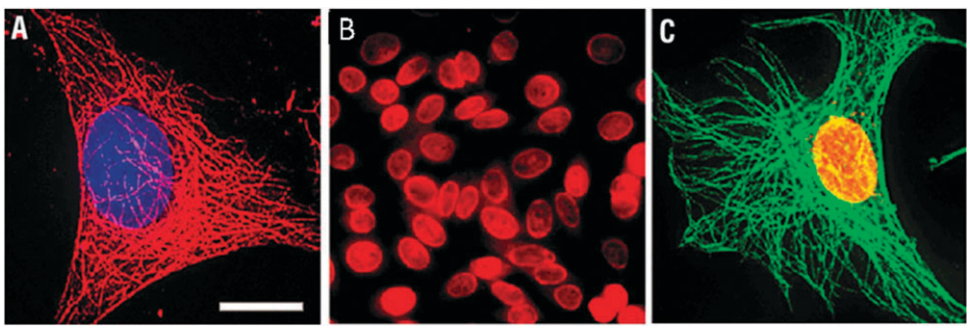

Fig. 19 (A) Fluorescence image of a 3T3 cell treated sequentially with monoclonal *anti*-α-tubulin antibody, biotinylated anti-mouse IgG, and QD630–streptavidin. (B) Fluorescence image of human epithelial cells treated sequentially with antinuclear antigen, *anti*-human IgG–biotin, and QD630–streptavidin. (C) Fluorescence image of a 3T3 cell treated sequentially with antinuclear antigen, *anti*-human IgG–biotin, and QD630–streptavidin (red); microtubules are labeled with QD535 as described in the case of (A). Reprinted by permission from McMillan Publishers Ltd: [Nature Biotechnology], ref. 12, copyright 2003.

epithelial cells (Fig. 19B) and 3T3 cells (Fig. 19C) was accomplished by sequentially incubating fixed cells with an antibody to human *anti*-nuclear antigen, biotinylated *anti*-human IgG, and QD-streptavidin conjugate. More recently, Yezhelyev *et al*. utilized QD-antibody conjugates for the simultaneous detection of cytoplasmic and nuclear marker proteins such as the target of rapamycin (mTOR), estrogen receptor (ER), and progesterone receptor (PR) in MCF 7, BT-474 and MDA-MB-231 cells.[131] Owing to the brightness and photostability of QDs, they could detect low levels of mTOR located in the cytoplasm and ER and PR located in the nucleus both with high-sensitivity.

In contrast to the above mentioned examples for intracellular labeling of fixed cells, QD-antibody conjugates have been extensively utilized for intracellular labeling of live cells through receptor-mediated endocytosis. For example, Tan *et al*. accomplished non-viral delivery of siRNA using chitosan nanoparticles that are conjugated with *anti*-Her2 antibody and doped with CdSe/ZnS QD.[166] Nanoparticles were first attached to Her2-positive SK-BR-3 cells, but Her2-negative MCF 7 cells, and subsequently taken up by receptor-mediated endocytosis. Similarly, Tada *et al*. accomplished the intracellular delivery of QD800-herceptin conjugate in Her2 positive human breast carcinoma cells (KPL-4).[167] Also, they utilized QD-herceptin conjugate for *in vitro* and *in vivo* detection of single-molecule transport of QD-herceptin conjugate along the cell membrane and into the perinuclear space. More recently, Zhang *et al*. successfully delivered CdTe QDs conjugated with an antibody (AVE-1642) to type 1 insulin-like growth factor receptor (IGF1R) in MCF 7 cells.[135] They found that the QD-antibody conjugate first attaches to the cell membrane, and then enter the cytosol and nucleus. Recently, Cambi *et al*. introduced somewhat different approach for the intracellular delivery of QDs by using virus-mimicking nanoparticles which are labeled with dendritic cell-specific pathogen-uptake receptor (DC-SIGN).[168] The nanoparticles were prepared by conjugating biotinylated *anti*-DC-SIGN antibody to streptavidin-coated QD525, QD605 or QD655. The QD-antibody conjugates could be readily attached to CHO cells and gradually delivered into the cytosol by DC-SIGN-mediated endocytosis. Also, contributions by clathrin- and caveolae-dependent endocytosis of the conjugates were evidenced by colocalizing the QD-nanoparticle assembly with labels for clathrin-coated pits and caveolin-1. Prasad and coworkers utilized Claudin 4 and PSCA, which are over-expressed antigens in pancreatic cancer cells (MiaPaCa, CoLo-357/Panc-1), as the targets for receptor-mediated endocytosis of QDs.[169,170] They found that CdSe/CdS/ZnS QDs conjugated with *anti*-claudin 4 antibody can be efficiently delivered in MiaPaCa, CoLo-357, and Panc-1 cells.[169] Here, receptor-mediated endocytosis of the conjugate was realized by colocalizing the conjugate with QD-transferrin conjugate. Recently, they extended investigations into intracellular labeling of pancreatic cancer cells by replacing CdSe/CdS/ZnS QDs with less toxic InP/ZnS QDs.[170] Conjugates of InP/ZnS QDs and *anti*-claudin 4 antibody or *anti*-PSCA antibody were efficiently delivered in MiaPaCa cells (Fig. 20) in which the corresponding antigens are over-expressed.

