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Review

Biological applications of quantum dots

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Abstract

Quantum dots (QDs) are a novel class of inorganic fluorophore which are gaining widespread recognition as a result of their exceptional photophysical properties. They are rapidly being applied to existing and emerging technologies, and could have an important role in many areas. Significant challenges remain, however, which must be understood and more fully defined before they can be widely validated.

This review provides on overview of QD technology, covering QD characteristics, synthesis methods, and the applications in which they have been put to use. The influence of synthesis methods on QD characteristics and their subsequent suitability to different applications is discussed, and a broad outline of the technologies into which they have been incorporated is presented, and the relative merits and weaknesses of their incorporation are evaluated. The potential for further development, and inclusion in other technologies is also discussed, and barriers restricting further progress specified, particularly with regard to the poorly understood surface chemistry of QDs, the potential for alteration of function of biological molecules when complexed with QDs, and on a larger scale the significant potential for cytotoxicity both *in vitro* and *in vivo*.

Contents

1. Introduction

Quantum dots (QDs) are nanometer-scale semiconductor crystals composed of groups II–VI or III–V elements, and are defined as particles with physical dimensions smaller than the exciton Bohr radius [1]. When a photon of visible light hits such a semiconductor, some of their electrons are excited into higher energy states. When they return to their ground state, a photon of a frequency characteristic of that material is emitted. Metal and semiconductor nanoparticles in the size range of 2–6 nm are of considerable interest, due to their dimensional similarities with biological macromolecules (e.g. nucleic acids and proteins) [1]. This review aims to explore the properties of QDs, and the role they may take in advanced medical imaging.

2. Optical properties of quantum dots

Quantum confinement effects give rise to unique optical and electronic properties in QDs, giving them numerous advantages over current fluorophores, such as organic dyes, fluorescent proteins and lanthanide chelates [2]. Properties that particularly influence fluorophore behaviour, and therefore applicability to different situations, include the width of the *excitation* spectrum, the width of the *emission* spectrum, photostability, and the decay lifetime.

Conventional dyes suffer from narrow excitation spectra, requiring excitation by light of a specific wavelength, which varies between particular dyes. QDs have broad absorption spectra, allowing excitation by a wide range of wavelengths, a property which may be exploited to simultaneously excite multiple different coloured QDs using a single wavelength (Fig. 1) [1,3]. Conventional dyes also have broad emission spectra, meaning the spectra of different dyes may overlap to a large extent. This limits the number of fluorescent probes that may be used to tag different biological molecules and be spectrally resolved simultaneously. In contrast, QDs have narrow emission spectra, which can be controlled in a relatively simple manner by variation of core size and composition, and through variation of surface coatings. They can be engineered to emit light at a variety of precise wavelengths from ultraviolet (UV) to infrared (IR). The narrow emission and broad absorption spectra of QDs makes them well suited to multiplexed imaging, in which multiple colours and intensities are combined to encode genes, proteins and small-molecule libraries [3,12,13]. It has been

suggested that a realistic scheme using 5–6 colours with 6 intensity levels could be used to yield approximately 10,000–40,000 different recognisable codes [3]. In combination with their good photostability, which is discussed below, they may provide the opportunity to monitor the long-term interactions of multiple-labelled biological molecules in cells.

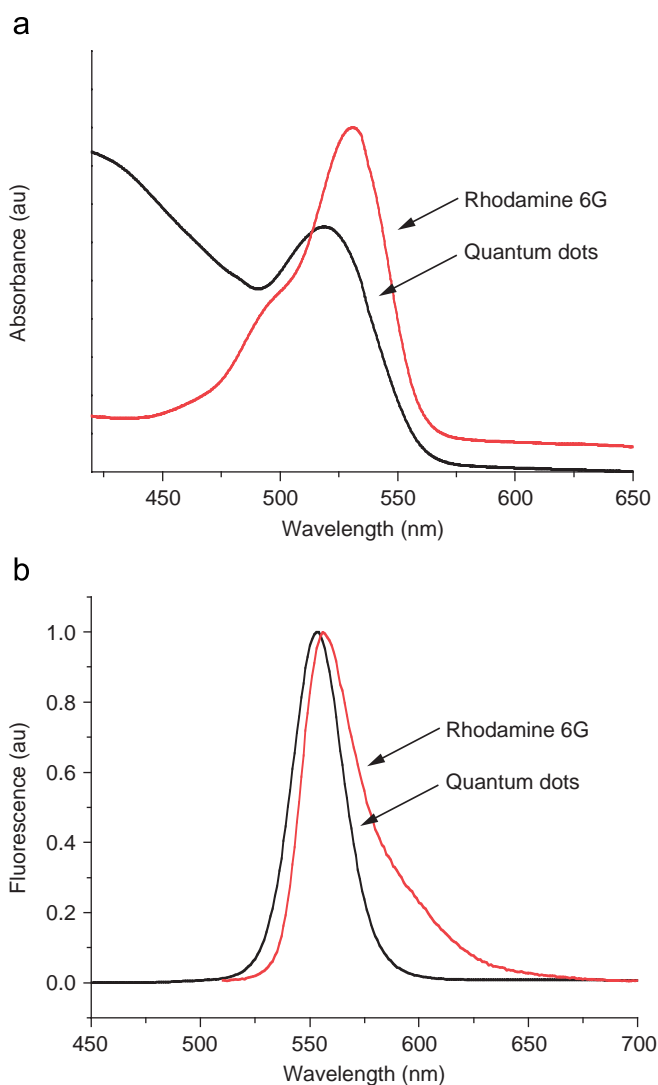Fig. 1. Excitation (a) and emission (b) profiles of rhodamine 6 G and CdSe QDs. The QD emission spectrum is nearly symmetrical and much narrower in peak width while its excitation profile is broad and continuous, meaning that QDs can be efficiently excited at any wavelength shorter than ∼530 nm. By contrast, the organic dye rhodamine 6 G has a narrow excitation profile and broad emission spectrum [1] (permission obtained).

Photostability is a critical feature in most fluorescence applications, and is an area in which QDs have singular advantage. Unlike organic fluorophores which bleach after only a few minutes on exposure to light, QDs are extremely stable and can undergo repeated cycles of excitation and fluorescence for hours with a high level of brightness and photobleaching threshold [3,4]. QDs have been shown to be more photostable than a number of organic dyes [5,6], including Alexa488, reported to be the most stable organic dye [7]. Dihydrolipoic acid (DHLA)-capped cadmium selenide-zinc sulfide (CdSe-ZnS) QDs showed no loss in intensity after 14 h, and were nearly 100 times as stable as, and also 20 times as bright as, rhodamine 6 G [6]. As previously discussed, this may be exploited in situations where long-term monitoring of labelled substances is required, and is an area in which QDs may find particular use.

