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Potentials and pitfalls of fluorescent quantum dots for biological imaging

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Fluorescent semiconductor nanocrystals, known as quantum dots (QDs), have several unique optical and chemical features. These features make them desirable fluorescent tags for cell and developmental biological applications that require long-term, multi-target and highly sensitive imaging. The improved synthesis of water-stable QDs, the development of approaches to label cells efficiently with QDs, and improvements in conjugating QDs to specific biomolecules have triggered the recent explosion in their use in biological imaging. Although there have been many successes in using QDs for biological applications, limitations remain that must be overcome before these powerful tools can be used routinely by biologists.

Progress in genetics, biochemistry and molecular biology has facilitated the identification of various molecules that are involved in cellular function. Fluorescence microscopy allows functional studies of these molecules in living cells and organisms. The utility of fluorescence imaging has generated a tremendous incentive to develop new probes for tagging molecules and for reporting changes in their cellular concentrations and activities [1].

Organic fluorophores such as genetically encoded fluorescent proteins or chemically synthesized fluorescent dyes are the most commonly used fluorophores. There are, however, two significant limitations of organic fluorophores: they cannot fluoresce continuously for long periods, and they are not optimized for multicolor applications. The latter limitation stems from two factors: each fluorophore can be optimally excited only by the light of a defined wavelength (which usually makes it necessary to use as many excitation sources as types of fluorophore), and each fluorophore has a relatively broad emission spectra (which often causes the signals from different fluorophores to overlap).

The serendipitous discovery of fluorescent SEMICONDUCTOR (see Glossary) nanoparticles called quantum dots or QDs (Box 1) has provided a potential means to surmount these limitations. These inorganic fluorophores are crystals made up of substances such as cadmium selenide (CdSe) (Figure 1a) and offer significant advantages over organic fluorophores, including brighter fluorescence, resistance to photobleaching and optical properties that facilitate the simultaneous imaging of multiple fluorophores. These features make QDs ideal for concurrently monitoring several intercellular and intracellular interactions in live cells and organisms over periods ranging from less than a second to over several days.

In this review, we describe the unique features of QDs that make them valuable for *in vitro* and *in vivo* imaging, and discuss the developments that have facilitated their use in bioimaging. We also discuss the limitations of QDs and the desired technical developments that could further enhance their utility for biological applications.

Utility of QDs in bioimaging

In this section we discuss the properties of QDs that make them a desirable alternative to organic fluorophores and highlight their advantages for biological imaging.

Unique spectral properties

The emission spectra of QDs can be tuned across a wide range by changing the size and composition of the QD core [2–4,35] (Figure 1c). The excitation spectra of QDs is very broad, whereas their emission spectra is fairly narrow (the full width at half-maximum intensity ranges from 20 to 40 nm; Figure 1b). By contrast, organic fluorophores usually have narrow excitation spectra and wide emission spectra that tend to spread out more towards the red region (Figure 1b, 'red tail').

These differences provide QDs with a few distinct advantages over organic fluorophores. First, a narrow emission spectrum reduces spectral overlap, which improves the possibility of distinguishing multiple fluorophores simultaneously. Second, the broad excitation spectrum of QDs facilitates the use of a single excitation

Glossary

Fluorescence blinking: a property of a single fluorophore to transit between a fluorescent (on) and non-fluorescent (off) phase, which is caused by its transition between a singlet (fluorescent) and a triplet (non fluorescent) state. Blinking occurs in quantum dots because a specific process causes them to switch between their ionized and neutralized states.

Multiphoton microscopy: a process in which more than one photon, each with a fraction of the energy needed to excite fluorescent molecules, is simultaneously absorbed by the fluorophore, resulting in fluoresce emission. This process facilitates the use of infrared light (which, owing to its longer wavelength, penetrates deeper into the tissue) for animal imaging.

Quantum yield: the ratio of photons absorbed to photons emitted by a fluorescent molecule. The quantum yield quantifies the probability that a molecule in an excited state will relax by emitting fluorescence rather than by decaying non-radiatively.

Semiconductor: a material that is an insulator at very low temperature but has considerable electrical conductivity at room temperature.

Stoke's shift: the separation in energy (and thus wavelength) between the excitation and emission spectra.

