Semiconductor Quantum Dots for in vitro Diagnostics and Cellular Imaging

Article *in* Trends in Biotechnology · May 2012

DOI: 10.1016/j.tibtech.2012.04.005 · Source: PubMed

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Review

Semiconductor quantum dots for *in vitro* diagnostics and cellular imaging

Zongwen Jin and Niko Hildebrandt

Université Paris-Sud, Institut d'Electronique Fondamentale, Orsay, France

The need for companion diagnostics, point-of-care testing (POCT) and high-throughput screening in clinical diagnostics and personalized medicine has pushed the need for more biological information from a single sample at extremely low concentrations and volumes. Optical biosensors based on semiconductor quantum dots (QDs) can answer these requirements because their unique photophysical properties are ideally suited for highly sensitive multiplexed detection. Many different biological systems have been successfully scrutinized with a large variety of QDs over the past decade but their future as widely applied commercial biosensors is still open. In this review, we highlight recent *in vitro* diagnostic and cellular imaging applications of QDs and discuss milestones and obstacles on their way toward integration into real-life diagnostic and medical applications.

QDs in biotechnology

Exploiting the bright and colorful photophysical properties of semiconductor QDs is an ongoing trend in biotechnology, and QDs are used, for example, in biosensors, cellular and tissue imaging, cancer cell targeting, *in vitro* diagnostics (IVD, see Glossary) and *in vivo* imaging [1–5]. Many QD-based biological studies have brought up many specific advantages as well as limitations. Although toxicity of QD materials has led to a controversial debate, researchers remain optimistic about this young field; material scientists as well as chemists are developing better packaging strategies and novel solutions to replace current troublesome compositions. Size-dependency is also a concern for most nanomaterials and has led to the new discipline of nanotoxicology. Nevertheless, the unique properties of QDs (Box 1) provide important advantages for fluorescence-based biotechnology. In this review, we highlight recent trends and applications of QDs in IVD and cellular imaging, and introduce the most important functionalization schemes and design criteria of QD bioconjugates (Box 1). We also highlight limitations and future challenges for facilitating QD applications in advanced biological and medical research.

QDs for IVD

The three most important characteristics of QDs for IVD are: (i) their high brightness and photostability; (ii) their color-tunability and narrow emission bands; and (iii) their shape and size. These unique properties provide significant advantages toward the paramount requirements of IVD, namely sensitivity, selectivity and multiplexing (the simultaneous measurement of multiple biomarkers). Several biomolecules can be conjugated to a QD, thus offering multiplexing of different biomolecules on the same QD and/or signal amplification. The color-tunable and narrow emission bands permit optical multiplexing of several markers with very high selectivity because the different QD emission bands show much less spectral overlap compared to conventional organic dyes or fluorescent proteins. Several QD-based assay methods [7–10] aiming at the detection of various biomarkers such as proteins [11–13], metabolites [14–16] or nucleic acids [17] have been developed over the past few years.

Point-of-care assays

An important field of IVD is POCT and/or small laboratory testing. One example of such assay formats are lateral flow test strip (LFTS) assays. Gold nanoparticles (AuNPs) are among the most popular signal transducers in LFTS assays. They are usually read out by colorimetric (absorption)

Glossary

FFPE – formalin fixed and paraffin embedded: FFPE tissues are usually thin slices of tissue, which are preserved directly after dissection or biopsy for histological investigations. The formalin-fixed tissue is embedded into paraffin to cut it into fine sections, which can then be stained for IHC analysis.
FISH – fluorescence *in situ* hybridization: A technique used in cellular fluorescence imaging to detect and localize target DNA sequences on chromosomes by hybridization with a specific fluorophore-labeled probe DNA fragment.
FRET, CRET, NSET, RET: See Box 2.
H&E – hematoxylin and eosin: H&E stain is a staining method for histological investigations using transmission microscopy. Hematoxylin stains the cell nuclei blue, whereas eosin stains other cellular structures such as proteins in red/pink.
IHC – Immunohistochemistry: Detection of biomarkers (e.g., proteins) in/on cells within a tissue sample by using biomarker-specific antibodies, which are conjugated to a signal transducer (e.g., enzyme or fluorophore).
IHF – Immunohistofluorescence: IHC using fluorophores as signal transducer
IVD – *In vitro* diagnostics: Medical or clinical tests that are performed outside a living organism (e.g., immunoassay with a human serum sample)
LFTS – Lateral Flow Test Strip: One of the most often used POCT devices, where the analyte and the reagents are transported via lateral flow to a certain area on a test strip, which indicates (usually by color or intensity) the test result (e.g., pregnancy test).
POCT – Point-of-care testing: Assays (usually in IVD) that can be performed near the site of patient care. Such formats require fast, inexpensive and easily applicable assays, which still need to provide sufficient selectivity and sensitivity for early diagnosis.

Corresponding author: Hildebrandt, N. (niko.hildebrandt@u-psud.fr).

 0167-7799/$ – see front matter http://dx.doi.org/10.1016/j.tibtech.2012.04.005 Trends in Biotechnology, July 2012, Vol. 30, No. 7

detection, therefore, such assays often lack sufficient sensitivity for the detection of low concentrated biomarkers (e.g., total prostate specific antigen (TPSA), with a cut-off value of 4 ng/ml in serum for prostate cancer screening), and they are therefore limited to visual qualitative on/off (e.g., pregnancy test) or semiquantitative measurements for relatively high concentrations of proteins [18]. Organic fluorophores have largely contributed to the evolution of fluorescence-based LFTSs (FLFTSs) toward quantitative assays that are capable of detecting trace amounts of proteins [19,20]. However, susceptibility to photobleaching, requirement of different excitation sources (and/or filters), and broad emission bands limit further development of organic dye-based FLFTSs for miniaturized low-cost solutions. Therefore, the application of stable and bright QDs with high extinction coefficients over a broad spectral range (efficient excitation of many different QDs with one single source) and color-tunable narrow emission bands in FLTFS assays provides great potential for sensitive and fast quantitative diagnostics. QD-based FLFTS assays still need to overcome challenges such as the preservation of physical stability and biological activity for dry QD formats. These are not fundamental limitations, therefore, QDs have great potential for their application in LFTS assays (e.g., for ultrasensitive biomarker detection), once the major challenges are overcome. Recently, different QD-based FLFTS assays have been developed [21,22]. For example, QDs labeled with anti-nitrotyrosine can capture nitrated ceruloplasmin, a relevant biomarker for cardiovascular diseases, lung cancer and stress response to smoking (Figure 1a) [21]. Here, QD-antibody probes are adsorbed to a conjugation zone on the test strip, where they are combined with the biomarkers, which migrate with the sample along the test strip. The resulting QD–antibody–biomarker complexes are then captured by different primary antibodies against nitrated ceruloplasmin, which are immobilized on a test line. The QD fluorescence intensity on the test line is detected with a commercial test strip reader 10 min after sample application. The QD-based FLFTS assay has a detection limit of 0.4 ng/ml in 20-fold diluted human plasma and can be stored for at least 3 months (sealed) at 4 °C without hindering its performance.