QDs also have a long fluorescent lifetime after excitation, which may be taken advantage of in time-gated imaging. The fast fluorescence emission of organic dyes upon excitation (<5 ns) coincides closely with short-lived autofluorescence background from many naturally occurring species, reducing the signal-to-noise ratio. Conversely, QDs emit light with a decay time in the order of a few tens of nanoseconds (30–100 ns) at room temperature, which is slower than the autofluorescence background decay, but fast enough to maintain a high photon turnover rate (Fig. 2) [8,9]. In time-gated analysis, photons hitting in the first few nanoseconds are disregarded to decrease background noise and increase sensitivity. The usefulness of this has been shown in producing images of 3T3 mouse fibroblasts with a high signal-to-background ratio [10], and in following erbB1 and erbB3 receptors. In this case,
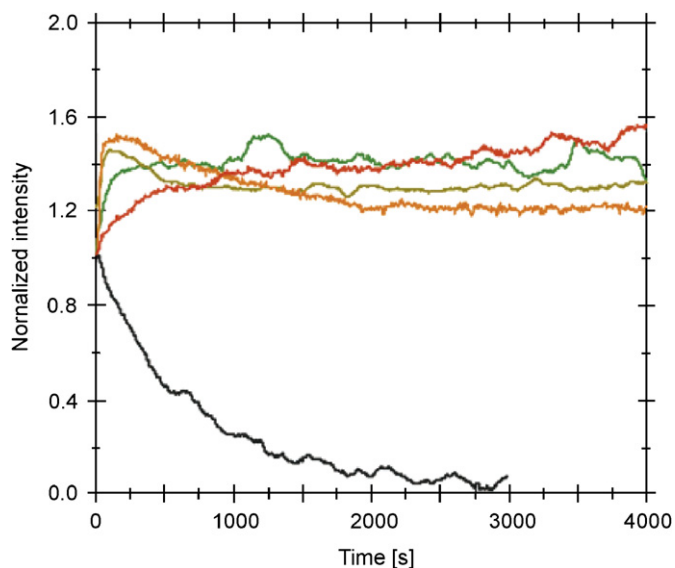
time-gating allowed distinction to be made between erbB3 receptors labelled with citrine and erbB1 receptors labelled with QDs, even though they could not be spectrally resolved [11].

3. Synthesis and surface chemistry

QD synthesis can be tailored to specific requirements, with core, shell and coating characteristics all affecting photochemical properties. QDs may be manufactured with diameters from a few nanometers to a few micrometers, and size distribution can be controlled within 2% [14] using precise growth techniques, involving high annealing temperatures [15]. Choice of shell and coating are gaining particular importance, as the shell stabilises the nanocrystal and to some extent alters the photophysical properties, whilst the coating confers properties to the QD which allow its incorporation into a desired application.

Bare core nanocrystals have proven impractical for two reasons. Firstly, the crystalline structure of the nanoparticle lends itself to imperfections [15], which results in emission irregularities, particularly blinking, in which single QDs switch between fluorescent and non-fluorescent states despite continuous illumination [16]. Secondly, the cores are highly reactive [15] due to their large surface area:volume ratio, resulting in a very unstable structure which is particularly prone to photochemical degradation. Capping core nanocrystals with ZnS has been shown to increase stability and performance, producing QDs with improved luminescence, higher photochemical stability and higher quantum yields at room temperature [17,18]. However, ZnS capping alone is not sufficient to stabilise the core, particularly in biological solutions, but a serendipitous byproduct of modification to render QDs biologically compatible, particularly with polyethylene glycol (PEG), is an increase in stability and a reduction in non-specific adsorption.

Solubilisation of QDs is essential for many biological applications, but presents a significant challenge. Non-water-soluble QDs can be grown easily in hydrophobic inorganic solvents, but solubilisation requires sophisticated surface chemistry alteration. Current methods for solubilisation without affecting key properties are mostly based on exchange of the original hydrophobic surfactant layer with a hydrophilic one [8,19,20], or the addition of a second layer such as the amphiphilic molecule cyclodextrin [21], which may also contain another functional group. Chitosan, a natural polymer with one amino group and two hydroxyl groups, has been used for intracellular delivery of specific molecules [22,23], and can be attached to the QD surface. Other methods for increasing solubility include encapsulation in phospholipid micelles [24], addition of dithiothreitol [25], organic dendron [26,27], oligomeric ligands [20], and the addition of a second layer of poly(maleicanhydride alt-1-tetradecene) to the QD's surface. Silica and mercaptopropionic acid (MPA) are also commonly used [8,19], and allow bioconjugation to ligands

Fig. 2. Time dependence of the fluorescence intensity of silanised nanocrystals and rhodamine 6 G at 488 nm. The nanocrystals exhibit a stable emission for at least 4 h, while the dye bleaches after 10 min, colours correspond to nanocrystal emission, R6G is in black [8] (permission obtained).

of interest. MPA achieves this through carboxyl groups, and silica through the presence of thiol groups on its surface. Fig. 3 lists conjugation schemes commonly used for attaching proteins to QDs [28]. The colloidal properties of solubilised nanoparticles, including the charge and hydrodynamic status, will be altered depending on the method used, meaning that the solubilisation strategy will need to be tailored according to the biological system being used [29]. The increase in diameter brought about by such modifications, and conjugation with biomolecules, may make intracellular delivery more difficult, and could increase toxicity [30]. Another challenge is that there is no technique which consistently allows preparation of QDs with control over the ratio of biomolecules per QD and their orientation on the surface. Current strategy (based on modifying COOH groups on the QD surface for covalent attachment of amine groups) is limited by problems of reproducibility and aggregation [31].

Although QDs have proven to be more photostable than conventional organic dyes in some protocols, a substantial loss of fluorescence has been noted upon injection into tissues and whole animals, and in ionic solutions [32–36]. This signal loss has been suggested to be due to slow degradation of surface ligands and coating, or to factors absorbed to the surface when subjected to body fluids, leading to surface defects and fluorescence quenching [17,37]. Some important technical problems remain, particularly in defining and characterising the surface coating chemistry. This must be controlled to develop a coating which provides minimal non-specific binding, whilst maintaining stability, avoiding oxidisation and withstanding salt concentration in cells. It must also maintain strong fluorescence without bleaching, quenching, or blinking.

4. Cytotoxicity

Cytotoxicity of QDs has been observed in a large number of in vitro studies [28,38–42], affecting cell growth and viability [43]. The extent of cytotoxicity has been found to be dependent upon a number of factors including size, capping materials, colour, dose of QDs, surface chemistry, coating bioactivity and processing parameters [42,44,45]. Even if not inducing significant alterations in cell physiology, QDs can produce subtle alterations of function which may affect the quality of data derived from their use [41,46,47].

A number of mechanisms have been postulated to be responsible for QD cytotoxicity. These include desorption of free Cd (QD core degradation) [28,38], free radical formation, and interaction of QDs with intracellular components. Examination of QD toxicity in a hepatocyte culture model showed that exposure of core CdSe to an oxidative environment causes decomposition and desorption of Cd ions. Such exposure during synthesis and processing played an important role in subsequent toxicity. Addition of a silica (SiO_2) and ZnS shell can reduce oxidation, but is unable to eliminate it, particularly under concomitant exposure to UV light [48]. The addition of ligand shells has also been observed to reduce Cd desorption, but again is unable to eliminate it under oxidative conditions, and ligand addition brings its own attendant problems as will be discussed.