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Box 1. History of biocompatible quantum dots

Ekimov and Onuschenko [46] carried out the first controlled synthesis of semiconductor crystals of nanometer size by heating glass containers with supersaturated solutions of copper and chlorine compounds at high temperatures to cause the controlled precipitation of copper chloride (CuCl). They used additional heating to create, systematically, collections of small crystalline CuCl particles ranging from tens to hundreds of Ångstroms, which were initially called quantum droplets and later given other names including nanoparticles, nanocrystals, nanocrystallites and Q-dots. This approach provided particles that remained trapped in the glass and thus could not be easily manipulated after synthesis.

In 1993, Bawendi's group [47] developed an approach for quantum dot (QD) synthesis that facilitated the production of high-quality (see Ref. [2]) monodisperse nanoparticle QDs. Their approach allowed the synthesis of QDs that could be dispersed in various solvents and whose surface could be derivatized. These QDs still had poor fluorescence quantum yields (~10%). A subsequent approach led to the large-scale synthesis of more uniform and monodisperse QDs with higher quantum yields (>20%) [48]. It was, however, the approach of coating the QDs with a few layers of zinc sulfide (ZnS) that provided the greatest enhancement of quantum yield (Figure 1a) [3,49].

Because ZnS-coated QDs are hydrophobic, several methods have been used to stabilize them in aqueous solution and to facilitate their conjugation to biomolecules to make them useful for biological imaging. These include (i) embedding them in a silica or siloxane shell with a thickness of 1–5 nm and with amine, thiol or carboxyl functional groups on its surface [17,50]; (ii) derivatizing their surface with mercaptoacetic acid [18]; (iii) encapsulating them in phospholipid micelles [16]; (iv) derivatizing their surface with dihydroxylipoic acid [2]; and (v) coating them with an amine-modified polyacrylic acid [13].

wavelength to excite QDs of different colors. Third, the large separation between the excitation and emission wavelength of QDs, the STOKE'S SHIFT, enables the whole emission spectra to be collected, resulting in improved sensitivity of detection.

These unique features of QDs allow the simultaneous detection of different-color QDs, enabling several cells to be tracked *in vivo* [5,7], and the simultaneous biosensing of multiple molecules *in vitro* [6]. Furthermore, owing to the efficient multiphoton excitation cross-section of QDs and the ability to synthesize QDs that can emit infrared or near-infrared light, QDs are highly suited for imaging cells deep within tissues [4,7–9].

Photostability and resistance to metabolic degradation

Quantum dots show exceptional photostability: for example, when illuminated constantly with a 50-mW light, QDs do not photobleach even after 14 h [5], whereas fluorescein photobleaches completely in less than 20 min. Moreover, unlike organic fluorophores, the inorganic nature of QDs makes them resistant to metabolic degradation: QDs have been shown to remain fluorescent and to be retained in live cells and organisms for several weeks to months with no detectable toxicity [5,10].

These properties make QDs effective not only for imaging QD-tagged proteins over long periods [11,12] but also for imaging the growth and development of organisms for periods ranging from weeks to months [5,10].

Universal approaches for conjugation to biomolecules

QDs of different color vary only with respect to the size of their core. Thus, their surface properties are identical (Figure 1). This makes it easy to use the same approach for conjugating a QD of any color to a biomolecule of interest. Although conjugation based on an avidin–biotin interaction has been the most commonly used method so far [5,11–15] (Figure 1d), other approaches such as the use of protein A or protein G to bind a protein-specific antibody (Figure 1e) are also available.

The QD can be conjugated to the linker (e.g. avidin, protein A or protein G, or a secondary antibody) either by covalent binding [16–18] or by self-assembly based on electrostatic interactions [2,5,19,26,55]. Commercially available QDs have the linker covalently attached to the QDs [13]; however, electrostatic self-assembly permits the researcher to choose their own linker molecule. In the latter approach, the linker is fused to a positively charged peptide or to an oligo-histidine tag, which enables it to be conjugated to dihydroxylipoic acid (DHLA)-capped zinc sulfide (ZnS)-coated QDs [2,19,20] (Figure 2a).