Homogeneous energy transfer assays

Apart from the replacement of traditional organic dyes by QDs for enhanced optical properties [23], an ongoing trend of QD-based diagnostics is their use in more sophisticated methods such as Förster resonance energy transfer (FRET), chemiluminescence resonance energy transfer (CRET) or nanosurface energy transfer (NSET) [24–34] (Box 2). These are nonradiative resonance energy transfer (RET) processes from an excited donor to an unexcited acceptor molecule (or nanoparticle) with a strong (r^{-6} for

Box 1. QD properties and bioconjugation [23,26,60]

Materials and dimensions

Colloidal inorganic QD nanocrystals are composed of III/V (ZnO, ZnS, CdS, CdSe, or CdTe) or II/VI (GaN, GaP, GaAs, InP, or InAs) semiconductors. The core material is typically coated with a shell material having a higher bandgap energy (e.g., CdSe/ZnS core/shell) in order to passivate the QD core for avoiding electron and/or hole trap states, thus improving the optical properties of the bare QD core. Core diameters are typically in the range of 2 to 8 nm and the shells add 1 to 4 nm to the diameter. Water-solubility of QDs is typically achieved by attaching compact organic ligands (thin coatings but often low long-term stability) or relatively large polymers and/or lipids (thick coatings but usually good long-term stability) to the QD surface. Single water-soluble QDs with thick coatings can reach up to ~30 nm in diameter. Although QDs are often considered spherical, they usually take different shapes (e.g., spheres, ellipsoids, pyramids, or disks), depending on the material and synthetic (crystal growth) conditions.

Optical properties

As a result of sizes (diameters) close to or smaller than the exciton Bohr radius of the core material, quantum confinement effects shift the absorption and emission wavelengths of QDs to the blue spectral region (compared to the bulk material) with decreasing QD size. Thus, the emission maxima of QDs can be controlled by their size from the blue to the near IR range (also depending on the material composition). Theoretically, a perfect QD has a distinct photon emission energy. As a result of size (distribution), shape and environment effects this emission energy is broadened but usually symmetric (Gaussian shape) and still narrow (typically down to 20–30 nm full width at half maximum) compared to other fluorophores such as organic dyes or fluorescent proteins. As a result of possible excitation with all energies higher than the bandgap energy, the absorption spectra of QDs are extremely broad (starting just below their emission maxima and continuously increasing toward the UV). Few distinct exciton peaks are usually visible in these continuous spectra. Multiplexing (different sizes = different colors) is possible with facile spectral separation of the different emission bands (e.g., bandpass filters) and large effective Stokes shift (spectral separation between excitation and emission wavelength). QDs can provide high fluorescence quantum yields and are usually photostable. Again, these aspects strongly depend on the shape, material composition and environment. QDs are known to show strong blinking effects (random switching between bright and dark states); most probably caused by Auger recombination. These effects can be suppressed by designing QD core/shell compositions that are less prone to Auger recombination or they can be turned into an advantage, for example, to distinguish single QDs (blinking) from QD clusters (nonblinking) in super-resolution imaging.

Bioconjugation

The most important aspects that should be considered for bioconjugation are: (i) the dimensions of the overall bioconjugate (a polymer/lipid coated QD with a long linker attached to a large protein might be large and disadvantageous for energy transfer applications or intracellular measurements); (ii) functionality (both the QD photophysical properties and the binding properties of the biomolecule should be conserved in the conjugate); (iii) reproducibility; and (iv) valence control (number of biomolecules per QD). The nanometric size of QDs allows single-biomolecule–QD conjugates, attachment of multiple biomolecules to one QD, as well as the embedding of multiple QDs in larger biological systems such as viruses. The first step of transferring the inorganic QDs into water-soluble and biocompatible fluorescent nanoparticles offers manifold possibilities as illustrated in Figure I. Once the functionalized QDs are stable in aqueous solution, many different classical as well as novel bioconjugation methods are available, ranging from random conjugation (mostly targeting primary amines or thiols with NHS-ester or maleimide-functionalized QDs) to controlled bio-orthogonal conjugation. Hexahistidine self-assembly directly to Zn-rich QD surfaces is another technique for attaching proteins, peptides, DNA and other biomolecules to QDs. It is also a relatively easy and stable ($k_d \approx 10^{-9}$ M) conjugation method. As a result of the convenient commercial availability of readily prepared QD bioconjugates and their stable binding ($k_d \approx 10^{-14}$ M), the probably most often applied conjugation is based on biotin–streptavidin recognition. Many other technologies such as HaloTag (Promega), SNAP-tag and copper-free click chemistry can also be used for QD-biolabeling.

