Quantum dots: bright and versatile \textit{in vitro} and \textit{in vivo} fluorescence imaging biosensors

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Semiconductor quantum dots (QDs) have become important fluorescent probes for \textit{in vitro} and \textit{in vivo} bioimaging research. Their nanoparticle surfaces for versatile bioconjugation, their adaptable photophysical properties for multiplexed detection, and their superior stability for longer investigation times are the main advantages of QDs compared to other fluorescence imaging agents. Here, we review the recent literature dealing with the design and application of QD-bioconjugates for advanced \textit{in vitro} and \textit{in vivo} imaging. After a short summary of QD preparation and their most important properties, different QD-based imaging applications will be discussed from the technological and the biological point of view, ranging from super-resolution microscopy and single-particle tracking over \textit{in vitro} cell and tissue imaging to \textit{in vivo} investigations. A substantial part of the review will focus on multifunctional applications, in which the QD fluorescence is combined with drug or gene delivery towards theranostic approaches or with complementary technologies for multimodal imaging. We also briefly discuss QD toxicity issues and give a short outlook on future directions of QD-based bioimaging.

1. Introduction

Semiconductor nanocrystals (quantum dots, QDs) have arguably affected bioimaging research more than any other nanomaterial. The versatility of QD-based application was not imaginable when the relationship between size and band-gap of semiconductor materials was described in the early eighties.\textsuperscript{1} It took until 1998 when two Science articles pointed out the advantages of this new material for biosensing and established QDs as a new class of fluorophores in the toolkit of biological researchers.\textsuperscript{2,3} In the following years scientists used QDs for inorganic ion sensing, organic small molecule and biological macromolecule sensing, bioconjugation and cell staining, cellular effectors and reporters, animal imaging, and therapy.\textsuperscript{4–11} In particular, their

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unique photophysical properties were exploited for in vitro\textsuperscript{12–20} and in vivo\textsuperscript{21–24} imaging applications. The published conjugation strategies and commercial availability of QDs enlarged their application field and led to an exponential increase of QD related research articles, which were reviewed over the last few years in numerous articles.\textsuperscript{2,25–33}

In this review we summarize the developments of QD-based imaging methods in relevant biological materials with a focus on the recent advances for in vitro and in vivo biosensing. Although QDs can be prepared with atoms from groups II–VI, III–V, or IV–VI of the periodic table and in many different alloyed versions, we mainly discuss the most popular Cd-based QDs as a model for all other QDs. Information about the usage of other types of QDs (InP, GaAs, etc.), gold nanoparticles, iron particles, carbon dots, and further nanometric imaging agents can be found throughout this Chemical Society Reviews Themed Collection “Imaging Agents” and in the following ref. 34–38.

We begin with a short overview about the preparation of QDs, their optical properties, and advantages as imaging agents over conventional fluorophores and then discuss important contributions of QDs in super-resolution microscopy and single-particle tracking. Although QDs have been used for imaging applications in bacteria and yeast\textsuperscript{39–44} and for monitoring biological processes in leaf cells of plants,\textsuperscript{45} we focus our review on mammalian material and survey recent studies of in vitro, tissue, and in vivo imaging. After a brief journey into the controversial topic of QD toxicity we conclude our review and give a short outlook on the bright future of QD-based imaging.

2. Preparation and properties of QDs

A bottleneck for the use of the first inorganic QDs in biological applications was the reproducible synthesis of highly luminescent, water-soluble, and monodisperse QDs. The synthesis approach of Murray et al. in 1993 was a milestone for the preparation of uniform colloidal QDs.\textsuperscript{46} Their method was based on a high-temperature organometallic process, which resulted in colloidal QDs with a low polydispersity but unfortunately also a low photoluminescence (PL) quantum yield (QY). To improve the PL properties passivation of the QD core by a shell of a few atom layers of ZnS or CdS was found to be advantageous.\textsuperscript{37–49}

An important property for the shell was that the material had a larger energy band-gap than the core, which led to a confinement of the excitons in the core and reduced the surface-related recombination in trap states. In addition to the enhanced PL properties, the core–shell QDs exhibited a better photochemical stability and a reduced Cd leaching from the core. Another important milestone, in particular for the later commercialization of QDs, was the improved synthesis protocol by Peng et al. The use of CdO as precursor instead of the sensitive and toxic Cd(CH\textsubscript{3})\textsubscript{2} was safer and allowed larger scale production.\textsuperscript{50} The use of toxic materials and the need for the transformation of QDs from the organic phase to the water phase, which comes at the cost of reduced QY, also led to the investigation of QD preparation in aqueous solution. Key features of the aqueous synthesis are direct water-solubility, facile preparation, good reproducibility, low costs, and improved biocompatibility.\textsuperscript{4,51} One of the first who demonstrated the aqueous synthesis of thiol-stabilized CdTe QDs were Rogach et al.\textsuperscript{52} and various alternative approaches have been developed to produce QDs in the aqueous phase with good QY and small size.\textsuperscript{53,54} In a recent study Au et al. compared the conjugation efficacy of aqueous and organic synthesized QDs and their stability in biological media. The results showed both 10-fold increased conjugation efficacy and better stability for the aqueous synthesized QDs.\textsuperscript{55}

For all synthesis approaches the experimental parameters, such as temperature, growth time, etc., are used to control the shape and size of QDs. For in vitro and in vivo imaging applications the size plays a major role and smaller QDs are most often beneficial for such purposes. Small hydrodynamic radii of QDs are an important issue for avoiding perturbation of QD-functionalized biological molecules or to access biological interactions (e.g. by Förster resonance energy transfer (FRET) or other energy transfer processes) at distances of only a few nanometers. Diameters of water-soluble QDs are usually in the range of 5 to 10 nm but aqueous QDs (aqQDs) with average diameters down to 1.6 nm have also been produced.\textsuperscript{56} Apart from classical approaches starting with nucleation and growth of QDs in solution, it was shown that QDs can be grown on, for example, peptide templates.\textsuperscript{57} QDs were also “naturally” produced by exposing standard wild-type Lumbricus rubellus earthworms to soil spiked with CdCl\textsubscript{2} and Na\textsubscript{2}TeO\textsubscript{3} salts for 11 days. An intrinsic heavy-metal detoxification strategy was responsible for the intra-worm production of luminescent QDs, which could be successfully used in live-cell imaging after the isolation from the chloragogenous tissues.\textsuperscript{58} A similar effect could be obtained with rats after treatment with CdCl\textsubscript{2}. The detoxification of the heavy-metal salt produced QDs, whose luminescence could be divided in three colours (red, green, and yellow).\textsuperscript{59} The production of solid-state material inside living organisms is an emerging field in nanobiotechnology.\textsuperscript{58}

On the interface between QD and the environment the surface molecules play an important role concerning water-solubility, biocompatibility, and bioconjugation. Different strategies of surface modification were used to render QDs biocompatible. These include surface cap exchange\textsuperscript{2,60–62} amphiphilic surface ligands,\textsuperscript{63,64} and encapsulation in micelles\textsuperscript{23} or silica shells.\textsuperscript{65–69} Regarding fluorescence imaging specific binding of the final biosensor to the target is highly important and the large and charged surfaces of QDs were a leading cause of non-specific binding. An established strategy to avoid this effect is the use of poly(ethylene glycol) (PEG) as surface coating, which was shown to efficiently reduce non-specific binding by using at least 12 to 14 units of PEG.\textsuperscript{70} Hydroxyl coated QDs showed a 140-fold reduction in non-specific binding compared to carboxylate QDs and 10- to 20-fold reduction relative to that of PEG- and protein-coated QDs.\textsuperscript{71} To obtain water-soluble QDs with reduced non-specific binding comes at the cost of their hydrodynamic radius and surface charge. Because traditional transmission electron microscopy (TEM) can only visualize the inorganic core, methods like dynamic light scattering, liquid chromatography, and laser...
Doppler velocimetry are important tools to investigate the intrinsic properties of QDs in the water phase. For three-dimensional size and shape analysis of QD-bioconjugates under physiological conditions and at low concentrations FRET has been successfully employed.\textsuperscript{72,73} Too large QDs can be problematic in biological application because of reduced diffusion in tissues and tumours.\textsuperscript{74} In order to provide water-soluble QDs with small hydrodynamic size, low non-specific binding and high QY a cap exchange with PEGylated-dihydrolipoic acid (DHLA) ligands showed to be advantageous. Additionally, DHLA-QDs also provided good solution stability over a large pH range. By manipulating the terminal functional groups of the DHLA-PEG ligands with amino, carboxyl, or hydroxyl groups, the surface charge could be altered and QDs efficiently bioconjugated.\textsuperscript{75–77} Designing QDs with small and compact ligands that provide biocompatibility, stability, and significantly reduced overall hydrodynamic radii of the final QD-bioconjugates is highly important for successful biosensing and cellular imaging.\textsuperscript{73,78} Another variant of surface coating is the use of soft-binding aminopropanol (APP) as cap exchange ligand for a rapid transfer of hydrophobic QDs into polar solvents. Due to the protonation of APP in aqueous solution, the ligand can be easily removed and the intermediate QD can be incorporated inside micelles under mild conditions.\textsuperscript{79}

The large surface of QDs offers the possibility for the binding of multiple biomolecules of interest (BOI), such as antibodies or enzymes, that can enhance the sensitivity and activity in biological applications.\textsuperscript{80,81} Among the most prominent methods for QD bioconjugation are the aniline-catalyzed hydrazine bond formation for binding to amino groups and the use of maleimide groups for the conjugation of thiol groups present, for example, in the hinge regions of antibodies.\textsuperscript{82} For specific covalent binding Schieber \textit{et al.} presented a method in which they used strain-promoted azide–alkyne cycloaddition reaction of azides with strained cyclooctynes. Therefore they conjugated azide-modified QDs with cyclooctyne-modified biomolecules for fluorescence imaging of tumour cells.\textsuperscript{83} For the preparation of a monovalent functionalized QD without chemical modification, Farlow \textit{et al.} used a functionalized oligonucleotide, which wrapped around the QD monovalently due to steric exclusion.\textsuperscript{84} These are only a few impressions from the vast choice of QD preparation and bioconjugation. An overview of the different surface coating strategies and functionalization pathways are summarized in Fig. 1 and plenty of detailed additional information can be found in the following reviews.\textsuperscript{27,29,33,85,86} Although QD bioconjugation is very versatile, one should keep in mind that QDs are relatively large objects compared to small organic dyes and to the biomolecule they are conjugated to. This can lead to alterations in the biological function of the QD-bioconjugate. Moreover, the large and often charged surfaces of QDs provide a large space for non-specific interactions, which can interfere with the specific recognition of the QD-bioconjugates.