Potentials of QDs as fluorescent probes in biology

In this section we discuss how the potentials of QDs for biological imaging are being realized. Box 2 describes how QDs have been used for sensitive multicolor assays of biomolecules *in vitro*. The techniques for using QDs to detect the presence and activity of biomolecules, for labeling proteins and cells with QDs, and for carrying out long-term live cell imaging are described below.

Specific labeling of cells and tissues

Labeling *in vivo* requires a high degree of specificity because of the abundance of background biomolecules that can generate false positives. Obtaining the same level of labeling specificity in cells that can be achieved *in vitro* has been a major challenge in applying QDs to cell biological issues. The first attempts to use QDs for labeling proteins in cells employed QDs conjugated to transferrin [18] or phalloidin, an actin-binding molecule [15]. To label live cells with transferrin-conjugated QDs, the cells required overnight incubation with the conjugates. By contrast, an incubation of only a few minutes is sufficient for the endocytosis of transferrin conjugated to organic dyes [21], which indicates either the poor affinity of the QD conjugate for the transferrin receptor or its nonspecific uptake. Thus, these early studies lacked the efficacy and specificity of labeling that are requisite for using QD bioconjugates for live cell studies.

These limitations have been overcome by the development of QDs that have superior stability in an aqueous environment and improved surface coatings that minimize nonspecific binding to the cell surface and the extracellular matrix, and by using bioconjugation approaches such as avidin–biotin, antibody–antigen and ligand–receptor interactions that provide a high specificity of labeling in fixed and live cells [5,11–13].

For example, the specificity of labeling has been demonstrated by experiments using QDs conjugated via avidin–biotin interaction to an antibody specific for the extracellular epitope of P-glycoprotein (Pgp), a plasma membrane