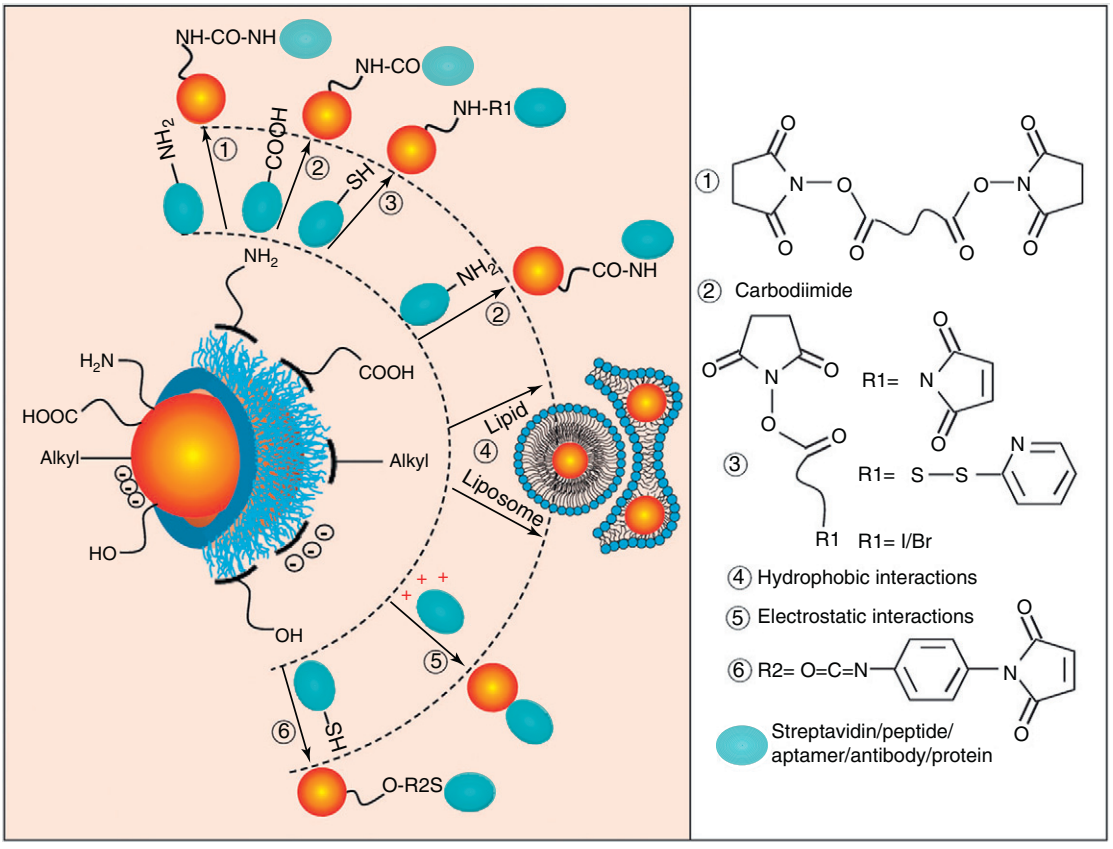

Figure I. Schematic presentation of different QD-bioconjugation methods. Left: The bare QD semiconductor nanocrystal (orange sphere) can be directly coated with organic ligands providing functional groups (NH_2, COOH, OH) for covalent conjugation or it can display negatively charged or hydrophobic (alkyl) surfaces for biomolecule adsorption. The same applies for QDs with thiol, polymer or silica-based coatings as protective layers (blue hair-like structures). Bioconjugation of the functionalized QDs can be performed by crosslinking amino, carboxyl or sulfhydryl groups of biomolecules to the QD functional groups, by lipid-based micelle formation around the QD using hydrophobic interactions or by electrostatic adsorption of proteins to the QD surface. Right: Examples of crosslinkers (1, 2, 3 and 6), interactions (4 and 5) and biomolecules (cyan ellipsoid) involved in QD bioconjugation. Figure reprinted with permission from [6]. Copyright 2010 The Royal Society of Chemistry.

FRET/CRET and r^{-4} for NSET) distance dependence on a 1–20 nm length scale. Many biological interactions can be monitored by such energy transfer methods if two (or more) biorecognition partners are labeled with adequate donor and acceptor molecules [26]. As the RET signal is specific for the biological recognition and different from the fluorescence signals of the single (unbound) binding partners, such assays can be performed directly in solution and do not require any washing and separation steps (homogeneous assay). A novel DNA sensor concept uses a combination of exonuclease (Exo) III-based signal amplification and QD-based FRET (Figure 1b) [24]. The first part of this sensing platform consists of black hole quencher (BHQ)-modified nucleic acids (on the 3′ end) labeled to a QD (on the 5′ end), QD-oligo-BHQ. The second part contains a nucleic acid hairpin structure hybridized to a specific target DNA. This hybridization opens the hairpin and leads to formation of a double-stranded DNA with overhangs at both 3′ ends. The 3′ end overhang of the opened hairpin then hybridizes with the 3′ end of the QD-oligo-BHQ, leading to a selective Exo III digestion of that latter oligonucleotide. This digestion has two main effects. First, it cleaves the BHQ, which has quenched the QD, and thus increases the QD luminescence, and second, it releases the double-stranded open hairpin target DNA for additional hybridization to other QD-oligo-BHQ (on the same or other QDs). This recycling and amplification process lead to an almost twofold luminescence intensity increase after 2.5 h for 1 nM target DNA and subnanomolar detection limits. The DNA sensor has strong multiplexing potential if combinations of differently encoded QD-oligo-BHQ and hairpin structures (for the recognition of different target DNAs) are used. Moreover, other analytes such as proteins might be detected by this method if, for example, aptamer–substrate complexes are applied for opening the hairpin structures.

QDs can also be used as energy acceptors. However, the strong and spectrally broad QD absorption usually leads to a more efficient light excitation of QDs compared to most potential donors. This results in a small ratio of excited donors and unexcited acceptors and thus FRET becomes very inefficient. Direct light excitation of QDs can be overcome by using CRET because it does not require any light for excitation. Luminol-based CRET to QDs (H_2O_2 oxidation of luminol catalyzed by the formation of a hemin/G-quadruplex by the self assembly of nucleic acid subunits) has been successfully applied in the detection of