The generation of free radicals, particularly reactive oxygen species has also been seen to contribute to toxicity [40,49,50]. Nicking of DNA was seen both in DNA incubated with QDs in the dark, and under UV exposure. This was attributed to photo-generated and surface generated free radical exposure [51]. CdSe core QDs induced apoptosis in neuroblastoma cells by activation of
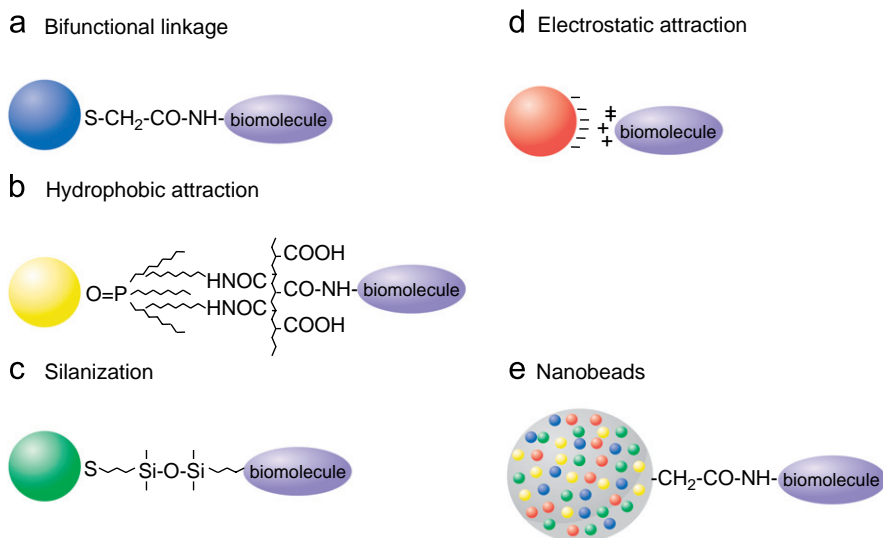
Fig. 3. A schematic illustrating different approaches of conjugation of QDs to biomolecules: (a) Use of a bifunctional ligand such as mercaptoacetic acid for linking QDs to biomolecules. (b) TOPO-capped QDs bound to a modified acrylic acid polymer by hydrophobic forces. (c) QD solubilisation and bioconjugation using a mercaptosilane compound. (d) Positively charged biomolecules linked to negatively charged QDs by electrostatic attraction. (e) Incorporation of QDs into microbeads and nanobeads [1] (permission obtained).

a number of apoptotic pathways, and downregulation of survival signalling molecules [52]. The composition of the core, and also the colour of the QD (a reflection of core size) appear to influence toxicity [40]. These studies also observed that addition of a ZnS shell was beneficial, and reduced free radical generation; however the DNA nicking observed was the result of incubation with CdSe/ZnS QDs with a biotin ligand. Whether or not the generation of free radicals is dependent on Cd desorption is unclear, but is a possibility given that Cd has been shown to generate free radicals [53], and that a similar reduction in free radical generation as Cd desorption is seen on addition of a ZnS shell.

In addition to the effects of the QD core, ligands added to render the probe biologically active may have toxic effects on cells. Mercaptopropionic acid (MPA) and mercaptoacetic acid, which are commonly used for solubilisation, have both been shown to be mildly cytotoxic [39]. MUA, cysteamine and TOPO have all been shown to have the ability to damage DNA in the absence of the QD core [54]. PEGylated QDs have been shown to have reduced cytotoxicity, but modification of these to produce PEG-amine for biological activity renders them cytotoxic once again [55].

Unfortunately, interpretation of information on cytotoxicity is difficult as a result of differences in cellular handling of QDs and the possible contribution of unexpected factors to toxicity. The reduced cytotoxicity seen with QD-PEG compared with unmodified QDs has been found to be related to reduced uptake of these modified QDs, and not necessarily to an inherently reduced toxicity [56]. The way in which QDs are handled by cells after uptake is also variable, and different intracellular fates are likely to contribute to different toxicity. Handling has been shown to be affected by size, colour and coating [53], and different handling has even been observed between QDs with the same coating but different emission wavelengths. With the limited data accumulated so far it is very difficult to estimate the true extent of QD cytotoxicity, which factors contribute, and the effects they may have.

Groups III–V QDs may provide a more stable alternative to groups II–VI QDs due to the presence of a covalent, rather than an ionic, bond, and have been reported to have lower cytotoxicity [57]. However these QDs are difficult to prepare on a competitive time scale, and tend to have much lower quantum efficiencies, meaning uptake has been slow. Data relating to cytotoxicity is understandably much more limited for these QDs, making it difficult to draw firm conclusions, and comment either way.

5. Biological applications of QDs

5.1. Fluorescence resonance energy transfer analysis

Fluorescence resonance energy transfer (FRET) involves the transfer of fluorescence energy from a donor particle to an acceptor particle whenever the distance between the donor and the acceptor is smaller than a critical radius, known as the Förster radius [58]. This leads to a reduction in the donor's emission and excited state lifetime, and an increase in the acceptor's emission intensity. FRET is suited to measuring changes in distance, rather than absolute distances [59], making it appropriate for measuring protein conformational changes [60], monitoring protein interactions [61] and assaying of enzyme activity [62]. Several groups have attempted to use QDs in FRET technologies [63], particularly when conjugated to biological molecules [64], including antibodies [65], for use in immunoassays.

QD-FRET has been used for monitoring protein interactions in the Holliday Junction [66], an intermediate in the recombination of DNA that undergoes conformational change on addition of Mg^{2+} ions [67]. Using QD585 as a donor on one arm of the DNA, and Cy5 as an acceptor on a perpendicular arm, movement of the arms on addition of Mg^{2+} could be detected as a change in the emission of both donor and acceptor. However, the changes were detected with considerably less efficiency than with the equivalent Cy3/Cy5 FRET.

Quantitative maltose sensing has provided an example of how QDs might play a role in enzyme assays. In a recent study, QDs conjugated to maltose binding protein (MBP) allowed binding of either maltose or a quenching molecule [68]. The quenching molecule, with a binding affinity similar to that of maltose, was readily displaced on addition of maltose, and a concentration-dependent increase in luminescence was observed. Several studies have exploited QD-FRET for imaging activity of proteases [69–72]. For this application a QD-probe conjugate is bound to a quencher probe by a peptide sequence which is recognised by a protease, in which state the fluorophore is quenched. On cleavage of the two molecules by a protease, emission is restored, allowing its activity to be visualised. Compared to previous results using organic fluorophores [73,74], QDs gave an increased luminescence of 52% over 47 h after incubation with a collagenase [69]. Subsequent studies have shown that QD-FRET can detect activity of caspase-1, thrombin and chymotrypsin [71], trypsin [75], and β-lactamase. A QD-FRET assay of collagenase has also been demonstrated to be able to distinguish between normal and cancerous breast cells [72].

A number of issues may affect the use of QDs in FRET applications. The physical dimensions of QDs, particularly after capping and the addition of further shells, such as DHLA, make close approach to the QD core difficult, reducing FRET efficiency. This may be partially overcome by the addition of a relay acceptor, but this reduces the overall efficiency, and may involve structural alteration to proteins to allow their incorporation, changing the physicochemical properties of the substances being used [68,76]. Peptide accessibility is also a concern, as in order to produce efficient probes, multiple energy acceptors need to be conjugated to a central QD, which introduces steric

hindrance to substrate accessibility for proteases [69]. Environmental conditions are also likely to have an effect on FRET changes, as it has been shown that fluorescence intensity in FRET applications changes as a function of both pH and ionic strength of the solution in which the system is placed. Displacement of peptide-dye conjugates from a central QD has also been reported, particularly when larger biomolecules are being used [75]. It should be noted that in all the above studies QDs have been used as energy donors. A comprehensive examination of the subject concludes that QDs make unsuitable energy acceptors for FRET applications [77].