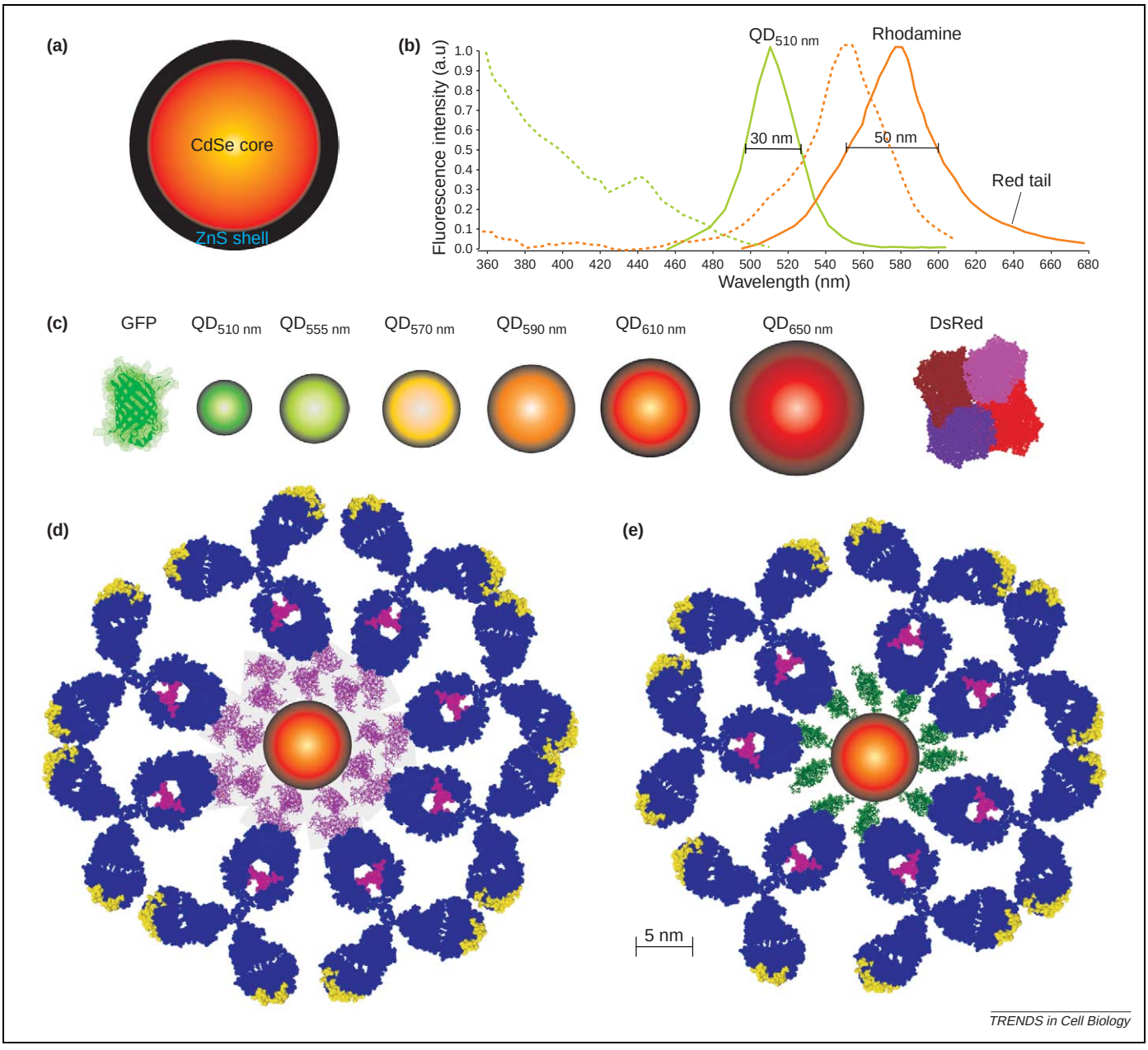

Figure 1. Properties of bioconjugatable quantum dots (QDs). **(a)** QDs are inorganic fluorophores and consist of a cadmium selenide (CdSe) core with several layers of a thick zinc sulfide (ZnS) shell to improve quantum yield and photostability. **(b)** The excitation spectrum (broken lines) of a QD (green) is very broad, whereas that of an organic dye (rhodamine, orange) is narrow. The emission spectrum (unbroken lines) is narrower for a QD (green) than for organic dyes (rhodamine, orange). Values indicate the full spectral width at half-maximum intensity (FWHM value). **(c)** The emission of the QDs can be tuned by controlling the size of the CdSe core: an increase in the size of the core shifts the emission to the red end of the spectrum. The combined size of the core and the shell of QDs emitting in the visible region of spectra are in the size range of commonly used fluorescent proteins such as green fluorescent protein (GFP) and DsRed. **(d,e)** To provide specificity of binding, QDs are conjugated with antibody molecules (blue) by using avidin (purple) or protein A (green) as linkers. Between 10 and 15 linker molecules can be attached covalently or electrostatically to a single QD, which facilitates the binding of many or a few (note the presence of free linker molecules) antibody molecules on each QD. Note that, although the QDs and molecules are drawn to size, their binding sites and relative topologies are shown hypothetically.

multidrug resistance transporter [5] (Figure 2). Here, a mixed population of HeLa cells, some of which expressed Pgp conjugated to green fluorescent protein (Pgp–GFP), was incubated with this QD bioconjugate, resulting in the specific labeling of all cells with detectable expression of Pgp–GFP but not in the labeling of neighboring cells with no detectable Pgp–GFP expression (Figure 2b, arrows indicate cells not expressing Pgp–GFP). This observation establishes that QD labeling in live cells parallels the sensitivity and specificity achieved by labeling via the expression of a protein fused to a genetically encoded fluorescent tag such as GFP.

The development of these live cell approaches has allowed several groups to use QDs for labeling proteins in live cells, where equally high levels of specificity have been achieved [11,12,22]. QDs conjugated to specific peptides and antibodies have been shown to provide specific labeling of tissues *in vivo* [23,56], as well as specific labeling of live bacteria and protist cells [24,25]. In addition to specific biomolecular labeling of cells, various generalized approaches are also available for tagging cells with single-color or several different-color QDs [5,26,55]. The specificity and ease of labeling cells with QDs has permitted the use of this technique by a growing number