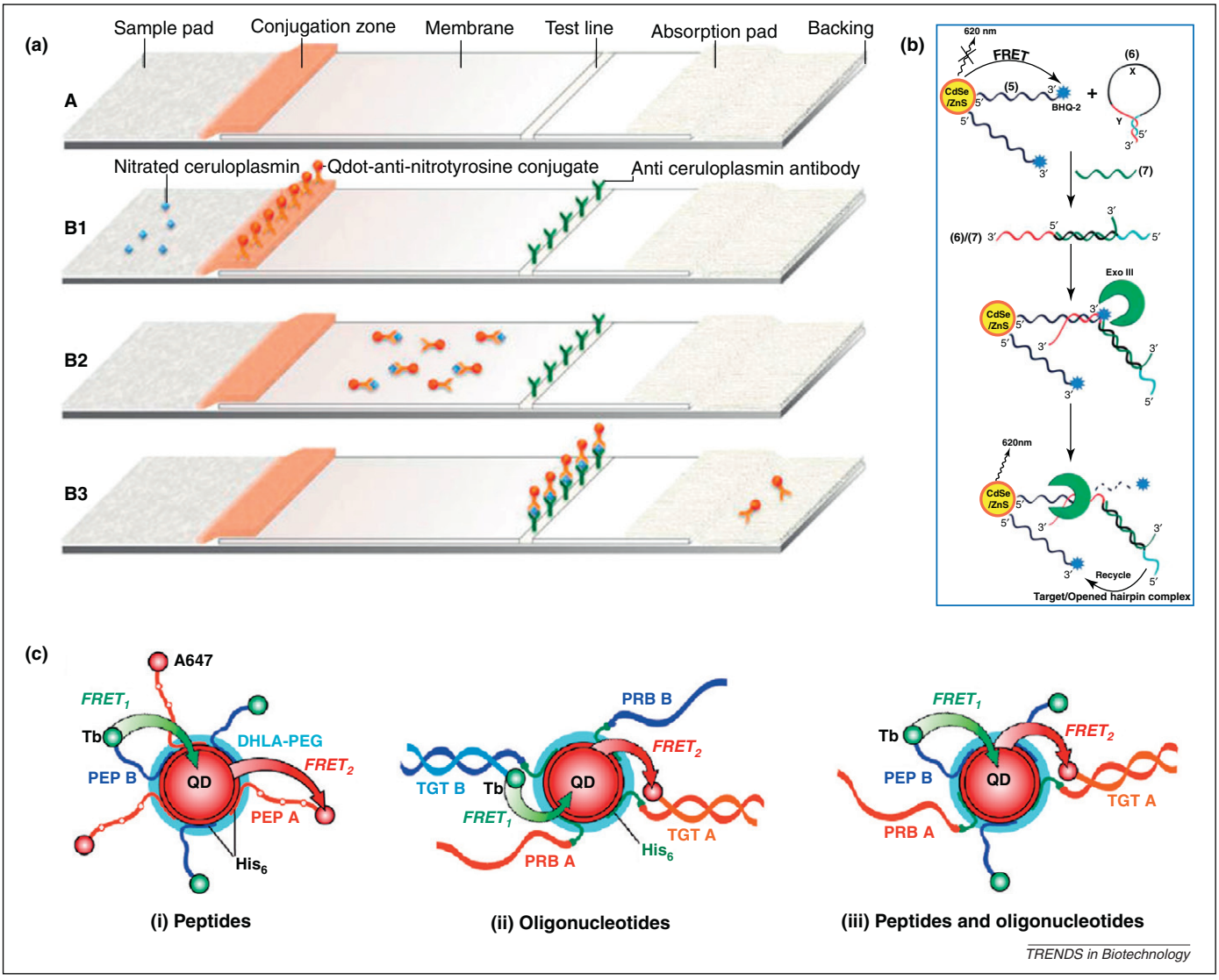

Figure 1. Examples (schematic diagrams) of quantum dot (QD)-based *in vitro* diagnostics (IVD). **(a)** Lateral Flow Test Strip (LFTS) assay (A – test strip description) for the detection of nitrated ceruloplasmin (NC). Aqueous sample containing NC is applied to the sample pad (B1) and transported to the conjugation zone, where the preabsorbed and dried QD–anti-nitrotyrosine antibodies (QD–AB1) are rehydrated and washed off and QD–AB1–NC biocomplexes are formed. The sample further migrates to the test line (B2) where anti-ceruloplasmin antibodies (AB2) are immobilized, leading to formation of AB2–NC–AB1–QD immunosandwich conjugates on the test line, whereas free QD–AB1 without NC further migrates to the absorption pad (B3). NC concentration is measured by QD fluorescence intensity on the test line using a commercial test strip reader. Reprinted with permission from [21]. Copyright 2010 American Chemical Society. **(b)** DNA sensor using Förster resonance energy transfer (FRET) and exonuclease (Exo) III catalyzed signal amplification. QD-Oligo-BHQ-2 probes (5) are mixed with hairpin DNA (6) that opens upon hybridization to target DNA (7). The 3′ overhang of the opened hairpin within the double-stranded DNA (6)/(7) binds to the 3′ end region of (5), which enables the selective digestion of the (5) oligonucleotide by Exo III within the sample. This digestion leads to the removal of the BHQ-2 luminescence quencher from the QD and a concomitant PL increase of the QD. Moreover (6)/(7) is recycled for repeating the process on another (5) oligonucleotide, which leads to signal amplification for a single target DNA. Reprinted with permission from [24]. Copyright 2011 American Chemical Society. **(c)** FRET-relay assays. Assembly of Tb-LLC (Tb)- or AlexaFluor dye (A647)-labeled peptides **(i)**, oligonucleotides **(ii)** or both **(iii)** around a QD. **(i)** was used for time-gated ($FRET_1$+$FRET_2$) kinetic protease activity assays; **(iii)** for time-gated ($FRET_1$+$FRET_2$) or nongated ($FRET_2$) single target nucleic acid hybridization assays; and **(ii)** for simultaneously time-gated ($FRET_1$+$FRET_2$) and nongated ($FRET_2$) duplexed nucleic acid hybridization assays. $FRET_1$: efficient FRET from Tb to QD becomes only possible after a microsecond time delay (after the excitation pulse). $FRET_2$: efficient FRET from QD to A647 is possible through direct QD excitation without time-delay (nongated) or through $FRET_1$ sensitization after a microsecond time-delay (time-gated). Reprinted with permission from [34]. Copyright 2012 American Chemical Society.

thrombin, ATP, DNA or metal ions [30,31]. If light excitation is required, luminescent lanthanide complexes (LLCs) in combination with time-gated detection are an elegant solution for biomolecular detection. Using time-gating (e.g., a detection window from 0.05 to 1 ms after an excitation pulse) has the large advantage that almost the entire autofluorescence from the biological sample as well as QD emission caused by direct light excitation have already decayed after some hundreds of nanoseconds, which enables nearly background-free signal detection in the time-delayed detection window. Such sensitive FRET probes can be further combined with different QD colors and proof-of-principle demonstrations for fivefold multiplexed diagnostics with subpicomolar detection limits, as well as multiplexed long-distance (five independent distances between 6 and 10 nm) and shape (QD elongation with increasing size) measurements at subnanomolar concentrations under physiological conditions have been reported [32,33]. Combining fluorescence color, intensity and lifetime within one multiplexed approach could provide extremely high order multiplexing (already the combination of only four colors, two lifetimes and three

Box 2. Energy and charge transfer using QDs [26,61–66]