5.2. Gene technology

A number of studies have shown that QD-conjugated oligonucleotide sequences (attached via surface carboxylic acid groups) may be targeted to bind with DNA or mRNA [25,78]. Comparison of QD performance against Texas Red and Fluorescein, traditional organic fluorophores, in hybridisation using total DNA as a probe gave mixed results. The optical qualities of QDs were superior, showing up to 59% greater photostability and 11-fold greater signal intensity, and QD probes could be used to detect the clinically useful ERBB2/HER2/neu locus, which is relevant to breast cancer. However, staining in centromeric regions, which was seen using organic fluorophores, was noted to be deficient using QDs, and fluctuation of signal intensity was observed, thought to be the result of blinking [79]. Further, attachment of oligonucleotides to the QD surface led to poor long-term stability. Oligonucleotide derivatised QDs were used, as building free carboxylic acid groups on the QD surface led to non-specific binding to target cells, making them far less useful than conventional organic fluorophore probes.

Using red, green and blue QDs in a number of combinations, it has been demonstrated that specific labelling and identification of target sequences of DNA can be achieved [3]. This was exploited by using QD microbeads for an assay of single nucleotide polymorphism (SNP). Authentic genomic samples, rather than clean model oligonucleotides, were amplified, producing biotinylated amplicons. These were subsequently incubated with QDbead-labelled oligonucleotides and then with streptavidin-Cy5, which interacts with the biotin on the amplicons. The combination of Cy5 and QD signals showed that hybridisation had occurred. Using this method, call rates of 100%, and 100% concordance with TaqMan in-house assays for 940 genotypes, were achieved [80]. These results suggest that QDs could be used to produce more efficient assays, requiring smaller quantities of DNA, to be developed. Others have also attempted to detect single point mutations using a similar protocol with favourable results [81]. A theoretical problem with such an assay is the effect that blinking might have on the intensity readings obtained. This was not addressed in either of these studies.

QD-FRET has also found a place in genetic applications. Use of QDs for determining the dynamics of telomerisation and DNA replication has been reported [82]. One group report the design of a DNA nanosensor which sandwiches a target sequence between a biotinylated capture probe and a reporter probe bound to Cy5. A target thus labelled binds to QD-streptavidin particles, with several oligonucleotides binding to each particle (Fig. 4) [83]. The efficiency of FRET when multiple molecules are bound is greater than when single molecules are bound [79,84], up to a maximum of 54 found in this study. The selection of QD650 and Cy5 as a donor–acceptor pair allowed negligible crosstalk and selection of a wavelength near the minimum of the Cy5 absorption spectrum. Compared to molecular beacons, which are commonly used in DNA hybridisation applications, this method produced a much higher sensing responsiveness at almost every target concentration tested. At 0.96 nM it was approximately 100-fold greater, and could detect signal at 4.8 fM, compared with 0.48 pM. Using an oligonucleotide ligation assay in the KRAS gene, mutation of which has been identified as an early event in tumorigenesis in ovarian serous borderline tumours [85], it was possible to discriminate between heterozygous and homozygous wild types with good efficiency. The authors of this study suggest that the detection limits of these sensors obviates the need for pre-target amplification and can be extended to non-DNA targets such as proteins and peptides.

Strong quenching (83±9% [86] and 85% [34]) using gold-conjugated DNA with QDs has also been shown [34,86], but the strength of quenching is affected by the interparticle distance, and with short interparticle distances additional non-radiative interactions affect quenching. It has also been noted that emission yield over time may be significantly reduced when QD-DNA complexes are complexed with clean oligonucleotides (i.e. those without Au attached in which no quenching should have occurred), with a greater than 50% decline being reported at 2.5 h. Although the reduction in yield is less than that seen with Au quenching, it nevertheless represents an important reduction in accuracy. Unfortunately, no data was given on emission yield at 1.5 h, the time at which the result of Au quenching was measured. The authors suggest that the reduced yield is due to the use of an ionic solution, as this has previously been observed in QDs in ionic solutions [35].

In addition to their role in DNA technology, QDs may find use in RNA technologies, in detection of mRNA molecules using ISH and in combination with siRNA in RNA interference applications. QDs have been successfully used in ISH techniques to study the expression of specific mRNA transcripts in mouse midbrain sections [87]. Labelling of up to four different mRNA transcripts in neurons in appropriate areas of the midbrain was possible, producing better results than the most sensitive organic fluorophore. Combining *in situ* hybridisation
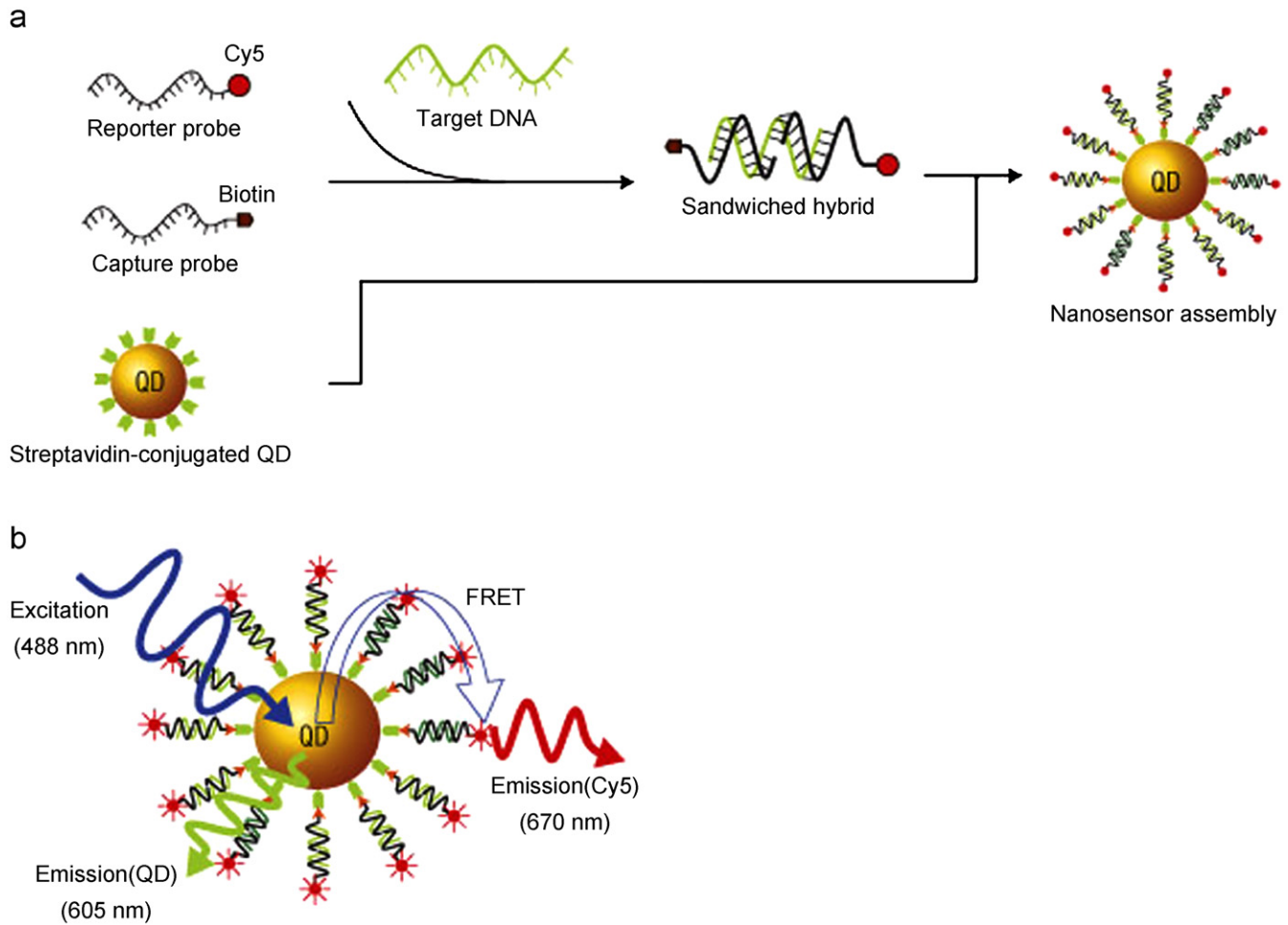
Fig. 4. Schematic of single-QD-based DNA nanosensors. (a) Conceptual scheme showing the formation of a nanosensor assembly in the presence of targets. (b) Fluorescence emission from Cy5 on illumination on QD caused by FRET between Cy5 acceptors and a QD donor in a nanosensor assembly [83] (permission obtained).