\textbf{Fig. 1} Overview of different bioconjugation (left side, BOI = biomolecule of interest) and surface coating (right side) strategies for QDs. Two surface coating strategies are presented: encapsulation with amphiphilic polymers (i, ii) and cap exchange with hydrophilic ligands exploiting the thiol-affinity of the ZnS shell of the QD (iii–v). Reprinted with permission from ref. 30. Copyright 2013 Society for Applied Spectroscopy.
2.1 Photophysical properties and advantages of QD imaging agents

The unique photophysical properties of QDs are the main cause of their popularity and versatile usage in biosensing. QDs are semiconductor nanoparticles that have physical dimensions close to or smaller than the exciton Bohr radius.\(^{87}\) The spatial confinement of intrinsic electron and hole carriers leads to an increased band-gap energy and to a splitting of the continuous energy bands in discrete energy levels. This effect makes QDs to an intermediate between bulk materials and molecules. The absorbance of photons with energies higher than the band-gap leads to the creation of an electron–hole-pair, an exciton. With increasing excitation energy (shorter wavelength) there is also an increased absorption probability, which leads to a very broad absorption spectrum and large effective Stokes shifts (difference between excitation and emission wavelength) of more than 100 nm if necessary.\(^{88–92}\) Trap states caused by a large number of defects (difference between excitation and emission wavelength) of more than 100 nm if necessary.\(^{88–92}\) Trap states caused by disturbed crystal structure (mainly on the QD surface) can lead to non-radiative deexcitation and is also one of the reasons for intermittent fluorescence (blinking) of QDs, which is visible on the single QD level.\(^{89}\) Another consequence of the quantum confinement effect is the size dependent emission, caused by an increased confinement of the excitons with smaller QD size and thus higher energy band-gap (Fig. 2B). This means that larger QDs of the same material exhibit a smaller energy band-gap and thus a PL emission in the red, whereas smaller QDs fluoresce in the blue (Fig. 2C). This effect allows to tune the PL colour of QDs by controlling their size and enables, in combination with different materials, to engineer QDs that cover the spectral range from the ultraviolet (UV) to the infrared (IR).\(^{93}\)

The PL spectra of QDs have a nearly Gaussian shape with a narrow full-width-at-half-maximum (FWHM) of ca. 20 to 30 nm and show a negligible tendency for photobleaching.\(^{63,93}\) QD PL is typically circularly polarized or non-polarized for spherical QDs, whereas quantum rods show a polarized emission.\(^{94}\) The PL decay is most often multexponential with average lifetimes ranging from ca. 10 to 100 ns. Important for imaging applications is the high brightness (product of QY and absorptivity), caused by high QYs and large molar extinction coefficients.\(^{95}\) Additionally, QDs provide large two-photon cross sections \(\sigma_{\text{2PL}}\) (ca. \(10^{-3}–10^{-6}\) Goeppert-Mayer units (GM)), which is advantageous for multiphoton excitation.\(^{96–98}\) Recently, CdSe/CdS-quantum-dots-quantum-rods with two-photon cross sections of ca. \(10^{5}\) GM and QYs of 78% have been produced.\(^{99}\)

One reason for the large interest in QDs for bioimaging is that fluorescent dyes and fluorescent proteins (FPs) have some shortcomings, such as narrow excitation spectra, small effective Stokes shifts, broad fluorescence bands, and susceptibility to photobleaching.\(^{33}\) Additionally, their PL lifetimes are in the same range (few nanoseconds) than biological autofluorescence, which does not allow time-gated detection for increasing the signal-to-noise ratio (SNR) and for better differentiation between different target molecules, as it was shown for QDs.\(^{13,100}\) The emission spectra of dyes show a pronounced shoulder in the red, which limits spectral multiplexing due to optical crosstalk.

Moreover, the narrow absorption spectra make it hardly possible to efficiently excite several dyes at once. In contrast, multiple-QD excitation with a single excitation source (wavelength) and simultaneous multiple-QD PL detection offer the possibility of a multiplexing capability with almost negligible spectral cross-talk.\(^{18,63,101,102}\) In a direct comparison concerning photostability it could be shown that QDs are 100 times more stable and 20 times more bright than Rhodamine 6G.\(^3\) Further comparison with other dyes showed the superiority of QDs concerning their photostability\(^{11,12,16,63,103–105}\) also against one of the most stable organic dyes AlexaFluor 488 (Fig. 3).\(^{106}\) Another advantage of QDs over traditional fluorophores is their almost two orders of magnitude larger two-photon cross section\(^{98,107}\) which is particularly interesting for in vivo applications with near infrared
for the development of super-resolved fluorescence microscopy,\textsuperscript{112} we will take a closer look at the impact of QDs on this particular field of imaging. Monitoring of biological processes at the subcellular level is important for the understanding of the relationship between cell compartment-triggered reactions. Super-resolution microscopy enables the localization of different proteins on the plasma membrane and in the cytoplasm and allows the investigation of how signals are transmitted within the cell or how molecules will be taken up and transported.\textsuperscript{28} The complexity of biochemical reactions in live cells cannot be fully resolved using standard wide-field ensemble measurements because many of these reactions are triggered by only a small number of molecules. For those reasons monitoring of nanometric subcellular structures or single-molecules is advantageous. The main challenges of measuring on the single-molecule level are the ability to detect a single-molecule in a dense medium and to distinguish two fluorophores that are separated by a distance below the diffraction limit of light (\( \sim 0.5 \times \) the detection wavelength). For super-resolution images of fluorophore ensembles the point-spread-function (PSF) of a single emitter can be spatially reduced (by using, e.g., stimulated emission depletion microscopy – STED) or the centre of each separated PSF can be localized with high precision by collecting a large amount of photons per single emitter (by using, e.g., photoactivated localization microscopy – PALM, stochastic optical reconstruction microscopy – STORM, or points accumulation for imaging in nanoscale topography – PAINT). Using super-resolution techniques such as STED or saturated structural illumination microscopy (SSIM) requires high excitation powers and can cause phototoxicity in biological systems. STORM and PALM require long imaging times up to several minutes and much effort has been put into the development of faster super-resolution techniques to allow imaging of live biological systems.

From the biological point of view three general key elements need to be considered for super-resolved microscopy. First, the probe must be transportable to the molecule or organelle of interest. This can be relatively easy when the target is a receptor expressed on the surface of the cell, but it can be more complicated when the probe has to be delivered inside the cytoplasm (cf. Section 4.1 for more details about cell delivery). Second, the fluorophore bioconjugate should be monovalent. Multivalency of the probe can be problematic because binding to more than one target can perturb the measurement. In one example of creating monovalent QDs Clarke \textit{et al.} used peptide coating and applied the peptide–QD conjugates in single-molecule measurements.\textsuperscript{111} Third, the affinity between probe and target should be very high to allow a binding time that is longer than the required observation time. This is one of the reasons why the biotin–streptavidin recognition has been so popular inside super-resolution techniques.\textsuperscript{114} Streptavidin (sAv) is (in its native form) a tetramer and can bind four biotins. The reduction of the tetramer to a monomeric protein also leads to the reduction of the binding affinity. To overcome this problem Howarth \textit{et al.} demonstrated the preparation of streptavidin with a controlled number of functional biotin binding subunits without decreased binding affinity.\textsuperscript{115–117} The three biological requirements mentioned

3. Super-resolution microscopy and single-particle tracking

3.1 Super-resolution microscopy

In view of the 2014 Nobel Prize in Chemistry, which was awarded to Eric Betzig, Stefan W. Hell, and William E. Moerner...
above (biological functionality, monovalency, and high affinity) argue for the use of small fluorescent dyes, which are in fact the most often applied fluorophores for super-resolution microscopy.

From the photophysical point of view fluorophores should be bright and photostable to allow highly sensitive single-molecule detection, which places QDs in an advantageous position compared to organic dyes or FPs. Because their fluorescence intermittency, also known as blinking, limits the application possibilities, QDs are mainly used in super-resolution techniques that rely on spatial and temporal localization of single-molecules, such as PALM, F-PALM, STORM, dSTORM, and GSD. In different studies using fluorescence fluctuation analysis it could be shown that the estimation of diffusional mobility can be highly influenced by blinking and that special analytical models are necessary to differentiate the contribution of photophysical fluctuations from those caused by transport.

Improvements in synthesis and surface passivation of QDs could alter and even suppress blinking, whereas variation of the excitation wavelength had only minor influence on the blinking behaviour. Initial investigations assumed that blinking of QDs had a characteristic timescale. In contrast to these results, Bachir et al. concluded that QD blinking fluctuations obey a power law distribution and that there is no single characteristic fluctuation time for this phenomenon.

In contrast to simultaneous ensemble measurements super-resolution imaging can take advantage of QD fluorescence blinking. Because complete on–off intermittency can be observed only for single QDs (and not for an ensemble of QDs because of a very low probability that the different QDs all blink simultaneously) it can be used to resolve closely spaced QDs. Dertinger et al. used a method, which relies on higher-order statistical analysis of temporal fluctuations (caused by blinking) recorded in a sequence of images. This method enabled a 5-fold improvement in spatial resolution and was demonstrated on QD-labelled microtubules of fibroblast cells. They coined their method super-resolution optical fluctuation imaging (SOFI) and achieved 55 nm spatial resolution (FWHM) in the x–y plane. With the synthesis of QDs with increased blinking rates also the temporal resolution of SOFI could be improved down to 80 ms. Chien and coworkers exploited the blinking phenomenon to map out the locations of individual QDs using total internal reflection microscopy (TIRF). The reconstructed image was in good correlation with the topographic image measured by atomic force microscopy (AFM).

Hoyer et al. showed the possibility of using a simple webcam, QDs, and ground-state depletion microscopy to obtain fluorescence images with single-QD resolution. Wang et al. exploited QD blinking to achieve three-dimensional (3D) super-resolution imaging with 8 to 17 nm in the x–y plane and 58 to 81 nm in the z-direction. After successful simulation they applied their approach to resolve the 3D distribution of EGFR molecules on breast cancer cells. However, the technique also had limitations, such as limited resolution in the case of a too dense QD distribution. Moreover, the method was based on frame-subtraction, which requires extremely high spatial stability (no motion) from frame to frame.

Most of the super-resolution techniques presented above used single-colour QD blinking. Shi et al. presented a spectral imaging nanoscopy approach for multicolour QD superlocalization. They used a transmission grating to disperse the emission light into zero and first order images. QDs in close proximity exhibited one overlapping zero-order spot but multiple (depending on the number of QDs) distinct first-order streaks (Fig. 4). The single QDs could be identified due to the disappearing of the first-order streak during the blinking off-state. The authors demonstrated their approach using up to three QDs connected with complementary oligonucleotides with different lengths and in human embryo kidney 239A cells.

As demonstrated in the aforementioned study a high spatial density of QDs requires special techniques to distinguish the blinking of single QDs. Another possibility of imaging dense spatial QD distributions at super-resolution was proposed by Deng et al. using a reconstruction algorithm named spatial covariance reconstructive (SCORE) algorithm. The key point of this technique was statistic monitoring of the covariance between the intensities of pixels, which led to a preference of high labelling densities. SCORE does not take into account temporally non-drifting background and thus enables the analysis of fluctuating emitters in a background of constantly fluorescing molecules. The authors demonstrated the advantages of SCORE applied to images of QD-labelled microtubules in HeLa cells and could obtain a resolution below 90 nm within a few seconds of image acquisition (Fig. 5).