Different energy or charge transfer (CT) techniques can be used to quench or sensitize QDs and to create a specific signal for biosensing. RET processes describe a nonradiative energy transfer by donor–acceptor dipole–dipole coupling without overlapping orbitals of donor and acceptor molecules. FRET, CRET and bioluminescence RET (BRET) efficiencies show an r^{-6} distance dependence, NSET an r^{-4} distance dependence, and dipole to metal particle energy transfer (DMPET) mainly follows the FRET mechanism for small distances but includes some distance 'correction factors' dependent on the QD emission wavelength. Electron exchange (Dexter transfer) and CT occur with overlapping orbitals of donors and acceptors, and show mainly an exponential decrease with distance. They are therefore limited to shorter distances. Mainly five transfer mechanisms are important for the use with QDs (Figure I).

i. **FRET using QDs as energy donors.** As a result of its broad absorption spectrum, the QD donor can be excited efficiently at an absorption minimum of the acceptor (e.g., organic dye or fluorescent protein), which minimizes direct acceptor excitation. The FRET efficiency increases with the number of acceptors around one QD. The QD is quenched and the acceptor is sensitized by FRET (and can then emit light in case it is a fluorescent acceptor).

ii. **FRET using QDs as acceptors.** If QDs are used as FRET acceptors there is efficient direct excitation of the QD at almost any wavelength used for donor excitation (due to the broad absorption spectrum of the QD). This will lead to a small ratio of excited donors to ground state QD acceptors, and therefore, inefficient FRET. The solution to this problem is the use of donors with long excited-state lifetimes such as LLCs (lifetimes up to several milliseconds) and pulsed excitation. Several microseconds after the excitation pulse, all QDs have decayed back to their ground states, whereas most of the lanthanide complexes have remained in their excited states and FRET becomes efficient. As a result of the large spectral overlap between lanthanide emission and QD absorption, such FRET systems allow extremely long Förster distances of >10 nm. The lanthanide complex is quenched and the QD is sensitized by FRET.

iii. **CRET or BRET using QDs as acceptors.** Another possibility to avoid direct excitation is to use chemiluminescent or bioluminescent donors. An advantage is the possibility of creating luminescence in deep tissues (which is not possible with external light excitation). However, a chemical reaction (usually the oxidation of luminol in CRET or luciferin in BRET) needs to be triggered at the desired position. The chemiluminescent or bioluminescent donor is quenched and the QD is sensitized by FRET.

iv. **NSET using QDs as donors.** This mechanism fits usually best (in comparison to FRET or DMPET) to describe energy transfer between QDs and Au-NPs. As the infinite surface of the Au-NP is taken into account, the r^{-6} distance dependence of point dipole–dipole interactions turns into an r^{-4} distance dependence. This allows energy transfer over larger distances compared to FRET (up to ~20 nm). The QD donor is quenched and the Au-NP acts as dark quencher.

v. **CT using QDs as donors or acceptors.** In CT, electrons or holes can be transferred from (or to) proximal molecules (e.g., Ru-complexes) with matching oxidation levels to (or from) mainly the QD surface states, which leads to QD photoluminescence quenching. This mechanism does not require spectral overlap, therefore, different QDs can be quenched by a single 'universal' CT quencher, which is a large advantage for color multiplexing. The QD is quenched and the CT-quencher acts as dark quencher.

The possibility of creating sophisticated (or smart) probes with high stability, high brightness, large (on an energy transfer scale) separation distances and various multiplexing possibilities predicts a promising future for QD-based energy and CT systems regarding biotechnology applications, especially for IVD and cellular imaging, where multiplexing, high sensitivity and high spatial resolution over long molecular distances are very desirable.

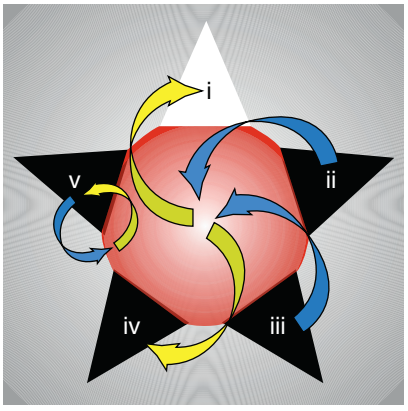

Figure I. QDs (red sphere in the center) are extremely flexible and powerful tools for energy or charge transfer both as donors (yellow arrows) and/or acceptors (blue arrows). A detailed description of the five main strategies is found within the box.

intensities would lead to $4^{(2x3)}$ = 4096 different barcodes), which shows the importance of combining QDs with many different other fluorescent materials. One interesting example of fluorescence lifetime multiplexing by time-gated detection uses a QD simultaneously as FRET acceptor and donor (Figure 1c) [34]. Tb-LLC and AlexaFluor dye-labeled peptides and oligonucleotides are ratiometrically assembled to a red emitting QD. After several microseconds of delay after the light excitation pulse, the Tb-LLC efficiently transfers energy to the QD ($FRET_1$) and subsequently the QD transfers a part of that energy to the AlexaFluor dye ($FRET_2$), which can be monitored by time-gated detection. Direct light excitation of the QD also results in $FRET_2$ to the AlexaFluor dye, which can be measured by steady-state (non-gated) detection without time delay. These two independent FRET mechanisms have been used for a kinetic analysis of protease activity with a detection limit of 200 pM trypsin and for single target (TGT-A) as well as duplexed (two targets TGT-A and TGT-B) nucleic acid hybridization assays with 16 nM (single TGT-A), 17 nM (TGT-A duplex assay) and 29 nM (TGT-B duplex assay) detection limits. These results demonstrate the possibility of very sensitive multiplexed detection of several individual biological events purely based on spectro-temporal discrimination without different QD colors.

Energy transfer for flexible POCT

Triggered by the desire to create multiplexed-detection platforms for easy-to-use POCT applications, several QD-based energy transfer biosensor concepts have focused on miniaturization and flexibility. An important aspect in this direction is the development of solid-phase assays, where biomolecules are immobilized on a solid surface. This concept might overcome solution-phase related problems concerning colloidal stability or nonspecific adsorption, and possibly allow few nanoliter sample volumes, minimal reagent consumption, and faster reaction times. One study toward a high-throughput microarray format has demonstrated QD/AuNP-based multiplexed detection of the disease relevant proteases matrix metalloproteinase-7,