techniques with immunohistochemistry allowed visualisation of the localisation of growth hormone and prolactin proteins in relation to their mRNA. Biotinylated oligonucleotide probes provided an attachment site for streptavidin-coated QD605 to target the mRNA molecules whilst QD685 conjugated to anti-rabbit IgG targeted the protein molecules labelled by immunohistochemical techniques. Using this protocol, mRNA and protein molecules could be distinguished, and the localisation of the molecules in relation to each other could be visualised in three dimensions, which is an advantage over current EM methods [88].

QDs have also found a use in RNA interference applications, where they allow monitoring of the extent of gene knockdown in a cell by measuring brightness [89]. RNA interference has become an important tool for determination of gene function, but inefficient and heterogeneous delivery of siRNA often observed in cell culture causes variable levels of gene silencing [15]. The ability to easily select cells with high levels of gene silencing is likely to be extremely useful if it proves feasible.

5.3. Fluorescent labelling of cellular proteins

External labelling of cells with QDs has proven to be relatively simple, but intracellular delivery adds a level of difficulty. Several methods have been used to deliver QDs to the cytoplasm for staining of intracellular structures, but so far these have not been particularly successful. Micro-injection techniques have been used to label xenopus [24] and zebrafish [90] embryos, producing pancytoplasmic labelling, but this is a very laborious task, which rules out high volume analysis. QD uptake into cells via both endocytic [91,92] and non-endocytic pathways has also been demonstrated, but results in only endosomal localisation. Two novel approaches have shown pancytoplasmic labelling, by conjugation with Tat protein, and by encapsulation in cholesterol-bearing pullulan (CHP) modified with amine groups [93]. Coating with a silica shell may also prove useful. An excellent report by Derfus et al. [94] compares some of the most commonly used methodologies.

Labelling of F-actin fibres demonstrated that QDs could be used to label proteins where preservation of enzyme

activity was desirable [47]. Streptavidin-coated QDs were used to label individual isolated biotinylated F-actin fibres. However, compared with Alexa488 (an organic fluorophore), a smaller proportion of labelled filaments were motile. Intracellular labelling of these filaments has also been shown to be possible (Fig. 5) [5]. QDs have also been used to label mortalin, and p-glycoprotein, molecules which are important in tumour cells [95,96]. Labelling with QDs was much more photostable than with organic dyes, with a 420-fold increase over Alexa488. Advantage was taken of this to image three-dimensionally the localisation of p-glycoprotein, with the long fluorescence lifetime allowing successive z-sections to be imaged [96].

A number of groups report multiple colour labelling of different intracellular structures [91,97]. Simultaneous labelling of nuclear structures and actin filaments with QDs of two different colours was demonstrated by one group, but variable labelling of nuclear structures was observed. Another group labelled mitochondria and nuclear structures, producing distinct red labelling of the nucleus and green labelling of the mitochondria. Single-colour labelling of Her2 has also been shown to be possible, and is of particular note, given that expression of this can be used as a predictive and prognostic marker for breast cancer. Specific labelling of both QD630 and QD535 to the receptor could be seen, and was possible even in fixed tissue specimens [5]. There are, however, limits on the number of independent signals achievable for multiplexed immunoassays. Emission spectra separated by 15 nm in

their intensity maxima (where the distributions have similar full-width at half-maximum, FWHM ~25–35 nm) can be resolved.

QDs have also been used in tyramide signal amplification (TSA), which uses horseradish peroxidase to attach tyramide to antibody targets in order to facilitate antibody binding. Use of this method allows increased fluorescence intensity and assay sensitivity [98]. Combination of QDs with electron microscopy techniques allowed labelling of nuclear promyelocytic leukemia protein (PML) and cAMP response element binding protein (CREB), and also made it possible to label multiple targets using a combination of QD and gold particles to show localisation of the two targets [99].

The photostability and advantageous signal-to-noise ratio achievable with QDs means they could be ideal probes for single molecule tracking studies. A number of groups have attempted to use QDs for following the dynamics of cell surface receptors involved in cell signalling. Early attempts using QDs to label serotonin transporters were limited by weak potency and an inability to discriminate between serotonin receptors and transporters [100]. However, QDs have subsequently been used more successfully to visualise and track the movements of glycine receptors [101], erb/HER receptors [102], AMPA receptors [103], GABA$_c$ receptors [104] and TrkA receptors in the interior of neural PC12 cells [105]. In these studies the dynamics of the receptors could be tracked, and were an improvement over organic fluorophores for long-term tracking. One group studying receptor-mediated signal transduction in erbB/HER receptors were able to follow the receptors during endocytosis, revealing a previously unknown retrograde transport mechanism [102]. In another study, QDs were used to track the movements of receptors within neural cells to demonstrate previously unknown receptor fates [105]. However, two groups reported that some receptors, which can be labelled with organic fluorophores, are inaccessible to QDs, probably the result of the large size of QD complexes in comparison [101,103]. This was despite the use of a novel targeting method in one, which reduced the size of the overall receptor-QD complex by replacing the anti-AMPA antibody with a small acceptor peptide [103]. The large size of QD conjugates when attached to target molecules may also interfere with the normal functioning of that protein, although no evidence of this was found when imaging TrkA receptors [105]. The optical superiority of QDs is likely to ensure their place in this area of research, but due acknowledgement must be given to the possible inaccuracies that may be inherent in their use.