Although blinking is one of the most often applied techniques for QD-based super-resolution, also other approaches, such as photon antibunching to narrow down the PSF or tri-exciton generation combined with spectral deconvolution and imaging.
can create super-resolved images. Another alternative approach is near-field scanning optical microscopy (NSOM), which allows simultaneous acquisition of topographic and fluorescence images and was used to identify organized nanosized domains of molecules on the cell surface.\textsuperscript{137–139} The resolution for this imaging technique can reach \textit{ca.} 50 nm.\textsuperscript{140} Zhong \textit{et al.} used NSOM and two different emitting QDs to visualize the distribution and organization of T-cell receptors and gangliosides (GM1) as well as their nanospatial relationship and their correlation during T-cell activation. The results showed that \textit{a priori} formation of GM1 nanodomains serves as a platform for the recruitment of T-cell receptor nanodomains.\textsuperscript{141} A very promising strategy for improving super-resolution microscopy is the use of controlled photoswitching of QDs. In a recent study Díaz and co-worker developed photoswitchable QDs based on FRET from QDs to photochromic acceptors that could be activated by UV radiation.\textsuperscript{142}

3.2 Single-particle tracking

In addition to obtaining highly resolved images of cellular components, it is of large interest to study the dynamics of molecules with subcellular resolution. Single-molecule tracking (SMT) usually follows a single-molecule, whereas in single-particle tracking (SPT) the track of a mesoscopic particle bound to an ensemble of molecules is recorded.\textsuperscript{143} Their aforementioned unique photophysical properties have enabled frequent use of QD nanoparticles for SPT.\textsuperscript{144} Because in single-molecule imaging the localization of individual spots is not limited by diffraction but by signal-to-noise ratio (SNR), uncontrolled expression of genetically encoded FPs and low photostability of FPs and dyes do not allow long-term measurements with high SNR, which is essential for high resolution images.\textsuperscript{145} Other advantages of single QDs over dyes and FPs in single-molecule \textit{in vitro} assays are for example their very narrow PL spectra with FWHM around 15 nm and a high SNR, which enable a significantly improved spatial resolution of 5 to 10 nm compared to 40 nm with organic dyes. The resistance to photodegradation allows SPT and SMT over several minutes (more than 20 minutes have been reported) compared to 5 to 10 s for standard organic dyes.\textsuperscript{146} Walter \textit{et al.} reviewed different SPT microscopy techniques and proposed a guideline to choose the best technique for each target of interest.\textsuperscript{147} In general, a standard wide-field microscope can be used for SPT, but it may be very beneficial to use the auto-focus function to keep a constant distance of the sample in the \textit{z}-position. The first step of identifying and selecting individual fluorescent particles is to reduce background via changing the intensity threshold. The \textit{x}- and \textit{y}-coordinates can be obtained by fitting a two-dimension (2D)-Gaussian function to the particle’s intensity profile. This localization of the particles will be done for each frame and is called segmentation. In the next step an algorithm can be used for the calculation of the corresponding trajectories. In practice the algorithm links the centres of fluorescent spots across the adjacent frames in the image series. Further analysis using mean-square displacement as a function of time allows analysing the type of motion and provides information about different parameters, such as diffusion coefficient, transport velocity, and size of the confinement domain.\textsuperscript{115,148,149} For a continuous tracking of the target the intrinsic blinking of QDs is problematic and special algorithms that consider the disappearing of targets need to be used. Such tools can be freely downloaded.\textsuperscript{150,151} To cover the complexity and dynamics of biological processes it is of great interest to obtain the trajectories in three dimensions. Holtzer \textit{et al.} enabled 3D-analysis of molecular mobility by introducing a cylindrical lens into the emission path and tracked passively diffused and actively transported QDs within life cells.\textsuperscript{152} In another study Ram and coworkers developed multifocal plane microscopy and used an algorithm allowing the 3D positioning of a point source. They demonstrated the strength of this technique by observing the complete endocytosis process of antibody-conjugated QDs to the sorting endosomes deep inside the cell.\textsuperscript{153} Wells \textit{et al.} presented a new approach for 3D tracking of molecular motion using QDs and four overlapping confocal volume elements with an active feedback every 5 ms. Their approach showed several advantages compared to 3D tracking using charge-coupled device (CCD) cameras, namely lower excitation power, capability of time-resolved detection of the tracked molecules, and measurement of trajectories for minutes and over distances up to 10 \textmu m in all spatial dimensions, which may enable tracking throughout the entire volume of many mammalian cells.\textsuperscript{154} The measurement of the angular components of a tracked molecule using polarization modulation techniques could provide information about conformational changes and add rotational orientation inside a living cell as another dimension to SPT.\textsuperscript{155,156} Similar to super-resolution one of the key factors for SPT measurements is the successful delivery of the conjugates into the cytoplasm, which can be problematic for QDs due to their large sizes or surface charges. Cellular delivery will be discussed in more detail in Section 4.1.

Most probably because of complicated cell delivery initial applications of QD-based SPT targeted membrane proteins and studied entry/exit kinetics of receptors such as glycine,\textsuperscript{157,158} GABA,\textsuperscript{159} HER2,\textsuperscript{14,160} EGFR,\textsuperscript{14,161,162} and HIV receptor CD4,\textsuperscript{163,164}
but also Interferon and NGF, various transmembrane proteins, and aquaporine were investigated by SPT. The high spatial resolution of QD-based SPT was used by Chung et al., who investigated the EGFR signalling processes with a novel time-dependent diffusivity analysis. Important steps within signal transduction are ligand binding and receptor dimerization. The authors were able to monitor single receptors in living cell and could observe that EGFR fluctuated continuously between monomer and dimer state and that preformed dimers were preferred for ligand binding.

More recent studies were also able to track single QDs inside cells. Lowe et al. used QD-labelled cargos to study the entry to and exit from the nucleus by the nucleus pore complex. They were able to decipher characteristic steps for the import, namely cargo capture, filtering and translocation, and release into the nucleus. The investigation of altered expression or regulation of presynaptic serotonin (5-HT) transporter (SERT) is very important due to the involvement in multiple neuro-psychiatric disorders. Chang and coworker used antagonist-conjugated QDs and monitored single SERT proteins on the surface of serotogenic cells and could identify two different pools defined by the lateral mobility of the proteins. In another study the authors investigated the diffusion kinetics of the lipid raft constituent ganglioside GM1, which acts as platform to facilitate neuronal signalling. Chen et al. developed an aptamer-based probe, which was capable to target membrane proteins (nucleolin and prion protein) and to provide a biotin functional group as versatile linker to different fluorescent probes including QDs. They investigated the endocytic pathway using SPT and trajectory analysis and observed three types of movements associated with distinct phases: membrane diffusion, vesicle transportation, and confined diffusion. Internalization into the cytoplasm was achieved through the clathrin-dependent/receptor-mediated pathway. Modification of the aptamer sequence could possibly allow to using this probe for the tracking of other markers. A further improvement for the investigation of the endocytic pathway was proposed by Ma et al., who were able to track the whole intracellular dynamic endocytosis process of phenylephrine conjugated to QDs via continuously filming fluorescent images in the same cell. The measured motion parameters and colocalization with specific fluorescent tags for different types of cell components enabled them to divide the endocytosis process into six stages after membrane passage: (i) directed movement along actin filaments and transportation to early endosomes; (ii) confined movement in early endosomes; (iii) directed movement along tubulin and transportation to late endosomes; (iv) confined movement in late endosomes; (v) directed movement along tubulin and transportation to lysosomes; (vi) confined movement in lysosomes.

Ram et al. investigated the transferrin receptor pathway in a 10 μm thick epithelial cell monolayer using 3D single-molecule tracking with the aim to improve drug delivery across cellular barriers at specific body sites. The complexity of the neuronal structure offers a large playground for SPT to obtain information about molecular dynamics of lipids and transmembrane proteins in correlation to synaptic membrane compartments. Biermann et al. investigated the dynamics and organization of surface molecules in brain slices and were able to image QDs bound to cell surface molecules at depths of up to 60 μm. Penetration depths of QDs inside the organotypic brain slices and measured molecular dynamics of surface molecules are shown in Fig. 6. Dong and coworkers used a combined approach of fluorescence correlation spectroscopy and confocal imaging to investigate the binding of differently sized N-acetyl-l-cysteine capped QDs within live cells. With a single-molecule analysis procedure they were able to demonstrate that the binding efficiency and the targeted membrane sites in A-427 lung cancer model system were dependent on the QD size. QD-based SPT can also be used to increase the sensitivity of a biosensing platform as shown by Liu et al. They demonstrated a fluorescent colocalization assay based on single-molecule recognition for the detection of a single thrombin protein at a solid-phase surface using TIRF. Without target amplification or probe separation they obtained a limit of detection (LOD) of 0.8 pM.

**Molecular motors.** A particular application of SMT and SPT is the investigation of molecular motors, which have an array of functions inside the cell, e.g., cell movement, muscle contraction, cell migration, and intracellular transport. The ability to perform mechanical work is provided by the hydrolysis of adenosine triphosphate (ATP). The major subjects of investigation are myosin V and kinesin. SPT allows tracking organelles transported by the molecular motors, whereas SMT can enable monitoring of individual motors. The measured parameters (e.g., average velocity, fluctuation of velocity, run length, and the single steps of motor molecules) can provide a better understanding of biological processes and their dynamics. QDs fulfill the requirements of a probe to investigate molecular motors because the system moves with ca. 1 μm s⁻¹ and trajectories consisting of discrete steps between 30 to 80 nm.

Myosin V was one of the first molecular motors to be investigated and it was demonstrated that the tagging of the head domains with two differently emitting QDs produced functional motors and that the position of the two heads during myosin stepping could be localized with a precision of ~6 nm. Courty et al. demonstrated SMT measurements with QD-tagged kinesin-1 molecules to track the motion of intracellular proteins inside HeLa cells for several minutes. Using a recombinant biotinylated kinesin-1 conjugated to SAv–QD and pinocytotic influx allowed them to evaluate the velocity of the motion estimated to a value of 600 nm s⁻¹. The unbinding–rebinding processes resulted in long directed trajectories, with an apparent processivity of several microns. By using QD–anti-phalloidin conjugates to target actin filaments and to image the motion of proteins Yoo and coworkers demonstrated the possibility of using antibodies for SMT. Watanabe et al. performed a systematic study of the molecular motors myosin, dynein, and kinesin in living cells. They monitored the movement of endocytosed QDs in vesicles along the microtubes towards the nucleus with a spatial precision of down to 1.9 nm and a temporal resolution of 330 μs. The influence of specific motor domains on their cellular routes and the visualization
of the rotation of single microtubules by immobilized motor domains were also demonstrated.\textsuperscript{186,187} Using QD tagged myosin V in living cells internalized via pinocytotic influx revealed that 95% diffuse isotropically within the cytoplasm whereas 5% showed a directed movement, which was proposed to be “walking” on cortical actin filaments with step sizes of 74 nm.\textsuperscript{188,189} Zhang \textit{et al.} reported a step size of 36 nm of individual myosin V motors with an accuracy of 2 to 3 nm in all three dimensions. The authors used 9 x 9 matrix excitation, an array detector, and QDs without intrinsic blinking. Their technique allowed an 80-fold increase of the imaging rate.\textsuperscript{190} Myosin V rotation around its own axis during the movement could be shown by Ohmachi \textit{et al.}, who developed a single-molecule fluorescence polarization technique for 3D orientation measurements. Their method allowed an orientation accuracy of 10° at 33 ms temporal resolution and revealed a myosin V rotation of 90° during each step.\textsuperscript{191} A single myosin step was characterized by Wang \textit{et al.} by using a super-resolution approach for the investigation of actin gliding and immobilized myosin.\textsuperscript{192}

4. \textit{In vitro} applications

An enormous amount of research studies using QDs for \textit{in vitro} imaging applications have been published over the last decade and many of those have already been reviewed in focused articles.\textsuperscript{37,193} Here we try to give a useful and interesting overview about the impact of QDs in different imaging fields and to provide a good starting point for the interested reader to continue their research for accessing more specialized studies. We will first outline different techniques for delivering QDs to different subcellular compartments, followed by the description of some general QD-based \textit{in vitro} imaging applications and then focus on investigations related to the characterization of the cellular environment, drug and gene delivery for theranostic purposes, and multimodal imaging.