Other antibodies tested for the intracellular delivery of QDs are *anti*-human α-fetoprotein (AFP) antibody and αCD3 antibody. AFP is a plasma protein produced by the liver, yolk sac, and gastrointestinal tract of human fetus, but it is a marker for liver cancer in adults. Chen *et al*. accomplished selective intracellular delivery of CdSe/ZnS QDs in human metastatic hepatocarcinoma cells (HCCLM6) by conjugating QDs with *anti*-human AFP antibody.[171] First, TGA-coated QDs were conjugated with the antibody by carbodiimide cross-linking, and then selectivity of the QD-antibody conjugate for AFP positive HCCLM6 cells was validated by using AFP-negative human colon carcinoma cells (SW480) as control. Recently, Bottini *et al*. utilized αCD3 antibody for the intracellular delivery of QD-doped silica nanoparticles in human Jurkat T cells.[172] The QD-silica-antibody conjugate was prepared by biotinylating the amino groups in both silica nanoparticles and αCD3 antibody, and subsequently bridging them together through neutravidin. Receptor-mediated endocytosis of the conjugate was substantiated by the intracellular fluorescence in Jurkat T cells at 37 °C; on the other hand, the conjugate was not delivered in Jurkat T cells at 4 °C or CD3-negative HeLa cells at 37 °C.

8.2 Ligands

Ligands for membrane proteins/receptors are potential candidates for the preparation of QD bioconjugates and receptor-mediated intracellular delivery of the conjugates. Activation of receptors by corresponding ligands results in the clustering of receptors followed by clathrin-mediated endocytosis of receptor-ligand complexes. Thus, ligands such

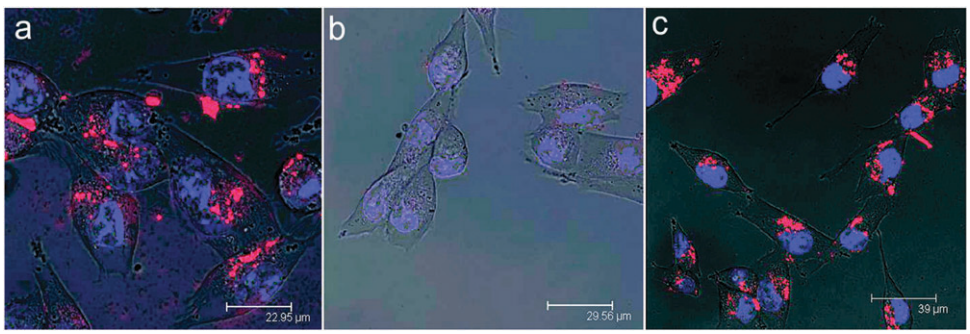

Fig. 20 Confocal microscopic images of MiaPaCa cells: (a) cells incubated with InP/ZnS QDs conjugated with *anti*-claudin 4 antibody, (b) cells incubated with unconjugated InP/ZnS QDs, and (c) cells incubated with InP/ZnS QDs conjugated with *anti*-PSCA antibody. Reprinted with permission from ref. 170; copyright (2009), American Chemical Society.

as transferrin, EGF, NGF, folic acid, wheat germ agglutinin (WGA), and certain sugars have been utilized for the intracellular delivery of QDs. Chan and Nie accomplished targeted intracellular delivery of bioconjugated QD for the first time by using CdSe/ZnS QDs conjugated to transferrin,[26] which is a carrier protein for iron. The QD-transferrin conjugate was efficiently taken up by HeLa cells by transferrin receptor-mediated endocytosis. On the other hand, control QDs conjugated with TGA remained stuck at the cell membrane. Transferrin receptor functions to maintain cellular iron homeostasis through intracellular delivery of transferrin-iron complex. Thus, transferrin receptor-mediated endocytosis is common for most cells. Because the endocytosis of transferrin receptor is well characterized, QD-transferrin conjugate can be used even a standard endosome marker.[105,170,173,174]