5.4. Cell tracking

In a landmark study, QDs encapsulated in phospholipid micelles were used to label individual blastomeres in xenopus embryos [24]. These encapsulated QDs were stable *in vivo*, did not become aggregated and were able to label
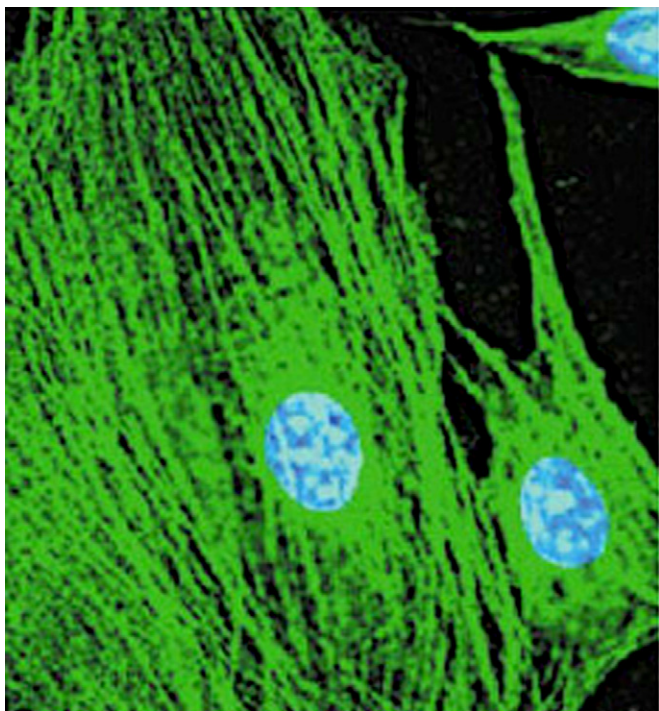

Fig. 5. Actin filaments stained with biotinylated phalloidin and QD 535–streptavidin, and nuclei counterstained with Hoechst 33342 blue dye in mouse 3T3 fibroblasts [5] (permission obtained).

all cell types in the embryo. At the levels required for fluorescence visualisation (2×10^9/cell) the QD-micelles were not toxic to the cells, but concentrations of 5×10^9/cell did produce abnormalities. The QDs were confined to the injected cell and its progeny, though unintended translocation to the nucleus was observed at a particular stage in the development of the embryo. Another group labelling *Dictyostelium discoideum* found that cell labelling for over a week was possible, and that QD labelling had no detectable effects on cell morphology or physiology [92]. Differently coloured QDs could also be used to label different populations in order to investigate the effect of starvation on *D. discoideum* development. These cells could be tracked for long periods with no discernible fluorescence loss. Zebrafish embryo blastomeres labelled with QDs and co-injected with CFP, a traditionally used lineage marker, showed passing of QDs to daughter cells in most cases, although some cells displaying CFP fluorescence did not show QD fluorescence. This was suggested to be due to aggregation of QDs, leading to unequal inheritance by daughter cells [90]. This is a recognised problem, along with fluorescence loss and instability in the QD structure in biological solutions [24].

5.5. Pathogen and toxin detection

QDs may find practical application for the detection of pathogens and toxins, and in defining their characteristics, including virulence. A number of studies have produced good results, and the opportunity for multiplexed imaging is particularly useful in this area. Several different pathogens have been targeted so far, including *Cryptosporidium parvum* and *Giardia lamblia* [106,107], *Escherichia coli* 0157:H7 and *Salmonella Typhi* [108] and *Listeria monocytogenes*. Simultaneous multiplexed labelling of both *C. parvum* and *G. lamblia* using immunofluorescent staining methods with QD fluorophores (Fig. 6) produced a good signal-to-noise ratio of 17, with better photostability and brightness compared with two commonly used commercial staining kits [107]. However, one study found that the QD-based assay was not as sensitive as ELISA-based techniques [109].

QDs conjugated to wheat germ agglutinin and transferrin have been used to label both bacterial and fungal populations. [110] Transferrin-bound QDs could provide a test for pathogenic virulence, as the presence of human transferrin is strongly correlated with virulence [111]. In this study, only pathogenic strains of staphylococci were labelled with transferrin-conjugated QDs, suggesting this could be used as a rapid test for invasive staphylococci. QDs have also been used for viral detection [112,113]. Using immunofluorescent techniques to detect respiratory syncytial virus (RSV) F-protein, it was possible to quantitatively analyse differences in F-protein expression between strains [113]. Application of *in situ* hybridisation techniques using QDs to the detection of Hepatitis B and C viruses has also been demonstrated. Using printed microarrays of
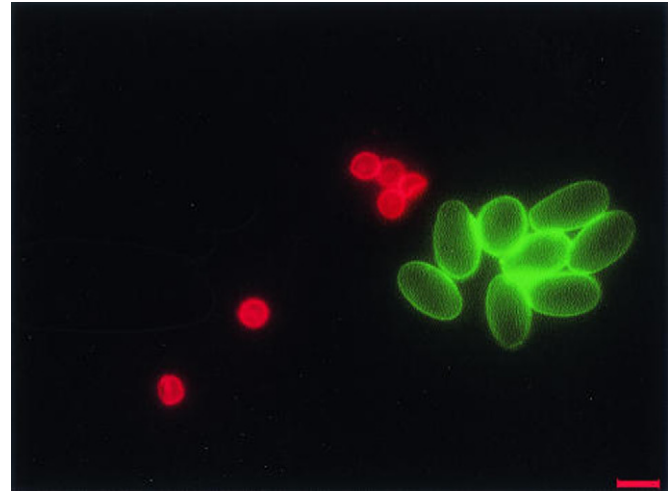

Fig. 6. Dual-color image of QD 605-labeled *C. parvum* (red) and QD 565-labeled *G. lamblia* (green) [107] (permission obtained).

sequences complementary to Hepatitis B and C virus genomes and also to p53 conjugated with QDs, multiplexed detection of HBV and HCV with a signal-to-nose ratio up to 150 was possible and required a short incubation time [112].

A number of studies have used QDs for detection of toxins [114–116]. QD immunofluorescence was used to label staphylococcal enterotoxin B (SEB), cholera toxin (CT), Shiga-like toxin 1 (SLT-1) and ricin. This proof-of-principle study showed specific detection of toxins could be achieved at concentrations as low as 3 ng/ml for SEB. Multiplexed detection using a mixture of the toxins showed that all four toxins could be detected. However, problems with cross-reactivity and possible non-specific binding were seen. Whether this was due to problems with the antibody, or the result of the incorporation of QDs requires further investigation.

5.6. In vivo animal imaging

At present there is relatively little work published on the use of QDs for whole body imaging. Whole-animal imaging presents a number of difficulties, the most important of which is the potential for toxicity in both animal and human applications. Much more work will need to be done before the usefulness or otherwise of QDs in this area can be established. Imaging in animal subjects introduces complications due to absorbance and scatter by tissues, and autofluorescence upon their excitation. Tissue absorbance and scatter is much lower in the near-infrared region (700–1000 nm) [117], so engineering of QDs to fluoresce in the NIR region can be used to increase the signal received. Tissue autofluorescence is also dependent on the wavelength of the excitation light [118]. As QDs have broad absorption spectra, a wavelength which minimises tissue autofluorescence can be chosen. Some studies have also investigated self-illuminating QDs [119]. These work by bioluminescence resonance energy transfer,

which acts in a manner similar to FRET except that in this system a bioluminescent molecule, such as a luciferase, acts as an energy donor upon activation by an enzyme (coelenterazine in the case of luciferase), which excites fluorescence in the QD. This has the advantage of eliminating the need for excitation light, and reduces tissue autofluorescence and background noise, but suffers from the disadvantages that it requires the introduction of two potentially immunogenic substances, relies on appropriate biodistribution of the enzyme, and given the large size of the complex, extravasation and penetration into many tissues, organs or tumours may be problematic [120]. Comparison between organic fluorophores and non-bioluminescent QDs has not yet been made, but these novel probes were visible in nude mouse models. Another important consideration for *in vivo* applications is clearance from the bloodstream. QDs, along with other nanoparticles, suffer from extensive reticuloendothelial uptake, which reduces the blood concentration [121]. Coating with PEG, which can prevent the opsonin–nanoparticle interaction, has been reported to increase the circulating lifetime of QDs, but does not eliminate non-specific uptake [32,122–124].