4.1 Cell delivery

There is no general strategy of QD cellular delivery and many different concepts have been proposed to deliver QDs to different...
places of interest inside various cells. An own comprehensive review could be written about this topic only. Here we limit ourselves to an overview of different concepts and strategies that were used for QD-uptake by cells. Because QDs are nanoparticles made of inorganic core materials the first challenge of using them for cellular imaging is the delivery into the cell. Cellular penetration of QDs can be achieved using different techniques divided in active and passive transportation. The major transport pathway of QDs into cells is endocytosis, which was illustrated by Osaki et al. as a drinking activity of eukaryotic cells, in which they ingest a part of their plasma membrane to swallow external objects.\textsuperscript{194} Passive transduction of QDs into the cell is mostly enabled via electrostatic interaction with the plasma membrane. For water-soluble QDs without functionalization with specific biomolecules different studies have shown the importance of the QD physical properties, such as type of surface groups, charge, and size, which determine uptake, transportation pathway, and accumulation inside the cell.\textsuperscript{195–198} Apart from the QD properties also cell type and cell incubation media and temperature can influence the uptake.\textsuperscript{199–201} In most cases amine surface groups, cationic charges, small sizes, normal cell culture incubation media, and human body temperatures are advantageous for cellular uptake. However, there is no general strategy of guaranteed efficient uptake and the experimental conditions need to be adapted to the cell type, the subcellular compartment of interest, and the imaging application itself.

In one example for the importance of surface charge Lee et al. investigated the influence of the charge of small-sized QDs (hydrodynamic radii of 7 to 10 nm) on their ability to penetrate the nucleus of a cell. They observed successful staining of the nucleus of fixed and live HeLa cells only with positively charged QDs, whereas the negatively charged QDs remained in the cytoplasm. The authors exploited this charge-mediated placement of QDs to specific subcellular regions for two-colour QD imaging.\textsuperscript{202} Other possibilities to enhance cellular uptake are the encapsulation of QDs with saccharides,\textsuperscript{194} in virus like particles,\textsuperscript{203} in nanogels,\textsuperscript{204} or in carriers based on lipid bilayer vesicles.\textsuperscript{205–209}

One of the main problems of passive QD transportation is entrapping inside the endocytic pathway and a minimal release into the cytoplasm. However, in order to allow bioactive QD-conjugates to target cell organelles, it is essential to release the QDs from endosomes or lysosomes. One approach is endosome disruption by using osmotic pressure.\textsuperscript{210,211} Bayles et al. used for the cytosolic delivery of QDs a cationic core–shell polymer, to whose surface anionic streptavidin–QDs were adsorbed via non-specific electrostatic interaction. The polymer colloids contained a pH-buffered proton sponge, which was able to increase its volume up to 50-fold at pH values below 6, due to the protonation of the tertiary amine groups. The volume increase but also the dramatic change of the zeta potential from +7 mV (pH 7.4) to +45 mV (pH 5.5) compromised the membrane integrity of late endosomes and led to their disruption.\textsuperscript{212} Kim et al. also exploited the local vesicular membrane destabilization for the escape of QDs into the cytosol. They used biodegradable polymeric nanospheres, which allowed endosomal to cytosolic translocation via pH-dependent reversal of the nanocomposite surface charge polarity. Once in the cytosol the polymer hydrolysed and released the QD-conjugates.\textsuperscript{213} In an effort to use the pH-responsive ability of polymers to enable target-specific delivery of QDs to tumour cells (solid tumours are known to have an acidic environment) Mok et al. modified the QD surface with grafted copolymers that exhibited charge reversal under acidic condition. They used poly(l-lysine) (PLL) whose backbones were post-modified using citraconanic anhydride, which is a pH sensitive primary amine blocker. In acidic environment the initially negatively charged QDs cleaved off the citraconylated amide linkages, which resulted in positively charged QDs with enhanced passive cell delivery.\textsuperscript{214}

Active transportation is characterized by ligand–receptor-mediated transportation using ligands, such as transferrin (TF)\textsuperscript{215} or antibodies.\textsuperscript{66,216–219} A very popular approach is the use of cell-penetrating peptides (CPPs).\textsuperscript{220} The peptide sequences, generally referred to as protein transduction domains, often include short segments from the human immunodeficiency virus 1 transcriptional activator Tat protein (HIV-1 Tat) and homopolymers of arginine.\textsuperscript{221–227} Ruan et al. and Chen and coworkers investigated the mechanisms of Tat-peptide-mediated delivery. Their results demonstrated a delivery via macropinocytosis, a fluid-phase endocytosis process triggered by Tat–QD binding to the negatively charged cell membranes. The Tat–QDs were tethered to the inner vesicle surface and then trapped in cytoplasmic organelles. After transportation along the microtubule tracks, they ended up in the microtubule organization centre.\textsuperscript{228} Surprisingly, FITC–Tat conjugates could internalize into the cell via clathrin-dependent and caveolae-dependent endocytosis and lipid-raft-mediated macropinocytosis. These differences in the uptake (by replacing a QD with a dye on the same type of CPP) showed that both the CPP and the QD play a significant role in the cell delivery mechanism.\textsuperscript{229} A deeper insight into the transduction kinetics of Tat-peptide-labelled QDs was provided by Suzuki et al., who used confocal microscopy, TIRF, and four-dimensional microscopy. They studied the kinetics of Tat initially and immediately before, at the beginning of, and immediately after entry into living cells. Their results demonstrated an energy dependency of the Tat-mediated translocation and that triggering of heparan sulfate proteoglycans (HSPGs)-mediated events on the cell membrane was necessary. Furthermore, the delivery efficiency was correlated with the number of bound peptides per nanoparticle.\textsuperscript{230} Medintz et al. utilized CPP-functionalized QDs conjugated with differently sized fluorescent proteins to investigate the influence on endocytosis efficiency, endosomal escape, intracellular stability and intracellular fate. To bypass the endocytic pathway they also tested microinjection, which lead to a more homogeneous distribution of conjugates throughout the cytosol.\textsuperscript{231} Kim et al. developed thermo-sensitive QDs, whose cellular uptake was controlled via temperature-induced shielding/deshielding of CPPs. Temperature control was established by using poly(N-isopropylacrylamide) PNIPAAm, which was co-attached with CPP via biotin–streptavidin recognition to the QD surface. Below the lower critical solution temperature (LCST) the PNIPAAm chains sterically hindered the cellular...
contact of CPPs. Above the LCST, the grafted PNIPAAm chains collapsed and the CPPs could induce cellular uptake.232 Boeneman Gemmill et al. studied different peptide motifs to enlarge the library of CPPs beyond the commonly used HIV-1 Tat-derived motif. Therefore they investigated four different peptides to deliver QDs into the cell. They found that the LAH4 motif, derived from a membrane-inserting antimicrobial peptide, and a chimeric sequence that combines a sweet arrow peptide with a portion originating from the superoxide dismutase enzyme, provided effective cellular delivery of QDs. They recognized that a small change within the peptide sequence can have a strong influence and can lead to inefficient cellular uptake. In comparison to the Tat-motif the new peptides have in common a strong positive charge at the N-terminii, which leads to a “halo” of positive charges favourable for the interaction with the cell and for an efficient cellular uptake.233 In an additional study they used the structure-activity relationship analysis for the investigation of key elements within the peptide sequence of different CPPs. They were able to identify the responsible regions for efficient endocytosis and delivery of QDs and other material inside the cytosol of different cell lines.234 Delehanty et al. exploited peptide-endocytosis, peptide-membrane interaction, polymer-based transfection, and microinjection in live cells over a four-day staining period demonstrating simultaneous five-colour fluorescence imaging of a cell using QDs.235 This long-term staining procedure nicely illustrated the versatility and stability of QDs under different cellular delivery conditions. To enable the targeting of QDs to cell components inside the cytoplasm it is important to release the entrapped QD–CPPs conjugates from the endosomes or lysosomes. One option is photo-induced release as demonstrated by Yaghiini et al. They used Tat–QDs conjugated to disulfonated aluminium phthalocyanine, which is an amphiphilic photosensitizer known to localize at phospholipid membranes. After the CPP-mediated internalization into the cell, the excitation of the photosensitizer via FRET with the QD as donor was leading to the photo-induced rupture of the endo/lysosomal membrane due to the creation of singlet oxygen. Successful release and efficient FRET was measured with steady-state confocal imaging and fluorescence lifetime imaging (FLIM).236 Liu et al. could circumvent the problem of QD–CPP conjugates entrapped in endosomes or lysosomes by using histidine- and arginine-rich peptides (HR9 peptides). Inhibition of typical energy-dependent endocytic pathways by CytD (an F-actin polymerization inhibitor) did not alter the transduction of the QD-conjugates and therefore the authors suggested a direct delivery through the cell membrane to be responsible for QD nanoparticle (NP) uptake.237 Another peptide used for cellular delivery is an insect neuropeptide, namely allatostatin 1, from Drosophila.238,239

In contrast to the frequent use of polyarginine-based conjugates, Chakraborty et al. utilized cholera toxin B as QD delivery agent. The advantage of this toxin is the direct delivery of QDs inside the cytoplasm in small vesicles, which circumvents the endocytic pathway. Intracellular QDs could be imaged even after several days.240 Based on this investigation, Tekle et al. compared the plant toxin ricin and bacterial toxin Shiga with the ligand Tf for receptor-mediated uptake into cells. All three components led to cellular uptake but also tended to be retained in the endocytic structure without efficient exocytosis or route to the Golgi apparatus.241 This passive retention of QDs inside the cell can be advantageous for imaging applications but can also increase the risk of severe health problems caused by QD toxicity. Wu et al. developed a route for the active retention of QDs inside the cell. Their concept was based on intracellular recycling of ligand–receptor complexes. QDs were conjugated with transferrin (QD–Tf) targeting the transferrin receptor (TfR) on the cell surface. In the presence of two Fe3+ ions the QD–Tf was binding to the TfR and was internalized into early endosomes via clathrin-mediated endocytosis. Acidification inside the early endosomes led to the release of the Fe3+ and the QD–Tf returned to the cell surface, where the Tf–TfR complex dissociated upon exposure to the neutral pH. The binding of the Fe3+ ions to Tf was established by the binding to four amino sites and two carbonate synergistic anions. Transformation of the latter ones to oxalate strengthened the binding to iron ions and thus led to a slower release inside the endosomes. The adjustment of carbonate-/oxalate-Tf ratio was used to control the intracellular Tf retention time.242 The differences in the Tf cycle and imaging as well as flow cytometry results of different ligand-dependent retention times are presented in Fig. 7.

Mechanical delivery techniques, such as electroporation and microinjection, can suffer from increased cell death and aggregration of QDs in the cytosol or a low throughput.243–245 Yum et al. presented a nanoscale mechanochemical method to deliver QDs into living cells using a membrane penetrating nanoneedle. This nanoneedle consisted of a chemically synthesized boron nitride nanotube, which was coated with a thin layer of Au. The Au layer facilitated the use of surface chemistry for attaching the QDs via disulfide bonds. Inside the cytoplasm these bonds were reduced into thiol-groups and led to the release of the QDs, which could also be delivered into the nucleus.246 Park and coworkers developed a novel platform for intracellular delivery of genetic material and nanoparticles, based on vertically aligned carbon nanosyringe arrays. Cargos like QDs could be loaded into hollow tubular compartments and simultaneously delivered to several cells without the need of external forces. The authors demonstrated this concept by QD delivery to the cytoplasm of cancer cells and stem cells.247

4.2 General applications

Successfully delivered QDs can be very useful fluorophores for investigating cellular structures and functions. One example is the measurement of cell motility by phagokinetic uptake of QDs and to correlate the migration of the cells to their metabolic state.248 Also of great interest is stem cell tracking to investigate their fate after differentiation and the behaviour of their daughter cells.249,250 QDs bound to specific targets can be used to gain insight into endocytosis, distribution and shuffling of receptors on the membrane, or the nanoparticles themselves.251–253