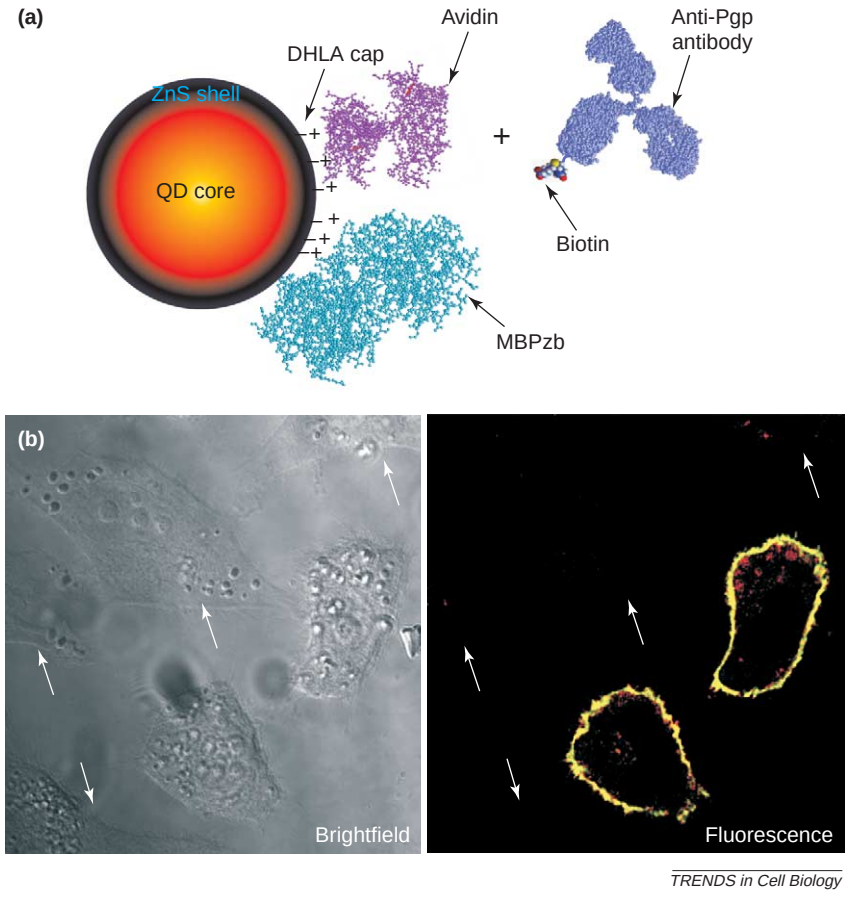

Figure 2. Specific labeling of live cells with quantum dots (QDs). **(a)** Positively charged avidin and maltose-binding protein containing a positively charged tail (MBPzb) self-assemble on the negatively charged surface of QDs capped with dihydrolipoic acid (DHLA) and can bind to biotinylated molecules such as antibodies specific for Pgp. **(b)** Transient transfection of HeLa cells with Pgp–GFP (green fluorescent protein) results in its expression in a subset of cells (not marked with arrows). The subsequent incubation of all cells with biotin-conjugated antibodies specific for Pgp, followed by avidin-conjugated QDs, leads to labeling of the cell membrane with the QD bioconjugates: only cells that express detectable levels of Pgp–GFP, and not those that do not express Pgp–GFP (marked with arrows), are labeled [48]. Yellow coloring in the fluorescence image indicates an overlap of green (Pgp–GFP) and red (QD bioconjugate) fluorescence emission. (See Ref. [2] for further details).

of researchers, making it widely accessible and practical for numerous applications.

Suitability for long-term imaging

Organic fluorophores are susceptible to photodamage and to metabolic and chemical degradation, making it difficult to tag cells for long periods. One way to surmount this problem is to use genetically encoded organic fluorophores – fluorescent proteins – which are continuously made and replenished in the cell. After their introduction into cells, however, these fluorophores require expression periods of up to a day or more before they can be detected. Similar delays are caused when they are photobleached, which can happen during continuous imaging.

Such limitations can be overcome by the use of QDs, which are resistant to photodamage [5,13], degradation by enzymes in live cells [5] and chemical damage [7]. Thus, QDs have facilitated the monitoring of molecules in live cells for several hours [11,12], and the monitoring cell fate during either a week of growth [27] or the whole developmental period of an organism [5,16].