Like transferrin receptor-mediated endocytosis of QD-transferrin conjugates, EGFR-mediated endocytosis can be utilized for the intracellular delivery of QD-EGF conjugates in almost all cells. For example, Derfus *et al*. accomplished efficient intracellular delivery of QD-EGF conjugates in rat hepatocytes by EGFR-mediated endocytosis.[120] The endocytosed QD-EGF conjugates were continuously tracked for analyzing the migration of micropatterned hepatocytes. Soon after, Lidke *et al*. utilized QD-EGF conjugate for targeting the transmembrane receptors erb1-eGFP or erb3-mCitrine in CHO or A431 cells.[151] Due to the exceptional photostability of QDs, they could follow receptor-mediated endocytosis of QD-EGF-EGFR complexes and intracellular trafficking of endosomes encompassing the complex. In addition to the intracellular delivery of QD-EGF conjugates, Kim *et al*. took a step ahead by accomplishing endosomal escape of QD-EGF using an endosome disrupting poly(D,L-lactide-*co*-glycolide) coat on QD (QDNC).[102] The NC coat facilitated pH-dependent endosomal escape of QD-EGF conjugate in SK-BR-3 cells.

Intracellular delivery of QDs in nerve cells can be accomplished by conjugating QDs to NGF and subsequent endocytosis mediated by TrkA- or p75.[153–155,175] Further, endocytosed QD-NGF conjugates can be utilized for extended imaging of neuronal differentiation, neurite growth, and intracellular receptor trafficking. Vu *et al*. found that QDs conjugated to the β subunit of neuronal growth factor (βNGF) activate TrkA receptors in PC 12 cells, initiate downstream signaling and neuronal differentiation, and uptake by TrkA-mediated endocytosis.[153] However, the level of downstream cell signaling by QD-βNGF was lower than that by βNGF. The low signal level was probably due to the bulkiness of QDs. Rajan *et al*. utilized QD-NGF and QD-RTA conjugates for TrkA-mediated endocytosis of QDs in PC 12 cells.[154,155] These conjugates, owing to the exceptional photostability of QDs, facilitated the imaging of endocytosis and spatiotemporal distribution and transportation of endosomes up to 4 days.[154] Nonetheless, considerable decrease in the density of intracellular QD was detected 18 h after incubation, which is due to either exocytosis or photobleaching of QDs. Also, they could detect active transport of QD-NGF-TrkA complex along microtubules to far-reaching subcellular regions and newly created regions. Cui *et al*. have shown that QD-NGF conjugates can be delivered in a compartmentalized culture of rat dorsal root ganglion and utilized for tracking retrograde axonal transport of vesicles.[175] Single-molecule studies in this report have shown that only a single NGF dimer is sufficient to sustain signaling during the axonal transport. Also, they found that vesicle transport along axons takes place in stop-and-go mode, without being affected by the concentration of QD-NGF.

Folate receptor over-expressed in many cancers including the cancers of the ovary, prostate, brain, column, nose, and throat is an ideal target for both receptor-mediated endocytosis of QD-folic acid conjugate and the detection of cancer cells. Bharali *et al*. accomplished efficient intracellular delivery of folic acid-conjugated InP/ZnS QDs in KB cells through folate receptor-mediated endocytosis.[48] On the other hand, cancer cells such as A549 and MCF 7, which are folate receptor-negative, were not labeled by QD-folic acid conjugate. Recently, Gao *et al*. have shown that CdSe/ZnS QDs conjugated with WGA can be delivered in heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2) by WGA receptor-mediated endocytosis.[176] Interestingly, the QD-WGA conjugates are taken up by caveolae- and clathrin-dependent endocytosis, which were verified by inhibiting clathrin-mediated endocytosis with chlorpromazine and caveolae-mediated endocytosis with filipin. The endocytosed QD-WGA conjugates were further transported by microtubules and actin filaments to the Golgi apparatus and lysosome. More recently, Kikkeri *et al*. found that D-galactose can effectively deliver CdSe/ZnS QDs into the cytosol of hepatocellular carcinoma (HepG2) cells by asialoglycoprotein receptor-mediated endocytosis.[84]