Many applications can avoid cellular delivery of QDs because several pathogen receptors are expressed on the outer membrane...
of cells. Those receptors can be targeted using ligands, aptamers, or antibodies. Because primary antibodies are relatively expensive compared to secondaries, most fluorescence imaging methods rely on a combination of primaries for receptor binding and fluorophore-conjugated secondaries (against the primaries) for fluorescence staining. Although very practical, this antibody combination causes larger sizes and lower specificity compared to direct staining with fluorophore-conjugated primaries, which can lead to limitations for nometric distance measurements (e.g., with FRET) or multiplexing (where high specificity for multiple targets is required). To overcome the size limitation but still provide facile conjugation, Howarth et al. used Escherichia coli biotin ligase for site-specific biotinylation of acceptor peptides that were genetically encoded at EGFR on HeLa cells and at AMPA receptors on neuron cells. This specific biotinylation allowed conjugation of QDs via biotin–streptavidin recognition instead of using the antibody approach. Chen et al. extended the application of biotin ligase using two orthogonal biotin ligases to enable duplex measurements with two QD colours. This allowed to simultaneously studying the trafficking and localization of two different cellular proteins for yeast-based applications. Le Gac et al. presented a study, in which they conjugated QDs with Annexin V to differentiate apoptotic cells due to the binding to phosphatidylserine (PS) moieties present on the outer membrane of apoptotic cells. Huang et al. developed QD-based quantification methodologies facilitating the analysis of subcellular distributions of multiple biomarkers, EGFR and E-cadherin (E-cad), which can help to predict the sensitivity to EGFR-targeted therapy. Zhang et al. presented a switchable probe to target folate receptors overexpressed on the membranes of different cancer cells. They used polyethyleneimine-coated CdS/ZnS QDs, whose fluorescence was turned off by adsorption of the folate receptor ligand folinic acid. After ligand–receptor binding on the cell membrane the ligand was released from the QDs and the emission was switched on. This method aimed at improving imaging of folic receptors due to the avoidance of false/positive results by using the “off/on” switch principle. Another possibility to label QDs to cells is the genetic manipulation of proteins to express a histidine tag, which can be targeted, e.g., by Ni-nitrilotriacetic acid (Ni-NTA)-modified QDs or carboxylated QDs. Furthermore, the use of the HaloTag protein was demonstrated by So et al. to mediate specific labelling of living cells. Sunbul et al. developed an efficient method for one-step covalent labelling of cell surface proteins with QDs based on enzyme-catalysed site-specific modification of short peptide tags. In another example of colour-multiplexing with QDs Orndorff and coworkers used two different neurotoxins and conjugated them to differently coloured QDs, which bound to two targets on glioma cells. Kang et al. used three QD colours for the development of a multiplexed cellular imaging system, which was capable of targeting three different molecular markers in a single cell. In another study of multiplexed imaging with QDs multiple cancer markers were visualized using small molecules such as aptamers or peptides. Zrazhevskiy et al. combined the specificity of antibodies with the multiplexing capability of five different QDs for single-cell molecular profiling (Fig. 8). This allowed them to simultaneously stain Ki-67, HSP90, Lamin A, Cox-4, and b-tubulin in HeLa cells with QD-antibody probes emitting at 525, 545, 565, 585 and 605 nm, respectively.

4.3 Cell environment

Internalized QDs can be used to discover factors for cell integrity and structure or metabolic processes and related consequences in a subcellular manner. One example is the study of integrin dynamics of human bone marrow derived progenitor cells (BMPC) during differentiation. Chen et al. used laser optical tweezers to show that the cytoskeleton is weakly associated with...
its cell membrane by measuring the integrin diffusion on the surface of BMPC at successive stages of osteogenic differentiation.\textsuperscript{277} Helmick et al. investigated the spatial orientation of actin filaments during cell motility. The ability to monitor the spatial and temporal organization of such biopolymers within a cell is essential to enable an understanding of the complexity and dynamics existing in biological processes.\textsuperscript{278}

QD-based imaging nanosensors have been developed for the measurement of oxygen,\textsuperscript{279} the superoxide radical,\textsuperscript{280} chloride ion,\textsuperscript{281} calcium,\textsuperscript{282} and proteolytic activity\textsuperscript{283} within the cell. QDs and QD conjugates that showed temperature and pH dependent PL intensities and lifetimes were used as intracellular nanothermometers and pH-meters.\textsuperscript{284,285} Yang et al. developed a QD-based nanothermometer to characterize the response inside single living cells upon external chemical and physical stimuli.\textsuperscript{286} Haro-Gonzalez et al. studied laser-induced thermal effects with optical traps containing single trapped microparticles.\textsuperscript{289} The application of QD–dopamine bioconjugates as an intracellular pH sensor was demonstrated by Medintz and coworkers, who exploited PL quenching resulting from pH-dependent electron-transfer from QDs to oxidized dopamine–quinone.\textsuperscript{290} Gui et al. used a polydispers quinone that showed a pH-dependent electrostatically tuned aggregation and disaggregation process with a simultaneous PL colour change.\textsuperscript{291} The combination of QDs and pH sensitive dyes in FRET was exploited by Snee et al. The authors observed a ratiometric response to pH owing to the modulation of the FRET efficiency. This approach can be a versatile method for chemical and biological sensing.\textsuperscript{292} Dennis et al. developed a QD-based sensor, comprising photostable QDs and pH-sensitive fluorescent proteins, which dramatically improved the sensitivity and photostability compared to common pH sensitive dyes. This FRET-based probe could be tailored by genetic engineering for different pH ranges.\textsuperscript{293}

Charge transfer between dopamine and QDs is not limited to the measurement of intracellular pH. Khatchadourian et al. used QD–dopamine as a tool for indicating QD uptake, breakdown, and processing in living cells. Blinking was used to detect single QDs and the effects of dopamine on the blinking behaviour were investigated under different biochemical conditions.\textsuperscript{294} Clarke and coworkers presented a study, in which they observed a redox-sensitive pattern of cellular staining with QD–dopamine conjugates. Under reducing conditions fluorescence was only seen in the cell periphery and lysosomes. Increasing oxygen concentrations led to QD PL in the perinuclear region including mitochondria. Under full oxidation QD labelling was detected throughout the complete cell.\textsuperscript{295}

QD-to-dye FRET can be a very useful technique to distinguish normal cells from cancer cells\textsuperscript{296} or to detect intracellular metabolic processes.\textsuperscript{297} Li et al. developed such a FRET biosensor for measuring the proteolytic activity of matrix metalloproteinase-2, which is heavily secreted by malignant tumour cells. The selective cleavage of the peptide resulted in the recovery of fluorescence from QDs. They developed a 535 nm emitting QD donor-based FRET probes (using Rhodamine B as acceptor) for\textit{ in vitro} and a 720 nm emitting QD donor-based FRET sensor (using an NIR emitting dye as acceptor) for\textit{ in vivo} use.\textsuperscript{298} Hu et al. developed a mercury biosensor based on FRET from N-acetyl-l-cysteine stabilized QDs to a Rhodamine 6G derivative–mercury conjugate. They could show intracellular imaging of mercury.\textsuperscript{299} QD/Au NP assemblies were used as fluorogenic substrate for BACE1 enzymatic assays by Choi and coworkers. BACE1 is a key enzyme for the production of amyloid-beta peptides, which are known to be related to Alzheimer’s disease (AD). Classical FRET approaches can suffer from a low solubility of the peptide substrate and low SNR for the use in cellular BACE assays. The QD–Au system could overcome such problems and its relatively small size (~ 12 to 15 nm) allowed efficient energy transfer from QDs to Au NPs. The latter are known as universal quenchers for diverse fluorophores due to long-range nanosurface energy transfer (NSET). NSET led to quenching of the luminescence of QDs that were assembled to the Au NPs via Ni–histidine interaction. Digestion of the BACE1 substrate cleaved the QDs off the Au NPs. This restored the QD PL and allowed the measurement of enzyme activity\textit{ in vitro} and in living cells.\textsuperscript{300} Wei et al. presented a DNA-templated heterobivalent QD FRET nanosensor. This probe exhibited dual motifs for extracellular and intracellular targeting and imaging of cancer cells. The first part of the DNA-based sensor (nucleolin-targeting motif – NTM) facilitated targeting of cancer cells and simultaneously stimulated macropinocytosis, which allowed the escape into the cytosol. Inside the cytosol the second part of the oligonucleotide (mRNA-targeting motif – MTM) targeted specific messenger RNA (mRNA), which was shown by switching off the FRET signal of a Cy5 dye.\textsuperscript{301} Probe preparation and the intracellular pathway together with fluorescence images demonstrating the specificity and versatility of the probe are shown in Fig. 9. This combination of specific targeting and cytosolic release of bioactive probes to measure tumour-associated mRNA is a promising tool for future imaging applications.
4.4 Drug delivery

Although the relatively large sizes of QDs (compared to small molecules) may interfere with an efficient recognition of and delivery into cells, their physical and chemical stability and nanoparticle character can afford long systemic circulation times and possibly an enhanced permeability and retention (EPR) effect. Moreover, the large surfaces of QDs offer an ideal "playground" to attach several similar or different functionalities. This physical property can be exploited to combine diagnostics and therapy to so-called theranostics. For the treatment of diseases like cancer, inflammation, and pulmonary diseases it is of great interest to monitor the pathway, uptake inside targeted cells or organs, and the therapeutic efficiency of the applied drugs. In particular, for cancer treatment persists the problem of a potential development of multidrug resistance, due to overdosed anticancer-drugs. One of the first applications of QDs inside a drug delivery system was demonstrated by Lai et al., who used QDs to cap the mesoporous channels of a silica nanosphere with entrapped drugs. Only by reduction of the chemically labile disulfide linkage between nanosphere and the QDs the drug could be released. A relative young research field is the use of QDs inside photodynamic therapy, in which for example a non-covalently bound combination of QDs and chlorin e(6), a second generation photosensitizer, could be used for light induced damage to cancer cells. Other therapeutic agents are chimeric single chain antibodies (scFv-Fc),

Fig. 9 (A) DNA-templated heterobivalent QD-to-Cy5 FRET nanoprobe, which consists of a central QD with attached mRNA-targeting motif (MTM) and nucleolin-targeting motif (NTM). mRNA binding replaces the hybridized Cy5 dye and can therefore "switch-off" QD-to-Cy5 FRET. (B) Intracellular delivery route. NTM triggers cell targeting and macropinocytosis, whereas MTM targets intracellular mRNA, which is probed by reduced QD-to-Cy5 FRET. (C) Brightfield and QD fluorescence overlay images after 1 h nanoprobe incubation at 37 °C. In both HEK 293 and HeLa cells lacking targeting motifs led to only very faint QD background fluorescence and no cellular targeting (left images) whereas the complete MTM-QD-NTM probe led to cellular targeting (right images). (D) mRNA targeting in HeLa cells. After 4 h of incubation both the complete nanoprobes (top images) and MTM-deactivated nanoprobes (bottom images) have entered the cells. Only the complete nanoprobe can target mRNA, which leads to replacement of Cy5 and reduced FRET inside the cell. This can be seen in the QD (reduced FRET – green colour) + FRET-Cy5 (QD-sensitized Cy5 PL – yellow colour) overlay images, in which the complete probe contains Cy5 only on the cell surface, whereas the MTM-deactivated probe contains Cy5 also inside the cell (centre images). Overlay of QD and Cy5 PL from direct excitation of the two fluorophores shows a significant replacement of Cy5 by mRNA only in the case of the complete probes (non-colocalized QD and Cy5 PL – red colour in the right images). Reproduced with permission from ref. 301. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.
which can act as target biomolecules for diagnostics but also serve for treatment. One of them is the antibody against insulin-like growth factor receptor (IGF1R). This antibody was conjugated to QDs and used for the investigation of IGF1R, which is involved in the proliferation, survival, and metastasis of breast cancer. With a simultaneous targeting and down-regulation of IGF1R activity after endocytosis in the cell, such scFv-Fc antibodies have a great potential as theranostic tools. 310