The suitability of QDs for such applications has been used to compare the behavior of *Dictyostelium* cells early and late in development [5] and more recently to study tumor cells during tissue extravasation *in vivo*, a process that is poorly understood owing to the lack of tools with which to study it *in vivo* [7]. The photostability of QDs has also been used to study tumor cell migration *in vitro* [28]. In addition to the utility of QDs for live cell imaging, the high photo and chemical stability of QDs are a boon for immunostaining clinical samples that need to be stored for long periods.

Lack of cytotoxicity

Because QDs are ideally suited for imaging live cells and organisms over long periods, it is imperative that QD labeling is not deleterious to cells. The presence of cadmium and selenium in the core of QDs led to the belief that QDs are toxic [29]; however, QDs have shown no toxicity in live animals when injected into the bloodstream of pigs [9] and for up to 4 months in mice [8,10,27]. Even when QDs were loaded in cells growing *in vitro*, no toxicity was detected after 2 weeks of growth [5].

Furthermore, no deleterious effects of QDs in cells have been observed *in vivo* in experiments using *Xenopus* [16], *Dictyostelium* [5] and mouse [7]. In these studies, various cellular behaviors including the growth, development, signaling, chemotaxis, differentiation and, in the case of

Box 2. Specific labeling of biomolecules *in vitro*

Quantum dots (QDs) have been used to tag molecules of interest both selectively and stably. One approach involves capping the surface of QDs with dihydroxylipoic acid (DHLA), which makes the QD surface negatively charged [2]; this enables QDs to bind to linker molecules, such as protein G engineered to carry a positively charged tail (PGzb) or avidin, which is innately positively charged. These linker molecules provide the specificity to bind the molecule of interest through interactions between either PGzb and antibody or avidin and biotin (Figure 2a). Such QD bioconjugates have been used to detect simultaneously as little as 10^{-9} g of single or multiple toxins and small molecules *in vitro* [6,20]. Specific biomolecules can be detected despite an excess of other nonspecific biomolecules; the specificity is limited only by the specificity of the antibody used [6]. Collectively, these results have proved that QDs can be conjugated to biomolecules without compromising their biological activity.

Because QDs are brighter than most conventional fluorophores, their use should increase the sensitivity of all fluorescence-based assays. In addition, QDs have been shown to be inert when conjugated via other approaches and when used to detect other molecules such as protein ligands [11,51]. For example, QDs have found a major application in the area of nucleic acid detection [52–54], where QD-tagged probes are being used for the simultaneous detection of multiple nucleic acids [52,53]. The ability to identify simultaneously (not sequentially) and specifically different molecules in a single solution significantly expedites high-throughput chemical screening and holds the potential to revolutionize microarray-based approaches for large-scale studies of the gene expression profiles of organisms.

tumor cells, extravasation and nodule formation of QD-labeled cells were indistinguishable from those of unlabeled cells [7].

These results indicate that QDs are inert and safe for live cell labeling and tracking over long periods. Nevertheless, whenever a new approach for QD synthesis or coating is used, or if QDs are used in an extreme environment that could compromise their integrity, it is important to test for their cytotoxicity.

Suitability for multicolor in vivo *imaging*

QDs have facilitated the simultaneous imaging of at least five populations of live cells, each labeled with a different-colored QD [7]. If combinations of different QDs are used to tag cells, then an ability to resolve five colors will enable the resolution of 36 populations of cells. This potential has been recently realized to generate ten unique codes using five different-color QDs [30]. The ability to generate such multicolor codes with QDs has been used to image simultaneously the fate of three different populations of *Dictyostelium* cells as they developed [5].

The high absorbance and scatter of visible light limit imaging in tissue beyond a depth of 100 μm. MULTIPHOTON MICROSCOPY can image at greater depths by using infrared excitation that does not scatter as much as visible light. QDs have been found to be two to three orders of magnitude brighter than the conventional fluorescent probes used in multiphoton microscopy [8]. Furthermore, all QDs are excitable with any multiphoton excitation wavelength between 700 and 1000 nm.

The lasers currently used for multiphoton microscopy do not allow a rapid switching of excitation wavelength. Thus, use of QDs offers a significant advantage for studies requiring the simultaneous imaging of multiple fluorophores. This approach has been used to compare the ability two populations of tumor cells to extravasate into the lungs of mice [7]. The ability to synthesize QDs that emit in the near-infrared spectrum has also facilitated *in vivo* imaging not only in mice [4,8], but also in bigger animals such as pigs [9]. Multicolor *in vivo* imaging is enabling cellular interactions to be examined during development and metastasis, and non-invasive surgeries to be carried out in a way that has have not been feasible without QDs.