Several groups report homing of QDs to biological targets *in vivo*. Targets have included tumours [32,33,125,126], vasculature in several different tissue targets [117], and also a number of targets in necropsy and tissue sections after *in vivo* injection of QDs [127]. One group used antibody-conjugated, PEG-encapsulated QDs to target a prostate-specific membrane antigen [33], a cell surface marker for prostate epithelium, which is also expressed in the neovasculature of a large number of non-prostatic primary carcinomas [128]. The particles bound specifically to human prostate tumour xenografts, and produced fluorescent signals significantly brighter than those produced by green fluorescent protein (GFP), which has previously been used for *in vivo* cancer imaging [129,130]. Another group report the synthesis of a tumour-specific chimera phage incorporating a streptavidin-binding site to which QDs may be attached, which is able to specifically target tumours [126]. Labelling of tumour vasculature has been shown to be possible, including multiplexed imaging of both vasculature and lymphatics in an MDA-MB-435 xenograft tumour system, which was evidenced by colocalisation with blood vessel and lymphatic markers. However, tissue penetration was reduced compared with organic fluorophores both *in vitro* and *in vivo*, which is likely to be the result of the relatively large size of the QD complex, and loss of luminescence was seen resulting from instability when transferred to living cells and tissues [32].

Imaging of vasculature in normal tissues has also been attempted. Using Type II QDs (with a CdTe core and also a CdSe shell) intraoperatively, coronary vasculature could be visualised with a signal-to-noise ratio of 5:1 under excitation with light similar to that which might be used to illuminate a surgical field [117]. Imaging of rat coronary vasculature was possible at a depth of 1.5–2.0 mm both before and after thoracotomy with sufficient resolution to allow identification of named blood vessels (Fig. 7) [125]. Imaging through intact skin and adipose tissue in mice allowed visualisation of vasculature at the base of the dermis 900 μm deep, and it was also possible to image capillaries through 250 μm of adipose tissue, producing images with greater detail at a greater depth using less power than needed for FITC-dextran, a traditional organic fluorophore (Fig. 7a) [131].

QDs could provide an alternative to traditional dyes in sentinel lymph node (SLN) mapping. SLN mapping allows the identification of the first node in the lymphatic basin into which a primary tumour drains, the status of which reflects the status of the entire basin [132]. Current practice involves extensive lymphadenectomy in many cancers, leading to significant morbidity. By removing only the SLN, this morbidity can be reduced, and a pathologist can examine the excised node in greater detail for micrometastases, particularly using specialised techniques such as PCR, which can detect one tumour cell in a background of one million lymphocytes, compared to one in ten thousand for standard haemotoxylin and eosin staining [132]. Current techniques for isolating the SLN using isosulfan blue [133] and radiolabelled dyes [134] suffer from several significant drawbacks [135–137]. QDs provide a possibly favourable alternative, as they can be engineered to fluoresce in the NIR region, and can be synthesised with an optimal size for lymphatic partitioning. Type II QDs coated in oligomeric phosphines are used to ensure localisation in the lymphatic system.

The first studies showed that injected QDs colocalised with isosulfan blue, a commonly used lymphatic dye in axillary nodes, after injection into mice [138]. Several studies on pigs have used QDs to identify the SLN in the pleural space [134,137], the oesophagus [139], the GI tract, and in melanoma drainage sites [140]. They showed that injected QDs rapidly localised to the SLN and could be imaged at a depth of up to 5 cm in lung tissue [137]. Allowing the QDs to remain *in situ* for 3 h did not show any migration beyond the SLN, or any reduction of fluorescence. Surgeons could be provided with image guidance with false colour QD images overlying the normal surface anatomy on a combined image (Fig. 8). The use of QDs allows identification of the SLN after resection, even if it is bloody and matted, and an idea of the completeness of resection is given. However, several studies on biocompatible near-IR-emitting QDs have reported a slight blue-shift [141], a low photoluminescent quantum yield (lower than 4%), and a broad emission greater than that of the visible-light emitting QDs [104,119]. The other drawback of this is the as yet unknown toxicity of QDs. The authors suggest that as much of the QD load is partitioned in the excised lymphatics, toxicity may be negligible, but this certainly cannot be assumed. These studies all noted the absence of acute effects in the pigs, but long-term toxicity was not addressed.
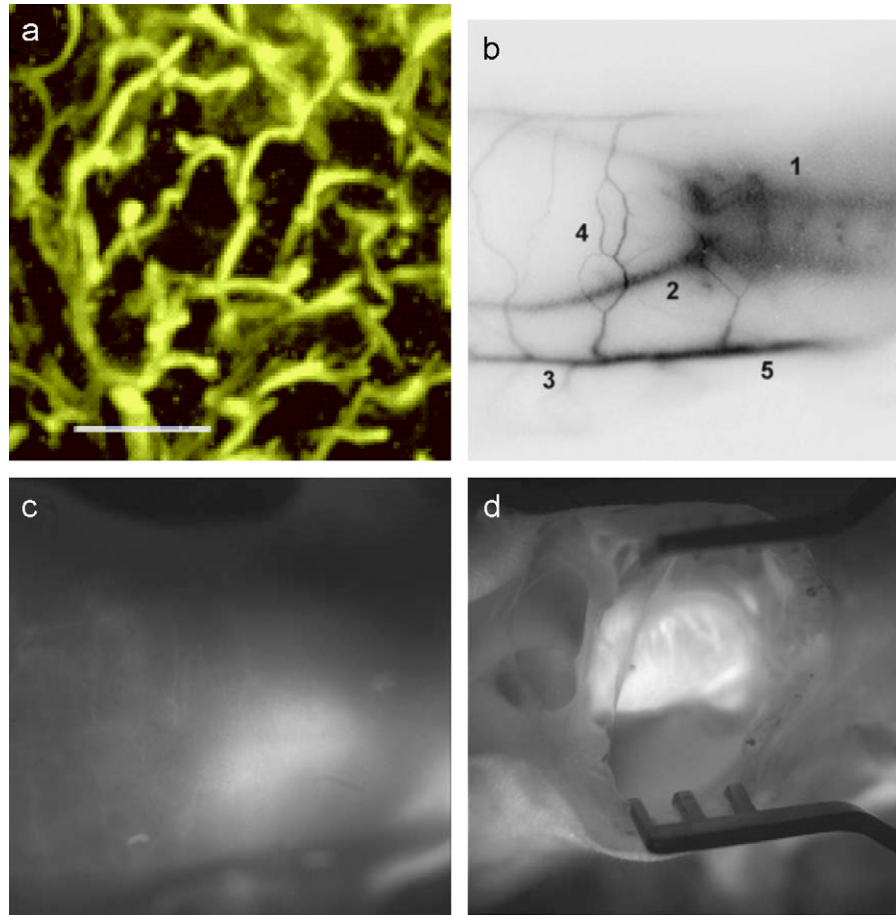
Fig. 7. (a) Projection of capillary structure through 250 μm of adipose tissue after intravenous nanocrystal injection into a mouse [131]. (b) Arterial and venous circulation visualised 40.5 s after administration of a nanocrystal bolus in a hyperinflated rat. Thoracic and abdominal regions of a mouse imaged after injection of a nanocrystal bolus, before (c) and after (d) thoracotomy [125] (permission obtained).
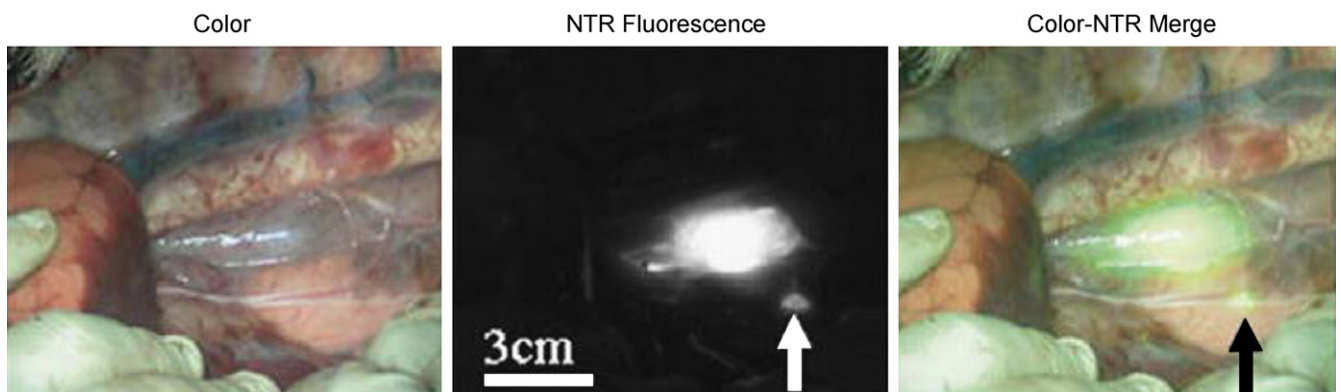