A more common application is the combination of QDs with anti-cancer drugs and in some cases QDs can provide a synergistic effect for the uptake of drugs, even for cells that show drug resistance. 306,307 Bagalkot et al. used the large surface area of QDs for the conjugation of an aptamer that targeted the extracellular domain of the prostate specific membrane antigen. The anticancer drug doxorubicin (Dox) was intercalated within the aptamer and thus in close proximity to the QD, which enabled FRET and quenched the PL of the QD. Successful binding of the aptamer to the cell membrane released Dox and re-activated the QD PL. 308 The preparation of such nanoparticles with dual functionality of tracking and therapeutic aspect often includes a multistep procedure. A mild aqueous ‘one pot’ method to prepare a QD-Dox conjugate wrapped with a PEG shell was presented by Chen et al. The condensation reaction of glutathione (GSH)–QD with Dox established the drug carrier whereas the shell stabilized the NP and prevented a fast clearance and drug release during systemic circulation. To increase the specificity folic acid was used as ligand to target the folic acid receptor, which was demonstrated in vitro. 309

In a recent study the advantageous properties of graphene (e.g., large surface area, low toxicity, and good stability) for improved delivery of Dox were combined with QDs as fluorescent reporters to monitor the drug delivery. A silica coating around the QDs suppressed potential toxicity and fluorescence quenching by graphene. One important advantage of using a graphene sheet was the large drug loading capacity of 1.4 mg mg⁻¹. A simple electrostatic layer-by-layer approach allowed facile preparation and for specific targeting a transferrin ligand (Trf) was attached to the surface of the graphene sheet. Delivery of the nanoassembly inside the cell could be monitored using the QDs. Because Dox fluorescence was efficiently quenched by the graphene sheet, delivery of Dox in the nucleus (release from graphene) could be probed by restored Dox fluorescence (Fig. 10). 310 The advantageous synergistic effects of QDs and carbon nanotubes for medical diagnostics and treatment have been reviewed by Madani et al. 311

Liposomes and micelles are among the most frequently applied nanocarriers. Their hydrophilic heads make them water-soluble and biocompatible, whereas the hydrophobic tails can be used for drug delivery via encapsulation of hydrophobic drugs. Gopalakrishnan et al. used the hydrophobicity to incorporate hydrophobic QDs into the bilayer membrane of lipid vesicles. They showed that these vesicles were able to fuse inside the cell but depending on the surface functionalization they could also stain the plasma membrane. The attachment and partial fusion with the cell membrane could be used to release the vesicles’ cargo into the cell. 312 Another possibility is to use co-encapsulated hydrophobic QDs and Dox in a phospholipid-based polymeric micelle. PEG was used to increase the circulation time and allowed a very efficient passive accumulation in cancer cells, revealing a sustained release of the drug over seven days. 313 Sigot et al. investigated the uptake and delivery capabilities of liposomes. Although PEG can prolong circulation times, the drawback is that the PEG coating can also prevent lipid–cell membrane association and the endosomal escape of the loaded cargo. The authors investigated the advantages of either fusogenic or pH-sensitive PEG-lipids inside the formulation of biotinylated lipid particles (BLPs) to promote the endosomal escape via PEG-lipid dissociation. Therefore they used a dual QD colour approach for targeting cancer cells and monitoring the release of the cargo. One type of QD was functionalized via biotin–streptavidin recognition with the epidermal growth factor (EGF), which targets the EGF receptor (EGFR) that is expressed on many cancer cells,
whereas a different emitting QD was encapsulated inside the BLP and indicated the successful endosomal escape into the cytosol.314

Passive drug transportation does usually not allow a controlled release of the drug and can therefore lead to a distribution throughout the body and accumulation in undesired areas. Controlled drug release can dramatically increase the efficiency of a treatment. Biodegradable micelles were used for such a controlled delivery strategy but only provided limited application for stimulated drug release.315 A more promising approach used an in situ preparation of CdSe QDs inside dual-responsive (pH and temperature) hydroxymethylcellulose–poly(acrylic acid) (HPC–PAA) hybrid nanogels. HPC was responsible for the sequestering and stabilization of the in situ formed QDs and PAA provided a pH responsive volume-phase transition. Using hydrogen bonding of the hybrid nanogel and the drug temozolomide (TMZ) was established at a low pH. With increasing pH the hydrogen bonds broke but this process was slowed down by a diffusion-controlled step provided by the mesh size of the nanogel. This approach avoided a random delivery of the drug. Additionally a change of the pH influenced the QD PL intensity, which could be used to gain information about the intracellular pH. The multifunctional hybrid nanogel offered optical pH sensing, cancer cell imaging, and controlled drug release within a single nanoparticle system.316 A more complex stimulated drug delivery system was proposed by Gui et al., who developed a thermo/pH-sensitive, magnetic/fluorescent, biocompatible, and low-toxic multifunctional microparticle. The magnetic core consisted of electrostatically adsorbed QDs on an iron oxide NP stabilized with thiodiglycolic acid and encapsulated in a silica sphere. A shell of a PNIPAM-g-chitosan copolymer provided temperature and pH regulated drug (adriamycin – ADM) release as a consequence of either an increased temperature or low pH, which led to the shrinking of the copolymer network (Fig. 11). These triggers could be controlled during the preparation of the probe. The authors demonstrated fluorescence imaging of in vitro cultured HepG2 cells and control experiments with free ADM and microparticles without ADM showed that the ADM anticancer drug retained its activity after release and that the microparticles did not show significant cytotoxicity.317

4.5 Gene delivery

RNA interference (RNAi) is a powerful tool to study gene functions and can be used for manipulating posttranscriptional inhibition of gene expression inside cells. Inhibition of protein translation, so called gene silencing, uses short, double-stranded, small-interfering (si) RNA and can be used as a therapeutic agent. A direct delivery of siRNA to the cell is not very efficient due to the negative charge of the nucleic acids, which prevents transfection to the cell. Nanocarriers that can be divided into viral and non-viral transporters, can be used to increase the transfection efficiency. Viral carriers offer precise and efficient exogenous nucleic acid transfection but they can also cause immune reactions by triggering the antiviral immune system.318 On the other hand non-viral carriers may suffer from low transfection efficiencies. One possibility of hiding the negative charge of nucleic acids is attachment to positively charged liposomes or polymers, which will retain their positive net charge after nucleic acid attachment. QD-liposome and QD-polymers conjugates have been successfully used for the imaging of siRNA transfection.319–321 A possibility to further improve cell transfection of QDs is surface-functionalization with peptides.318,322,323

For optimized transfection efficiencies the delivery steps and the fate of siRNA and other nucleic acids need to be monitored. Because protein knockdown by RNAi can be delayed over 48 h after administration, the use of organic dyes or fluorescent proteins, which often suffer from photobleaching and do not allow efficient multiplexing, is limited.324 The unrivalled stability and colour-tuning capability of QDs are very advantageous for RNAi because they allow precise and multiplexed long-term tracking, while simultaneously using the QDs as nanocarriers. In an example of long-term imaging with QDs Ishihama et al. investigated the movement of mRNA within the nucleus. They could track mRNA–QD conjugates inside the nucleus for more than 60 s after microinjection with a temporal resolution of 30 ms.324 In another study Shao et al. studied the interplay and timing for suicide gene therapy, in which tumour cells are transfected with a gene that triggers the production of a specific enzyme that metabolizes an applied drug, which then triggers a cascade leading to tumour cell death. The authors used QDs labelled to plasmid DNA and tracked them at different time-intervals. It could be shown that the conjugates were shuttled to the nucleus after 24 h, which was identified as the perfect time point to administer the drug.325

Another crucial step in nanocarrier-based cell transfection is the dissociation of the nucleic acid from its carrier, also known
as unpacking. Studying the dissociation kinetics is therefore highly important for designing efficient nanocarriers. Both a premature dissociation and an overly stable binding would be detrimental to the overall transfection efficiency. The binding of nucleic acids mainly occurs by electrostatic interactions with the carrier surface material or labile covalent binding. Cationic polymers can condense nucleic acids through electrostatic interactions to form polymer–nucleic acid nanocomplexes. Their dissociation could be studied by FRET from QD-donor-labelled nucleic acids to dye-acceptor-conjugated polymers. The dissociation led to reduced FRET, which was used to gain information about the unpacking and distribution of individual nanocomplexes. Chen et al. used the QD-FRET pair to compare unpacking of three different cationic polymers for gene transfer, namely condensed DNA in chitosan, polyethylenimine (PEI), and polyarginine (PA). The authors observed that the dissociation kinetics and the type of intracellular compartment where dissociation occurs have a significant impact on transfection efficiencies for the different gene carriers due to potential enzymatic barriers such as nuclease-mediated DNA degradation.

A follow up study demonstrated simultaneous tracking of DNA dissociation from NPs and subsequent DNA degradation in a non-invasive manner. By using a QD-based FRET relay (consisting of QD, nuclear dye, and Cy5) three distinct states of DNA condensation and integrity could be observed. Inside this FRET relay the QD donor excited the nuclear dye that served as first acceptor and simultaneously as donor for the second acceptor Cy5. The nuclear dye acted as indicator for the DNA integrity, whereas the FRET to Cy5 displayed the dissociation of the DNA from the carrier. Condensed, free, or intact or partially degraded pDNA were identified in a single-particle manner within cells.

One very promising application of RNAsi is chemotherapy. siRNA can sequence-specifically inhibit the expression of targeted oncogenes, minimizing side-effects, such as immune suppression, caused by conventional chemotherapies. Jung et al. developed multifunctional siRNA–QD constructs, which fulfilled the challenging chemotherapeutic demand of target-oriented delivery. They demonstrated a selective and efficient inhibition of the expression of the epidermal growth factor receptor variant III (EGFRvIII) in target human U87 glioblastoma cells by monitoring the down-regulated signalling pathway. For the target of siRNA delivery, they used the arginine–glycine–aspartic acid (RGD) peptide, which is known to bind to the integrins overexpressed in various cancer cells. Additionally they increased the cellular uptake using the HIV-Tat derived peptide.