Suitability for FRET-based sensing

The tunability of QD emission, the resistance of QDs to photobleaching, the ability to conjugate a single QD to several acceptor dyes, the narrow emission of QDs and the ability to excite QDs far from their peak emission make QDs significantly better donors than organic fluorophores for fluorescence resonance energy transfer (FRET)-based applications. The ability of QDs to function as energy donors for FRET has been tested *in vitro* [31,32] and has been exploited for *in vitro* monitoring of the dynamics of telomere formation [33] and maltose biosensing [19,34]. The maltose sensor designed by using maltose-binding protein (MBP) provides a specificity and sensitivity of detection similar to that of free MBP molecules in solution; thus, conjugation to QDs does not affect the properties of this biomolecule [19].

Recently, QDs have been synthesized by changing the structure and/or composition of the QD core to tune the fluorescence emission with no change in the size of the core [35]. Because the transfer of energy between a FRET pair is sensitive to the distance between the fluorophores, this application of QDs opens the possibility of designing multiple FRET pairs that have QD donors with different peak emissions but that transfer their energy with similar efficiency. Having multiple FRET-based sensors each with a high efficacy of energy transfer will permit highly sensitive and simultaneous monitoring of multiple molecules in live cells. For example, changes in the level of sugar molecules, such as maltose or glucose, or changes in the activity of multiple proteins involved in a signaling cascade, can be detected simultaneously in a single cell.

Pitfalls of QDs as probes in biology

Despite their potential and their success so far in biological applications, QDs also have limitations associated with their use. Box 3 discusses one of the chief

Box 3. How to get quantum dots into cells

Owing to their size and chemical nature, quantum dots (QDs) cannot diffuse through the cell membrane. To use QDs for labeling and imaging cytoplasmic proteins, the QDs must be delivered by invasive approaches such as microinjection [16], cationic lipid-based reagents [7] or conjugation to membrane-permeable peptides [30]. However, these approaches can cause the intracellular QDs to aggregate in punctae or to end up in endosomes [26,55], instead of being dispersed in the cytosol.

Crucial challenges to using QDs for intracellular imaging are (i) the development of non-invasive approaches for the efficient intracellular delivery and dispersal of QDs; (ii) the development of methods to label intracellular proteins that are located in an environment vastly different from the extracellular space; and (iii) the development of QDs that either are inert to the cytoplasmic environment or respond in a defined manner to selective changes of the cytoplasmic environment.

impediments to their use – that is, their effective delivery into cells. In this section we discuss the properties of QDs and QD bioconjugates that need to be improved before the full potential of these inorganic fluorophores can be realized in terms of biological applications.

Sensitivity to environment

For biological studies, QDs must be inert to the environment inside and outside the cells. QDs are inherently hydrophobic in nature and thus different strategies have been used to make them hydrophilic. These modifications unfortunately decrease both the stability and QUANTUM YIELD, owing to the sensitivity of QDs and their fluorescence to environmental factors such as pH, salts and oxidation [2,29,36,37]. Moreover, the colloidal nature of QDs in aqueous environment makes them susceptible to irreversible aggregation, which could happen during long-term storage, changes in pH, heating or even freezing of the QD solution. Because the environment around each QD cannot be regulated at will in live cells, there is need for continued improvement in synthesis such that QDs are impervious to environmental changes.

Size, valency and purity of bioconjugatable QDs

Unlike organic dyes, which are usually small molecules, QDs are the size of average to large proteins (Figure 1c). To construct the QD, the core is first encapsulated and capped to provide water stability and to increase the quantum yield. The QD is then conjugated with avidin (67 kDa), protein A (30 kDa) and/or protein G (42 kDa), or secondary antibodies (150 kDa) to provide specificity [2,5,13,14,38]. A single QD has been measured to be bound to as many as ten proteins of this size [2] (Figure 2d,e). Thus, the resulting QD with the conjugated proteins is comparable to a protein of 500–750 kDa. This raises potential concerns over the influence of the QD-based tags on protein mobility and functionality. One way to overcome this concern is to reduce the valency (the number of linkers per QD), another would be to use smaller linkers for bioconjugation. As a step towards the latter, a penta-histidine motif inserted at the terminus of the protein of interest has been recently used to bind the QD surface [39].