Fig. 8. Esophageal sentinel lymph node mapping in pigs. Showing original colour, QD fluorescence and false-colour QD fluorescence merged with original image [139] (permission obtained).

5.7. Barriers to use in vivo

The value of QDs for *in vivo* applications is controversial. Although these studies have produced some successful results, predictable problems were noted. The size of QD complexes limits tissue penetration [32], and instability in biological tissues has been noted [24]. The only data currently available comes from observation of experimental animals over the short term. Significant problems can be anticipated. Firstly, QD complexes, including their capping materials may be immunogenic, which could result in both dangerous immune reactions in subjects, and could also render the QDs ineffective as a result of antibody binding. Secondly, the heavy metals contained in the core, and the

materials used for capping (e.g. MPA) may be toxic to the host. Thirdly, the size of QD complexes precludes renal excretion, making clearance from the bloodstream unlikely. This will result in eventual uptake and concentration in the liver, which is particularly sensitive to cadmium toxicity. A large number of high-quality and high powered trials specifically addressing these issues will need to be undertaken before QDs can be considered for human use, and such a process is likely to be lengthy.

5.8. Tumour biology investigation

Tumour vasculature plays an important role in determining tumour pathophysiology, and drug delivery. Combination of QD imaging with second-harmonic generation (SHG) [142], which has been used for collagen imaging in normal and cancer tissue [143,144] has allowed imaging of the distribution of blood vessels within the interstitium, of which collagen is a major component [145]. Using QD microbeads of different sizes, with a different wavelength QD embedded in each size of bead, an assessment of tissue penetrability can be made, with infusion of these microbeads into the tumour vasculature showing differing distribution of the differently sized microbeads between intravascular and extravascular compartments. The authors suggest that this could be used to provide an in vivo assay for assessing drug delivery in tumours. QDs have also been used to study tumour cell extravasation and seeding [146], with five distinct populations of cells being labelled and tracked using differently coloured QDs. The role of bone-marrow derived precursor cells in tumour vasculogenesis has also been investigated using QDs, producing images which showed the blood flow, rolling and adhesion of these cells [145].

An application in which QDs might find a more immediate application is in the assaying of cell motility, which is widely accepted to correlate strongly with metastatic potential [147]. One method for measuring this involves measuring phagokinetic tracks left when cells pass over a layer of markers and ingest them. Gold particles have been used previously, but provide practical difficulties in making up the substrate, and are so large that ingestion of a relatively small amount of markers may perturb cell motility. QDs have been investigated as an alternative, and with substrate incorporating QDs, phagokinetic tracks created by human mammary epithelial cells and non-tumour cells have been observed [148].

A recent publication reports a protocol for quantitative measurement of expression of cancer antigens in various tumour tissues [149]. They were able to provide protein expression measurement on a continuous scale, which they suggest is an improvement over the current most commonly used Pathology Scoring method. Interestingly, they found that multiplexed measurement of different antigens was unreliable as a result of what seemed to be a FRET process occurring between different wavelength QDs. QDs

may have potential for treatment as well as investigation of cancers. Whilst the cytotoxicity of QDs has been a major barrier to their use in vivo it may prove to be key in their role against cancer cells. The CdTe component of the QD structure has been shown to produce reactive oxygen species which activates Fas R, a tumour necrosis factor, inducing apoptosis and cell death [150]. Although this has not been investigated, this could be used to provide therapeutic options in cancer treatment.

6. Discussion and conclusions

A number of useful results have been generated using QDs, particularly in the field of single-molecule tracking, where their long fluorescence lifetime and photostability are particularly advantageous. Multiplexed imaging for which QDs could provide ideal probes, is also attractive for a host of applications, and presents an opportunity for significant progress in many fields. There has been speculation over possible uses of QDs in a large number of applications, but care must be taken not to be overly optimistic, as a number of important problems have not yet been solved, and QD behaviour has yet to be fully characterised.

A number of significant barriers prevent widespread uptake of the technology at present. There is evidence of cytotoxicity and alteration of cell function, and of the function of molecules labelled by QDs. The large size of QDs relative to current fluorophores reduces their ability to access and label cellular molecules, and may reduce tissue penetration on a larger scale. Uncertainty over the toxicity and fate of QDs in vivo, particularly regarding distribution and breakdown precludes their use in human applications until much more data is available. In addition to this, many fundamental characteristics of their surface chemistry and physicochemical properties in varying situations are poorly understood. Many assays incorporating QDs, particularly those based on immunofluorescence have been reported to be less sensitive than other assays. Whether this is due to inherent weaknesses in the assay design or antibodies used, or the result of the incorporation of QDs, requires further investigation.

The superior optical properties of QDs compared with currently used imaging molecules are indisputable, and the studies presented here have shown that QDs do have potential for usefulness in a number of areas. However, only when the significant concerns apparent have been fully addressed will it be possible to make a considered judgement on the applications into which they can usefully be incorporated.

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