Because nanocarriers are mainly endocytosed, the use of endosomal disrupting agents can improve endosomal release and enhance siRNA-mediated knockdown. Also QD-based siRNA nanocarriers that use the proton-sponge effect to deliver the carrier into the cytosol have been used. Yezhelyev et al. demonstrated 10- to 20-fold improved gene silencing activity and a 5-fold decrease of cytotoxicity compared to common delivery agents. Qi et al. used proton-sponge coated QDs to improve silencing of JAM-2, which belongs to a subfamily of junctional adhesion molecules and can be found around tumours and sites of inflammation. The proton-sponge coating consisted of amphiphol and PEI, which allowed (through electrostatic interaction) the transport of siRNA. By applying siRNA concentrations of 15 nM and 7.5 nM QDs in serum-containing medium the authors achieved a gene silencing efficiency of JAM-2 of more than 90% (Fig. 12). In an earlier publication Qi et al. used amphiphol to overcome possible limitations of conventional siRNA delivery agents, such as lipofectamine, for in vivo applications. Amphiphol is a linear polymer with alternating hydrophilic and hydrophobic side chains that are often used for solubilizing integral membrane proteins and delivering them into cell lipid bilayers, without disrupting the integrity of the cell membrane. In combination with NPs coated with hydrophobic surface ligands they can form stable complexes that are capable of carrying siRNA. At the same time
they avoid enzymatic degradation, aid endosomal escape, and mediate siRNA delivery into the cytosol.332

4.6 Multimodal imaging in vitro

Similar to a double-functionality of theranostic QD-conjugates for therapy and diagnostics one can add multiple other physical, chemical, or biological functions to a QD for so-called multimodal imaging. This is a very challenging approach because one single nanoparticle may need to provide very different functions simultaneously. Research studies on QD-based multimodal imaging range from pure proofs of concept of double-functionality without any improvement of either function to actual biosensing applications that exploit multiple improved imaging modes or imaging and separation properties combined within one nanoparticle. Probably the most frequently investigated QD-based hybrid-NPs are magnetic-QDs. Various approaches have been used for the preparation of such particles. In one of the first studies Gaponik and coworkers encapsulated iron oxide NPs and QDs in a polymer microcapsule and demonstrated the possibility of the external manipulation of these capsules by a magnetic field.333 In other approaches, semiconductor material was grown on the surface of iron oxide NPs334 or on a cobalt core.335 Selvan et al. published a protocol for the preparation of nanocomposites with superparamagnetism and tuneable optical emission properties.336 In an extensive study Gao et al. investigated the factors, which lead to core–shell nanocrystals or to nanosponges in a one pot synthesis and they observed that altering the sequence of adding the different reagents is the most important factor.337 It was also shown that a too close proximity of QD and magnetic materials can influence the photophysical properties and lead to QD PL quenching.338 Silica-encapsulation of iron oxide NPs and QDs leads to larger particles but preserves the intrinsic properties of both materials. In addition, the silica shell provides a good biocompatibility and a large surface for further functionalization with biomolecules.339,340 A method which led to even higher QYs than those prepared with silica co-encapsulation was the covalent binding of silica-coated Fe₂O₃ and poly(styrene-co-maleic anhydride)-coated QDs via a ring-opening reaction. The functionalization of the resulting nanocomposite with the PEG derivate Jeffamine M-1000 polyetheramine resulted in a high colloidal stability over a wide pH range (pH 2–13).340 Cho et al. embedded iron oxide NPs in a spherical polystyrene matrix on which QDs were conjugated. They also showed the possibility to use the same system for drug storage. For this purpose the chemotherapeutic agent paclitaxel was loaded on the surface using a layer of biodegradable poly(lactic-co-glycolic acid) (PLGA) and antibodies were used for specific targeting and cancer treatment.341 To create bright probes a high concentration of the imaging agent is very beneficial. Shibata et al. presented a smart solution using photo-uncaging ligands for site-specific delivery of bimodal nanoparticles. Photoactivation of such ligands resulted in systematic degradation and led to a localized release of QDs and superparamagnetic iron oxide nanoparticles (SPIONs) for fluorescence and magnetic resonance imaging, respectively. The small contrast agents also facilitated removal after the imaging process.342

Another approach is layer-by-layer assembly using differently charged materials to form hybrid structures.343 Because magnetic QDs provide advantages for imaging, sensing, and bioseparation they had a large impact on biomedical research. In particular, applications in labelling and sorting of cells demonstrated the benefits of such type of material.344,345 Zhang et al. developed a multi-shell structured multi-functional nanoprobe, composed of Fe₃O₄–SiO₂–QD–SiO₂–polydopamine core–triple-shell nanoarchitecture, which displayed high fluorescence intensity and rapid magnetic separation. They demonstrated the separation efficiency by sorting HL-60 cells from K562 cells on a microfluidic chip using a HL-60-specific aptamer (Fig. 13).346

Although in vitro measurements are most often an intermediate towards the application of multimodal probes in vivo, it is a crucial step for the acquisition of information concerning probe functionality, cell delivery, and cytotoxicity effects. Multimodal probes that combine fluorescence and magnetic resonance imaging (MRI) belong to the most important dual-imaging agents and SPIONs can be used as both a magnetic

![Fig. 13](image-url)
separation tool and a MRI contrast agent. Qiu et al. combined QDs, SPIONs, and Au NPs within a single PLGA-based NP. This particle was used to investigate the uptake of neutrophils, which are leukocytes and an integral part of the innate immune system. It is of great interest to understand the spatiotemporal aspects that relate neutrophils to the sites of infection and inflammation. QDs and SPIONs were embedded inside the PLGA core and Au NPs were assembled to the surface. As SPIONs can act as mediators in magnetic hyperthermia and as MRI contrast agents it is desirable to achieve a localized concentration for efficient cancer treatment and enhanced MRI contrast. Highly concentrated Au NPs are advantageous for the production of localized heat for photothermal therapies. Using the hybrid NPs the authors were able to image, track, and manipulate neutrophils.

More common for MRI measurements is the use of Gd-complexes as contrast enhancers. One approach for the preparation of a bimodal probe was the encapsulation of QDs inside a paramagnetic micelle where Gd-complexes were embedded inside the micellar coating. These particles were functionalized with cyclic RGD to target \( \alpha_v \beta_3 \) integrins, which were overexpressed on the surface of both angiogenic endothelial and tumour cells. They were also used with annexin A5 to specifically target asymmetrically distributed PS, which is exposed on the cell surface of apoptotic cells. PS is one of the main targets for the investigations of programmed cell death (PCD). Acute myocardial infarction, heart failure, or unstable atherosclerotic plaques lead to increased PCD, whereas cancer results in suppressed PCD. The measurement of apoptotic activity may greatly enhance the capability for rating the status of a disease and can serve as an early indicator for the effect of therapeutic interventions. A relatively facile method for bimodal probe preparation was demonstrated by Prinzen et al., who used biotinylated Annexin A5 and biotinylated gadolinium–DTPA to bind to streptavidin-coated QDs for the visualization of cell death and activated blood platelets. Activated platelets also expose PS, which is necessary to trigger the blood coagulation process. Other approaches used silica-embedded QDs with paramagnetic molecules inside the silica sphere or immobilized on the surface. As an alternative of using Gd-complexes as paramagnetic molecules Wang et al. doped the shell of QDs with Mn. These particles showed a high QY and high MRI relaxivity values.

In particular the ability of exciting several QDs of different PL (QD-IHF) compared to conventional detection methods showed the advantages of QD-based immunohistofluorometry (QD-IHF) compared to conventional detection methods. In particular the ability of exciting several QDs of different PL colours with the same excitation source (wavelength) for the detection of different biomarkers is extremely interesting for tissue imaging. Jennings et al. used five different emitting QDs conjugated with antibodies that targeted different components of the immune system present in a mouse spleen tissue. They performed a five-colour immunohistochemical staining and the specificity of each antibody was maintained under multiplexed conditions. Ferrara et al. demonstrated the unique possibilities of QD-based multiplexing for the investigation of coronary arteries. They combined the photostability and large two-photon absorption cross sections of QDs with the advantage of three-dimensional fluorescence imaging using two-photon excitation laser scanning microscopy. The results showed the feasibility of multicolour profiling and a 3D visualization of the vascular endothelium with outstanding resolution. Giepmanns and coworker exploited the tight packing of atoms in QDs for combining fluorescence microscopy with electron microscopy (EM). Due to different shapes of the QD cores they could differentiate three different QDs with EM and fluorescence microscopy in a single mouse tissue.

Tissue microarrays (TMA) enable fast and high throughput screening of biomarkers. Different studies showed the possibility of developing convenient data analysis software that can subtract autofluorescence, normalize tumour expression to cellular content, and produce a comprehensive profile of tumour derived antigens on a TMA. Another important aspect is the affinity of the antibody used for detection. Xiao et al. showed that a chicken-derived antibody, IgY, could be used for QD-IHF and provided high sensitivity and specificity for relative quantitation of cancer proteins. In direct comparison to conventional

5. Tissue

In an interlacing state between in vitro and in vivo applications the imaging of pathogens expressed in tissues is an important research field. The qualitative and quantitative measurement of biomarkers in tissue biopsy samples provides important information for the diagnosis, staging, and treatment efficiency of a disease. A sensitive and specific diagnosis is very hard to achieve by evaluating only a single biomarker and multiplexing is a very important issue, in particular for immunohistochemistry (IHC). The evaluation of a pool of biomarkers from a single sample affords multiple and therefore deeper disease information and takes into account the limited amount of biopsy material. Classical approaches of fluorescence IHC are based on organic dye antibody conjugates (e.g., FITC or rhodamine-based dyes), which often provide excellent biological and imaging properties and are readily available in a large spectral range. Drawbacks of such common IHC conjugates are photo-bleaching, spectral crosstalk, and low SNR caused by strong autofluorescence. Because efficient, sensitive, and selective fluorescence staining and imaging is more difficult for relatively complex tissue samples compared to cultured cells, the established IHC methods based on enzymes or dyes are still much more common than the use of QDs, which can suffer from more complicated bioconjugation and non-specific binding. Nevertheless, the physical benefits of QDs, in particular for multiplexing, have motivated a number of QD-based IHC research studies that showed the advantages of QD-based immunohistofluorometry (QD-IHF) compared to conventional detection methods. In particular the ability of exciting several QDs of different PL colours with the same excitation source (wavelength) for the detection of different biomarkers is extremely interesting for tissue imaging.
antibodies IgY showed an enhanced affinity to antigens measured by western blotting and IHC on normal and cancer cells. Further comparison in TMA for the quantitation of the human growth factor receptor HER2 using IgY and QDs showed a good agreement with chromogenic in situ hybridization.365

Yezhelyev and coworkers compared the use of multiplexed QD-IHF detection of hormone receptors and HER2 to traditional methods such as IHC, western blotting, and fluorescence in situ hybridization (FISH) in single paraffin embedded clinical tissue sections. Their results were in very good agreement with the already established clinical applications, which showed the translational potential of QDs for clinical tissue diagnostics. However, the authors also pointed out the need for more compact QD probes, which would allow deeper tissue penetration, controlled and site-specific conjugation, and an inclusion of housekeeping markers for standardization.366 A next step towards clinical applications was performed by Chen et al., who developed a QD-based HER2 kit for image acquisition in breast cancer tissue. The kit included an analysis software that allowed automated, sensitive, quantitative, and convenient detection and was successfully tested on 94 clinical samples.367

Li et al. used QD-IHF for image deconvolution and image analysis to improve Akt-1 quantification. The Akt/protein kinase B signalling pathway is implicated in tumour genesis and progression. The study of 840 TMAs of radical prostatectomy cases showed that high levels of Akt-1 could be associated with a higher risk of biochemical recurrence and prostate cancer-specific death.368 A general concern that could hamper the translation of QD-IHF in clinical practice is batch-to-batch variation of QD bioconjugate production. Xu et al. compared different batches of QDs concerning their consistency in tissue IHC using four-dimensional spectral imaging. The authors developed a quantitative method for evaluating the efficiency of QDs in staining tissues based on Hadamard transform spectral imaging. The Hadamard transform is a mathematical transform based on square waves and provides information about location and qualitative features. The four dimensions were location (the two coordinates x and y), fluorescence intensity (z) and wavelength (λ). For a proof-of-concept the authors used proliferating cell nuclear antigens (PCNA) in breast cancer tissues labelled with different batches of bioconjugated QDs. The results demonstrated that it is possible to maintain uniform bioconjugation efficiencies with different QD bioconjugation processes.369 Xu et al. investigated optimization of the labelling protocol for QD-IHF probes. In their study they pointed out the advantages of QDs over organic dyes and evaluated different staining methods as well as the variation of the number of antibodies per QD. The results demonstrated the multiplex detection and quantification of different biomarkers (which were validated by western blotting and conventional IHC) using QD-IHF.370 A direct comparison of conventional IHC, single-labelled, and double-labelled QD-IHF was demonstrated for a semi-quantitative in situ analysis of the nuclear markers estrogen receptor and progesterone receptor in ductal carcinoma in the breast (Fig. 14A). The results showed a concordance rate of 100% for the detection of both receptors between conventional IHC and both types of QD-based IHF using multispectral imaging (MSI).371