caspase-3 and thrombin with detection limits of 10 ng/ml, 20 ng/ml and 1 U/ml, respectively [27]. This was achieved by attaching three different QD–streptavidin conjugates (with QDs emitting at 525, 606 and 655 nm, respectively) to a glass slide using a microarrayer, and subsequent incubation with biotinylated peptide-AuNPs. Initially quenched by the AuNPs (most probably due to NSET, although the authors used FRET theory to describe the energy transfer) due to the formation of QD–streptavidin–biotin–peptide–AuNP complexes, QDs recover their photoluminescence (PL) upon specific proteolytic cleavage of the peptide–AuNPs, which can be measured with a fluorescence slide scanner. The conjugates can be spatially immobilized on different microarray spots, therefore, color and space might be combined for achieving high-order and high-density multiplexing; one advantage of microarray technology. Various concepts of solid-phase QD-immobilization (e.g., to glass beads or optical fibers) for FRET-based nucleic acid detection with 1–3 nM detection limits have been developed [28]. Alternative solid support materials, such as microparticles or microfibers, could possibly allow the integration of solid-phase QD-FRET biosensors in tissues or cells. Among various applications, a reusable electrokinetically controlled microfluidic platform has been developed [29]. Functionalization of a glass-polydimethylsiloxane (PDMS) microfluidic channel with probe oligonucleotide-labeled QDs and subsequent delivery of Cy3-labeled target nucleic acids into the microchannels has allowed the detection of 5 fmol nucleic acids within minutes. Such versatile approaches demonstrate the flexible abilities and unique advantages of QDs for solid-phase IVD applications.

Main disadvantages of QDs in IVD

Despite the many advantages of QDs, the main disadvantage for IVD remains the lack of reproducible production and easy long-term storage, which is of utmost importance to integrate the nanocrystals into real-life clinical diagnostics [4]. As careful optimization is a long and labor-intensive process, the proof-of-principle concepts (demonstrated by many different researchers) need to be picked up by the industry for taking the final big step forward into commercial QD-based IVD. Regarding RET, another problem remains the large size of QDs. This is especially true for the commercially available nanocrystals, which have thick polymer or lipid-based surface coatings. This coating results in stable and very bright QDs, however it adds several nanometers to the semiconductor particle diameters, leading to relatively long center-to-surface distances of up to more than 10 nm, which can make RET inefficient. Therefore, the development and reproducible production of QDs with thin coatings, still providing the necessary stability and brightness, are clearly necessary for a successful future of QD-based energy transfer in IVD.

QDs for tissue and cellular imaging

The application of QDs within tissues and cells, due to the complex environment, morphology and function is very challenging. Size, shape and surface properties can be serious obstacles for a sensitive and reproducible observation of subcellular functions and interactions. Another often-discussed issue is cytotoxicity, which usually depends on structure and material related properties, and ranges from completely harmless to strong cytotoxicity, depending on the applied QDs and investigated biological systems. No clear and especially no general conclusion has been found concerning QD toxicity, therefore, this discussion will be ongoing in the future [4]. High brightness and photostability (high signal to noise ratios during long observation times) as well as color-tunability and narrow emission bands (facilitated optical multiplexing) are interesting QD features for fluorescence imaging of tissues and cells.

Immunohistofluorescence (IHF)

One promising application for QDs is IHF, in which QD–antibody conjugates are used for tissue staining. Tissues are usually preserved as formalin fixed and paraffin embedded (FFPE) or frozen and then cut into thin tissue sections (5–10 μm) for clinical imaging-based diagnostics. FFPE sections are well suited for high quality histological staining. However, the sample recovery process (including mounting, heating, paraffin removal, tissue rehydration and antigen retrieval) is very time-consuming. Preparation of frozen tissue sections for immunohistochemistry (IHC) is much quicker than for FFPE samples, which gives access to immediate medical decisions (e.g., during surgery). However, cryosectioning leads to lower quality tissue slices compared to FFPE. The combination of IHF with conventional hematoxylin and eosin (H&E) staining (for transmission-based microscopy) can provide molecular precision of biomarkers as well as structural features of tissue. Multiple-fluorophore-based staining allows higher-order multiplexing for maximum information retrieval from patient tissue biopsy to uncover the underlying genetic and phenotypic characteristics, which are important for establishing personalized medicine approaches. Several recent studies have utilized QD–antibody conjugates for improved signal to noise ratio and multiplexing in IHC applications [35–43]. In most cases, secondary antibody–QD conjugates are used because QD antibody labeling does not provide high conjugation yields and is therefore much more costly because primary antibodies are expensive. QD-based molecular mapping of four protein biomarkers (E-cadherin, high-molecular-weight-cytokeratin – CK HMW, p63, and α-methylacyl CoA racemase – AMACR) has recently been used in clinical tissue specimens from 16 prostate cancer patients [35]. The four different proteins can be targeted by sequential staining using four different primary antibodies and two different secondary antibodies. First, two biomarkers are recognized by primary antibodies followed by their staining with two secondary antibody–QD conjugates. After washing, the other two markers are recognized and stained by the same method, using two different primary but the same secondary antibodies (the latter are labeled with different QDs than before). The four color (525 or 705, 565, 605 and 655 nm emission wavelength maxima) QD staining method has revealed extensive tumor heterogeneity at the molecular, cellular and architectural level by direct visualization of human prostate glands undergoing structural transitions from double layer of basal and luminal cells to a single layer of malignant cells. The multiplexing capability of this

method is particularly useful to draw clear pathological conclusions within specific tissue areas that can only be identified as 'suspicious' (so-called complex foci) using conventional H&E or single-color IHF staining (Figure 2a). This has been demonstrated by the identification of single malignant cells within a predominantly benign prostate gland by multiplexed QD staining (Figure 2b). Although the four-color QD staining procedure was labor-intensive and time-consuming (~8 h) and the patient number was insufficient for achieving significant clinical outcomes, the results demonstrate the consistency and reproducibility of QD-based molecular pathology for clinical translation. QD-based IHF has been extended in FFPE tissue sections to a threefold simultaneous staining using primary antibodies of three distinct species origins (mouse, rabbit and goat) against E-cadherin, epidermal growth factor receptor and β-catenin and the three matching secondary antibodies conjugated to different QDs (565, 605 and 655 nm emission wavelength maxima, respectively) [36]. A comparison with sequential staining using biotinylated secondary antibodies and streptavidin–QD conjugates of the same three different colors revealed higher signal intensities for sequential staining but more consistent and stable signals using the directly labeled secondary antibody–QD conjugates. It is practically difficult to find more than four distinct species antibodies, therefore, simultaneous staining with this method is limited in multiplexing. In any case, it is advisable to consider both the simultaneous and sequential staining depending on the tissue system to be investigated. QD-based phosphoprotein staining can be used in formalin-fixed tissues for therapy monitoring and cancer diagnostics [37]. For example, tissues from tumor xenografts of enzastaurin-treated and untreated mice were investigated with streptavidin–QDs + biotinylated secondary antibodies + primary antibodies against phospho-glycogen synthase kinase (GSK)3β. The treated samples showed a significant reduction of the phosphoprotein. The diagnostic model (using the same QD-staining principle) detected phospho-signal transducer and activator of transcription (STAT)5 (STAT5 phosphorylated by internal tandem duplications (ITD) of Fms-like tyrosine kinase 3 – FLT3-ITD) in 14 bone marrow biopsies from patients with acute myeloid leukemia. The six ITD-positive cases showed significantly elevated levels