8.3 RGD peptide

RGD peptides are ligands for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Therefore, peptides carrying RGD sequences were utilized for integrin-mediated intracellular delivery of QDs.[149,174,177–179] Among these two integrins, $\alpha_v\beta_3$ is over-expressed in the membrane of angiogenetic endothelial cells and many cancer cells, in particular, metastatic cancer cells. Mulder *et al*. conjugated RGD peptide to CdSe/ZnS QDs which was coated with paramagnetic ligands and PEGylated lipids, and used the conjugate as a bimodal probe for imaging $\alpha_v\beta_3$ integrin in HUVEC cells.[149] The conjugate was initially bound to the membrane of proliferating HUVEC cells, and then internalized, which was detected by fluorescence and MRI imaging. Subsequently, Lieleg *et al*. found that the efficiency of intracellular delivery of QD-RGD conjugate depends on the spacer in between QD and RGD.[179] More recently, Koole *et al*. utilized RGD peptide for the intracellular delivery of microemulsions encompassing silica/PEG shelled CdSe/CdS/CdZnS/ZnS QDs in HUVEC cells.[174] They found that the efficiency of intracellular delivery can be improved by conjugating multiple copies of RGD to QDs.

8.4 Toxins

Bacterial toxins such as Shiga toxin and cholera toxin B (CTB) and the plant toxin ricin bind to certain receptors in mammalian cells and facilitate receptor-mediated endocytosis of QDs. Tekle *et al*. found that Shiga toxin subunit B or ricin toxin subunit B can be an ideal candidate for the intracellular

delivery of QDs in HeLa cells.[173] Conjugates of QDs with these toxins were initially attached to corresponding membrane receptors in HeLa cells and subsequently taken up by receptor-mediated endocytosis. Despite their efficient intracellular delivery, these conjugates showed poor intracellular trafficking and recycling, warning that QD-toxin conjugates delivered in the cytosol can cause adverse physiological consequences. Chakraborty *et al.* accomplished efficient intracellular delivery of CdSe/ZnS QDs in a number of cell lines such as NIH3T3, mouse muscle derived cells (MDSC), human mesenchymal stem cells (hMSC), human melanoma cells (M21), and mouse tetra carcinoma cells (MH15) by conjugating QDs with CTB.[180] Like proteins, amino groups in CTB can be conjugated to carboxylic acid-functionalized QDs by simple carbodiimide cross-linking. The QD-CTB conjugate initially binds to gangliosides, which are complexes composed of a glycosphingolipid and exist in lipid rafts in the cell membrane of essentially all mammalian cells, and then taken up by caveolae-dependent endocytosis. They utilized QD-CTB conjugate for extended imaging of mesenchymal stem cells, and realized that the endocytosed QD does not affect the differentiation potential of the cells. The advantages of QD-CTB conjugate are two fold: the extent of its intracellular aggregation is less than that of QDs conjugated with several other peptides, and endolysosomal degradation of QDs can be bypassed by caveolae-dependent endocytosis.

9. Conclusions and prospects

This review article introduces a variety of methods for interfacing QDs with cells, and the versatility of QDs conjugated with polymers, proteins, peptides, antibodies and ligands as bright and stable fluorescent markers for targeted and nonspecific imaging of cells, subcellular organelles, and single-molecules. We have included summary of bioconjugate reactions as well as general strategies for labeling cells in this article with an intention to draw an overall picture about the current status and usefulness of QDs for bioimaging. Yet another aim of this review is to gather information about bioconjugate methods, biomolecules, and strategies for extracellular and intracellular labeling in the field of QDs so that it can provide nano reviews on each topic to be utilized for various applications in the fields of cell biology and biotechnology. Now, synthesis, optical properties and surface chemistry of QDs are all well-optimized that preparation of bioconjugated QDs is not a problem anymore. Also, ready-to-label QD-bioconjugates of any color have become commercially available. Having such a well-built background for QDs, we expect that the information provided in this review will be useful for introducing novel *in vitro* and *in vivo* biological applications of QDs, utilizing QD bioconjugates for investigating complex biophysical and biochemical problems, and deriving novel hybrid nanomaterials by interfacing QDs and bioconjugated QDs with organic, semiconductor and metal nanoparticles.

It is needless to state the influence of semiconductor QDs in the nanoscience, nanotechnology, nanobiotechnology, and single-molecule research areas, the 3NS field. The property of QD important for bioimaging is its bright and stable fluorescence of our choice, which provides us confidence to surf on cells, excavate through the cell membrane, swim in the cytoplasm, light up subcellular organelles, and untangle complex structures and functions of cell organelles and biomolecules. For this endeavor, QDs offer ample surface area for gathering multiple tool boxes such as sensors and tags. Although toxicity of QDs is a matter of considerable discussion, the focus of QDs in biology has been changing to multiplexed and multimodal imaging *in vivo*, in particular, in the field of non-invasive imaging of cancers. Priceless efforts are being invested by many researchers with uncompromised intentions to resolve toxicity of high-quality QDs and develop non-toxic QDs in order to offer all the advantages of QDs to biomedical imaging and therapeutic interventions.

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