There is also a need for approaches to purify the bioconjugated QDs efficiently from the unconjugated components. Mixed-surface self-assembly can be used for purification and can also reduce the valency of the QDs. In this approach to reduce the valency of the QD, either avidin or protein G engineered to contain a positively charged domain (PGzb) is allowed to bind the negatively charged DHLA-capped QDs in the presence of MBP containing a positively charged tail (MBPzb). Thus, there is competition between the linkers (avidin or PGzb) and MBPzb to bind the surface of the DHLA-capped QD. Varying the ratio of linkers to MBPzb during QD self-assembly can regulate the number of linkers on the QD surface and thus the valency of the QD bioconjugate. Because MBP can reversibly bind amylose, the presence of MBPzb also facilitates purification of the bioconjugated QDs on an amylose column [2]. Because these approaches make use of noncovalent interactions, there remains a need to develop strategies that use more stable covalently linked QD bioconjugates.

Single-molecule imaging in live cells

Owing to their high photobleaching threshold and the large separation between the excitation and emission spectra, QDs provide a very high signal-to-noise ratio, making them suitable for the long-term tracking of single molecules [12]. However, many of the approaches used to distinguish whether or not a signal comes from a single fluorophore, such as single-step photobleaching, cannot be applied to QDs owing to high photostability.

The use of another diagnostic approach, FLUORESCENCE BLINKING of a single QD under continuous wave illumination, is also hindered by the sensitivity of QD blinking to changes in excitation intensity [40], temperature [41] and the surrounding environment [42]. These features alter blinking characteristics to the extent of eliminating blinking under some conditions, such as those found in a reducing environment [43]. Because the cellular environment is highly reducing, it could potentially make blinking a poor criterion for identifying single QDs in live cells. In addition, unlike organic dyes, for which the blinking interval is about 0.5 ms [44], for QDs this interval is around 500 ms [40]. In fact, it has been recently reported that this interval could be as much as 100 s for commercially available avidin-conjugated QDs [43]. Such a long intermittency will prevent the use of QDs to track single molecules in the cytoplasm of live cells where they diffuse at about 3 $\mu m^2/s$ [45]. The above features limit the ability to establish when a single QD molecule is being observed.

The multivalency of currently available QD bioconjugates further precludes their use for labeling only a single molecule in live cells (Figure 2d,e). This is a major impediment to studying single molecules because it can result in a change in the behavior and even functionality of the molecules. Nevertheless, the ability of QDs to avoid photodamage and metabolic degradation, their high quantum yield and the potential to develop approaches to label single molecules without affecting their functionality provide the motivation to design QDs and to develop imaging strategies that will overcome these limitations.

Concluding remarks – what lies ahead?

The potential value of QDs in bioimaging is due to (i) their photostability, which facilitates the long-term tracking of QD-labeled cells and molecules; (ii) their ability to tune emission wavelength, which enables them to tag simultaneously several different population of cells and molecules; (iii) their broad excitation and narrow emission spectra, which facilitates the simultaneous detection of different QD-tagged cells; and (iv) the availability of common approaches to bioconjugate them without compromising molecular function, which allows the specific labeling of biomolecules *in vivo* and *in vitro*. These features also make QDs ideal donors in FRET – a feature that has been already tested *in vitro* and is likely to find widespread applications *in vivo*.

Apprehensions about the toxicity of QDs have been an impediment to their biological application; however, several independent studies have recently demonstrated

the lack of toxicity of QDs *in vivo*, which will enable harnessing of the potentials of QDs for *in vivo* applications. Before QDs can find wider use in biological research, several improvements must be made, including alterations of the surface properties that affect their stability in cellular environments, and developing methods for their delivery and efficient targeting in cells, without altering their properties. Although there is much that we still need to understand about these little wonders, with the current interest and the concerted efforts of physicists, chemists and biologists, it is likely that they will soon become a standard tool for biological applications.

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