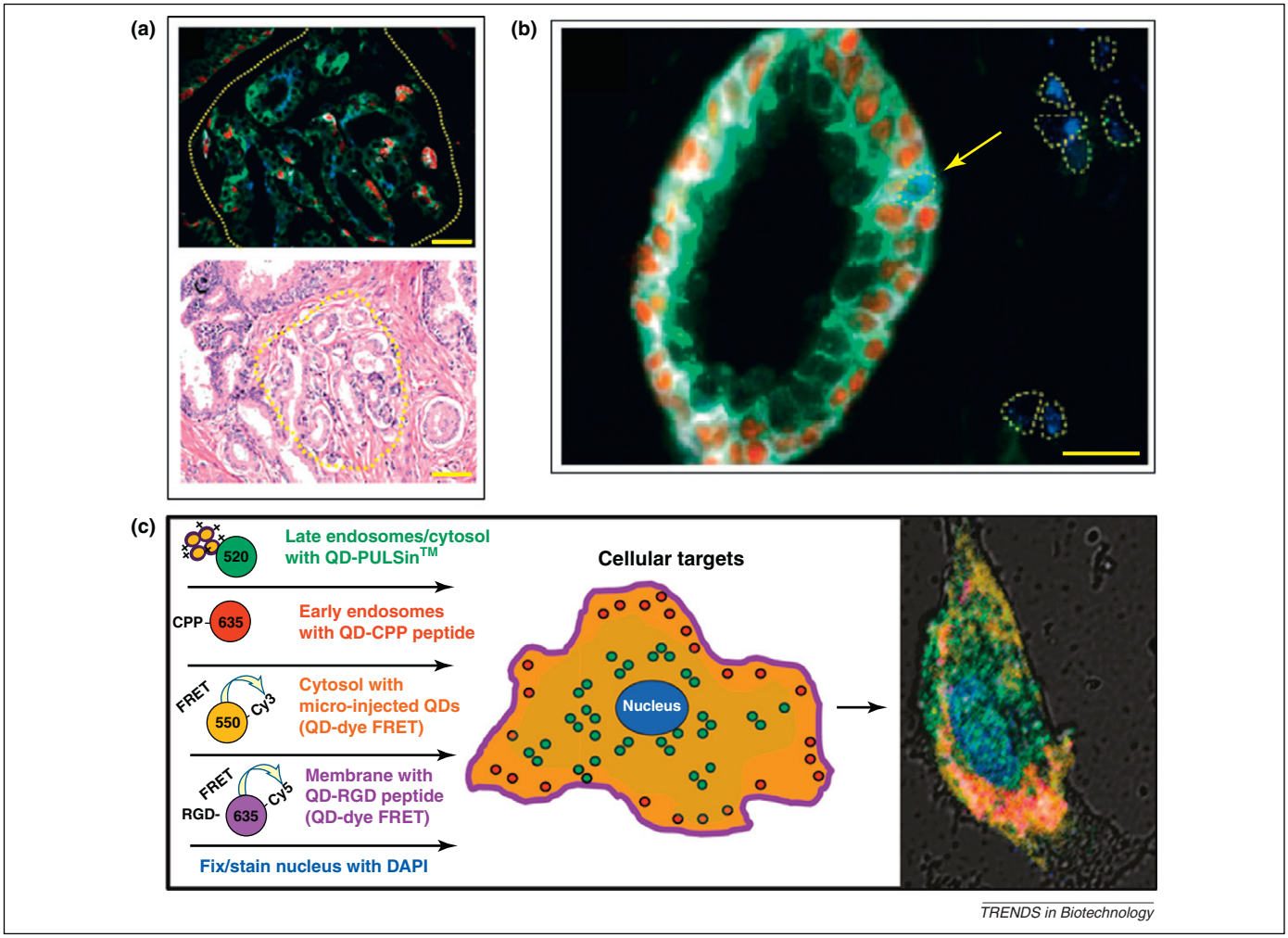

Figure 2. Examples of quantum dot (QD) application in cellular imaging. **(a)** Comparison of multiplexed QD mapping (top scale bar 50 μm) to hematoxylin and eosin (H&E) staining image (bottom scale bar 100 μm) of a histopathologically complex focus (within the yellow broken line). **(b)** Identification of a single malignant tumor cell (indicated with an arrow) in a predominantly benign prostate gland and surrounding malignant cells (blue signals in dotted circles) by multicolor QD tissue staining of four protein biomarkers (E-cadherin, green; high-molecular-weight-cytokeratin (CK HMW), white; p63, red; and α-methylacyl CoA racemase (AMACR), blue). **(c)** Schematics presentation for multicolor QD delivery into different compartments of live cells (left) and a corresponding multicolor fluorescence image (right). (a) and (b) reprinted with permission from [35]; (c) reprinted with permission from [45]. Copyright 2010/2011 American Chemical Society.

of phospho-STAT5 compared to the eight ITD-negative cases. Such examples demonstrate the large progress of QD-based tissue staining for real-life clinical applications and their implication in larger studies with important clinical outcome can be expected in the near future.

Fluorescence in situ hybridization (FISH)

Although the photophysical properties of QDs should make them ideal candidates for FISH, especially for multiplexed applications, the current status of QDs within FISH is rather disappointing. An overview of different QD-based FISH approaches is given in a recent review [44]. Within that article, the authors also compare QD to organic dye (Cy3)-based FISH using biotinylated oligonucleotides and chromosome paints and commercially available QD and Cy3-conjugated streptavidin, respectively. For indirect detection (streptavidin conjugates added after hybridization) QD-based FISH was successful but the experiments were not as reproducible as Cy3-based FISH (only 25–30% success rate for QD compared to >95% for Cy3). Although some QD samples showed higher brightness and less photobleaching than the Cy3 controls, most of the preparations displayed a higher background signal than Cy3. Preparation of the same biotin–streptavidin-based FISH probes before hybridization did not lead to any successful QD-based FISH experiments, whereas the Cy3 controls worked reliably. These results show the possibility of successful application of QDs in FISH but also demonstrate their reproducibility-related drawbacks, which have excluded them from being used in commercial FISH applications so far.

Live cell imaging and single particle tracking

Cellular function is maintained by interaction with its external environment and its internal organization. Therefore cellular studies can be categorized under molecular interactions on the cytoplasmic membrane and intracellular and subcellular interactions. Much of the understanding of cellular function has been obtained by microscopic observation of single live cells. Cells, with adequate buffering of cell culture media, maintain their cellular functions during imaging as a function of temperature and gaseous environment. QDs can preserve their photostability and high brightness inside living cells. These optical properties clearly depend on different factors such as the semiconductor material, the QD structure, biological coating or functionalization, and the excitation conditions (such as single or multiple photon excitation) [45]. The investigation of detailed cellular processes by single particle or single virus tracking is an important application of QDs in live cell imaging [46–53]. In one interesting example, vesicular secretion of neurotransmitters in neuronal communication was investigated by size and pH-dependent single QD fluorescence [47]. Individual synaptic vesicles (lumen ~24 nm) were loaded with single QDs (diameter ~15 nm) and their fluorescence intensity tracked over time during neuron stimulation. Consequently, full–collapse–fusion (FCF) of the vesicles was distinguishable from transient fusion and retrieval (so-called 'kiss-and-run' – K&R). Thus, the authors could reveal the functional impact of K&R, which had not been accessible before due to limited signal-to-noise ratios of other optical probes. K&R fusion pores are so small (1–5 nm) that they only allow protons but not the incorporated QDs to escape, which leads to a transient pH change (5.5 intravesicular to 7.3 extracellular) and a concomitant QD fluorescence intensity increase of ~15%. An occurrence of ~25% of K&R for all fusion-exhibiting vesicles with a prevalence of K&R over FCF for the earlier and FCF over K&R for the later stimulation phase was determined by this method.

Cellular uptake

Cellular uptake of QDs and their controlled delivery to different intracellular organelles remains a challenging task. An important aspect is again QD stability. A new family of compact cap-exchange ligands for biological applications of QDs provides the nanocrystals with thin coatings and high stability in a large range of pH and high salt concentrations [54]. Microinjection into adherent COS-1 cells leads to well-dispersed QDs in the cytosol without aggregation. No significant changes in brightness and stability could be detected over a period of 6 h. The same QDs were also self-assembled with hexahistidine-labeled cell penetrating peptides (CPPs), leading to efficient endocytotic uptake of the QDs after incubation for 1–2 h. However, the punctuated QD fluorescence as well as the colocalization with the endosomal marker AlexaFluor 647-labeled transferrin suggested that the QDs were mainly distributed in endosomes. No cytotoxic effects could be found over a period of 72 h. Nonconjugated commercial QDs (Qdot655 – Invitrogen/Life Technologies) with different surface coatings have been successfully taken up by two different human mammary cells [55]. Mainly the coatings (possibly due to the different surface charges) had a strong influence on QD uptake. Polyethylene glycol (PEG)-coated and PEG-amine-coated QDs did not penetrate the cells at all, whereas the carboxyl QDs were efficiently internalized in both cell types with a slightly stronger uptake in the cancerous cells. As in many other studies, most of the endocytosed QDs end up in late endosomes or lysosomes. There are several different strategies for QD delivery into live cells [56–59]. In one example, multicolor QD labeling of distinct subcellular compartments was investigated in human cancer cells growing in culture over a 4-day period [59]. First, green QDs (520 nm) were delivered with PULSin™ (Polyplus-Transfection) to target late endosomes. After 4 days in culture, only a few QDs appeared within the cytosol and most remained sequestered within late endosomes. Subsequently, early endosomes were labeled with QD–CPPs (635 nm), QD550–peptide–Cy3 conjugates were microinjected into the cytosol, and membranes were stained with QD635 coassembled with Cy5-peptides and the membrane-integrins-specific peptide RGD (Figure 2c). Combination of QDs with Cy dyes allowed efficient FRET (from QD to Cy), thus avoiding direct excitation of the Cy dyes and a concomitant suppression of photobleaching and long possible imaging periods. Moreover, only one excitation wavelength was necessary. In another publication it was demonstrated that palmitoylated peptides can be used for endocytotic uptake and subsequent delivery of QDs into the cytosol with minimal cytotoxic effects [57]. All these QD cellular uptake and

Box 3. Outstanding questions

Important outstanding questions or future tasks for establishing QDs as outstanding fluorescence tools in biotechnology are:

- Large-scale production of stable, reproducible and long-term-storable QDs.
- Nontoxic QDs and near-IR-emitting QDs with high fluorescence quantum yields and narrow emission bands.
- Stable QDs with thin biocompatible coatings (especially for FRET applications).
- Bioorthogonal labeling strategies for QD bioconjugates with both the full biomolecular and optical functionality.
- Development and optimization of sophisticated detection methods (e.g., QD-based energy transfer in connection with many different other fluorophores) and their integration into complex biomolecular systems.
- Industrial optimization of biocompatible QDs and their detection methods for their application in clinical laboratories and the commercial IVD market.

detection strategies demonstrate the large versatility of delivering QDs to different intracellular organelles. However, they also demonstrate that efficient endosomal escape for useful applications of QDs inside the cytosol has not yet been achieved and further research is necessary to realize this important step and to avoid active microinjection or electroporation methods (Box 3).

Summary and future challenges

Although QDs have not yet been established as commonly used commercial products for widespread clinical applications, these semiconductor nanocrystals look into a bright and colorful future concerning their use in nanobiosensors. Many recent applications make use of the unique photophysical and structural properties of QDs and they exist today in many different biocompatible and biofunctionalized forms. Large-scale production of QDs with stable and compact protective surface coatings as well as novel and optimized concepts for controlled bioconjugation are challenging tasks for the near future in QD material development. From the spectroscopic and microscopic point of view, many sophisticated technologies have been developed and/or improved by using QDs, which has provided indisputable evidence for their outstanding properties for advanced biosensing. QDs will continue to be an important trend in biotechnological research and if large industry-oriented projects will improve and optimize the production of reliable and reproducible QD-bioconjugates, commercial clinical applications will also jump on that trend and ride it into a successful future.

Acknowledgments

The authors acknowledge the European Commission (FP7 project NANOGNOSTICS), the Innovative Medicines Initiative (IMI project OncoTrack) and the Agence Nationale de la Recherche France (ANR-Investissements d'avenir, Project NanoCTC) for financial support.

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