Multiplexed QD-IHF and spectrally resolved fluorescence imaging can also be used for deciphering the molecular, cellular, and architectural heterogeneity of cancer specimens. In two studies Liu et al. showed the use of four protein biomarkers for the identification of single malignant tumour cells within complex tissue. This method provided new molecular and morphological information that was not accessible by traditional hematoxylin and eosin (H&E) staining and IHC.372,373 In contrast to conventional methods QD-IHF can provide both patterns and quantities, which are necessary for personalized diagnostics. Chen et al. used QD-based quantitative spectral analysis of hormone receptors and HER2 to establish a new molecular classification system of breast cancer. This classification could better reveal the breast cancer heterogeneity and identify five molecular subtypes with different five-year prognoses, which may help to formulate a more personalized and comprehensive therapy strategy as well as prognosis.374 QD-IHF also showed to be a useful tool to investigate the controversial question of the prognostic potential of the epidermal growth factor for breast cancer.375 Andrade et al. employed lectin to evaluate cell surface glycoconjugates, which play an important role in differentiation–dedifferentiation processes. They evaluated and compared the expression and distribution of glycoconjugates in normal human breast tissue and benign (fibroadenoma) and malignant transformed breast tissue. Fluorescence images showed that different regions of breast tissue expressed particular types of carbohydrates. This helped elucidating the glycoconjugate profile in biological processes and provided new insights into cellular and extracellular matrix structures, molecular content, and tumour environment for a better understanding of biological processes including the development and progression of cancer.376 Another interesting study was published by Fang et al., who investigated cancer invasion and metastasis. The mechanisms for these two properties of cancer have not yet been fully understood. In the focus of the investigation was the basement membrane (BM), which is a specialized form of the extracellular matrix (ECM) and has important cellular functions. The degradation of BM by metastasizing cancer cells leads to structural remodelling, which is characterized by increased deposition of, for example, collagen. Because of its role as a physical barrier to resist cancer invasion collagen, specifically collagen IV, is potentially of great interest. The authors used QD-based imaging to gain new insights in the dynamic changes of collagen IV during cancer invasion (Fig. 14B).377

In addition to provide complementary information to IHC, multiplexed QD-IHF was also used for the detection of mRNA using FISH assays. In a comparative study concerning the expression of specific mRNA in mouse brainstem sections, Chan et al. could measure a significantly increased sensitivity in FISH using QDs instead of organic dyes. Their approach provided ultrasensitive simultaneous detection of multiple mRNA and protein markers in tissues and histological sections.378 Tholouli et al. described a methodology for multiplexed QD-based in situ hybridization (QD-ISH) using QD-labelled oligonucleotides.
and spectral imaging for routinely processed, formalin-fixed paraffin embedded (FFPE) human biopsies. Single and multiplex QD-ISH was performed in samples with acute leukemia and follicular lymphoma. The advantage of multispectral imaging is the separation of spatially co-localized signals. The method relied on the generation of a complete optical profile for each pixel in the image field, from which multiple spectral distributions could be reconstructed via at least square fitting linear unmixing approach, enabling signal quantitation.379 mRNA displayed one cogwheel inside the whole protein production cycle. The understanding of the relationship between proteins and mRNA is an important aspect for different cellular functions and processes. Matsuno et al. tried to illustrate this relationship by visualizing the processes of transcription, translation, transport, and secretion of hormones. In situ hybridization and immunohistochemistry under electron microscopy provided sufficient ultrastructural resolution for two-dimensional images of subcellular localization of hormones and their mRNA. The combination with QDs and confocal laser scanning microscopy added the third spatial dimension.380,381

Tissue and collagen matrices, also called phantom tissue, can be used to optimize probes for later in vivo measurement. Investigations of the preferential uptake of QDs in tumour tissue,382 or the evaluation of the possible penetration depth of multiphoton excitation383 were performed to test the limits of the probes prior to in vivo use. Wong et al. presented a study in which they exploited the increased protease activity inside tumour microenvironment for enhanced intratumour delivery of a probe using a tissue model of collagen gel. They proposed a multistage system in which 100 nm NPs were degraded due to protease activity and led to the release of 10 nm QDs. They observed that the size change was efficient in the enhancement of diffuse transport.384

Conventional multiplexed tissue staining methods are based on fluorophores that are attached to secondary antibodies, which necessitates a sequential multicolour staining. Sweeney et al. demonstrated a more facile and less time-consuming staining method using primary antibodies labelled via biotin–streptavidin recognition to QDs. Although one should keep in mind the elevated costs of primary antibodies compared to secondaries, the direct use of labelled primaries enabled multiplexed staining of three antibodies for triplexed measurements in tonsillar tissue within eight hours.385

6. In vivo applications

Once an imaging agent has been successfully applied in vitro the transfer to in vivo systems is the consequent objective. First publications of QD applications in vivo appeared in the beginning of this millennium31,23 and since then the number of articles has increased tremendously as witnessed by many
review articles. It should be noted that all in vivo applications of QDs have been limited to animals. However, this is a general limitation for fluorescence imaging and is mainly related to the limited tissue penetration depth of light and the very few imaging agents that have been approved for humans. Therefore only NIR-fluorescence based proof-of-concept studies, imaging of body parts, and endoscopic investigations have been performed so far. Other limiting factors for QD in vivo imaging in humans are their undefined biocompatibility and toxicity and a concomitant unavailability of QD probes that have been approved for applications in humans. Toxicity issues are reviewed in the last section of this article. In this chapter we give a short overview of QD properties and requirements for in vivo applications, discuss the use of QDs in studying the lymphatic system and for tumour localization, and report about QDs in multimodal imaging probes, a research field that has gained a lot of interest in the recent years due to the nanometric combination of fluorescence with other imaging techniques.

6.1 Properties, requirements, and applications of QDs for in vivo imaging

The different available in vivo imaging technologies can be identified by mainly five properties: (i) resolution (spatial and temporal), (ii) penetration depth (in tissues), (iii) excitation energy (ionizing vs. non-ionizing), (iv) availability of injectable biocompatible molecular probes, and (v) the detection threshold of probes for a given technology. In contrast to other imaging techniques, such as CT or positron emission tomography (PET), fluorescence imaging uses non-ionizing radiation and is usually less hazardous. The unique photophysical and photochemical properties and large surfaces of QDs offer many in vivo imaging advantages that are hardly realizable with common fluorophores. The synthesis of QDs with emission in the NIR window and therefore deeper tissue penetration depths. Larson et al. exploited the large two-photon absorption cross section of QDs for non-invasive multi-photon microscopy of the capillaries through the skin of living mice. The QD-containing vasculature was clearly visible and they were able to measure blood flow velocity and heart rates. In another study Lim et al. investigated the biological effects and tissue absorbance, scattering, and thickness related to the performance of two-photon-excited NIR-QDs. Apart from imaging QDs inside the tissue they can also be used for coating glass pipettes that are applied to target visually identified neurons for electrophysiological studies. Andrásfalvy et al. showed that such QD-coated glass pipettes can provide better two-photon excitation signals in deep brain tissues compared to other methods and they demonstrated the utility of their QD-pipettes in vitro and in vivo.

In one of the early QD in vivo applications Akerman et al. faced some unexpected problems when using CdSe/ZnS QDs functionalized with lung targeting peptides. Although the QD bioconjugates showed excellent targeting specificity for the relevant vascular lung tissue, accumulation of QD fluorescence within the targeted tissue was not detectable. This was mainly attributed to a low pH in the microenvironment, to oxidation of the surface, or to adsorbed proteins. Dubertret and coworkers circumvented fluorescence quenching by encapsulating individual QDs in phospholipid block-copolymer micelles, which led to stable and non-toxic nanoparticles used for microinjection in early stage Xenopus embryos. Embryo development up to the tadpole stage could be followed by QD fluorescence imaging, which allowed lineage-tracing experiments in embryogenesis. These results demonstrated the importance of appropriate
surface coatings and materials to produce efficient QD in vivo imaging agents. Many surface coating strategies have been developed, which used compact ligands electrostatic interactions (e.g., with hyaluronic acid), immobilization on oligomeric nanoparticles, and encapsulation in micelles, to name only a few.

Advantages of PEG-containing QD surface coatings for in vivo imaging have been presented in a number of studies. Ballou et al. investigated the influence of different coatings on the distribution and circulating half-lives in vivo. Localization was determined by fluorescence imaging of living animals and by necropsy. Short circulating half-lives were found for amphiphilic poly(acryl acid), short chain (750 Da) methoxy-PEG, and long chain (3400 Da) carboxy-PEG QDs, whereas approximately 70 min were found for long-chain (5000 Da) methoxy-PEG QD. Al-Jamal et al. investigated the length of PEG chains on commercially available Qdot 705 ITK amino (PEG2000) QDs (Life Technologies) and studied their fate after tail injection in an anesthetized mouse by fluorescence imaging. They found that the speed of the first pass extraction towards the liver could be slowed down with increasing the hydrodynamic diameter (longer PEG chains) of the QDs.

Supporting this study Daou et al. investigated the length of PEG chains in anesthetized mice by fluorescence imaging. They found that the speed of the first pass extraction towards the liver could be slowed down with increasing the hydrodynamic diameter (longer PEG chains) of the QDs. Al-Jamal et al. examined the modulation of surface and bilayer characteristics of functionalized QD-liposomes vesicles in terms of their blood circulation and retention time in tumour tissues. They could show that cationic hybrid vesicles have a rapid blood clearance, whereas PEG-incorporation on the QD surface dramatically prolonged the blood circulation half-life after systemic administration. The longer circulation time also led to a higher accumulation in the tumour. These results were supported by Papagiannaros and coworkers, who used QDs encapsulated in PEG-phospholipid micelles. Compared to commercially available QD-PEG they accumulated much faster (1 h instead of 4 h) and only half of the dosage was necessary to reach the same SNR. This QD complex presented a rapidly acting nanosized imaging agent that allowed an effective visualization of tumours using NIR imaging.

Alternative synthesis approaches circumvent the organic phase by preparing QDs directly in water and sometimes using targeting molecules as stabilizers. The possibility to use such aqueous QDs for in vivo imaging has been shown in different studies. Also biocompatible alloyed QDs have been developed and CdTeSe-Cds core–shell QDs were successfully applied as NIR in vivo imaging probes. Injected to rodents the QDs did not show any apparent toxic effects over three months, which was confirmed by histological sectioning. Additionally, the number of QDs in the liver and spleen reduced over time, which suggested a clearance of the nanoparticles from the body. To introduce a paramagnetic effect in such alloyed QDs Yong et al. doped them with Mn, thus creating a magnetic semiconductor system. Based on the small size, NIR PL wavelengths, high QY, and paramagnetism such Mn doped QDs may be attractive multi-functional in vivo imaging probes. Apart from QDs also elongated Cd-based quantum rods (QR) have been used for in vivo imaging.

The indefatigable amount of work in improving distribution, circulation time, and specific targeting of tissues and organs has led to a multiplicity of NIR-QD-based in vivo imaging applications. NIR-QDs have been used to track labelled stem cells, neutrophils, and viruses to improve the knowledge about their behaviour inside living animals by non-invasive imaging. In this context Pan et al. contributed an interesting study about monitoring of in vivo dynamics of avian influenza viruses, which are important for understanding viral pathogenesis and developing antiviral drugs. Bioorthogonal chemistry was used to label CdTeSe/ZnS alloyed QDs to avian influenza H5N1 pseudotype virus. This conjugated complex demonstrated bright and sustained PL signals in mouse lung tissue and allowed visualization of respiratory viral infection in a non-invasive real-time manner. The biodistribution in lungs and other organs could be quantified by measuring fluorescent signals and Cd concentrations of virus-conjugated QDs in tissue. This approach provided a simple, reliable, and quantitative strategy for tracking respiratory viral infections and antiviral drug screening.

Due to the large surface area of QD nanoparticles they can also be used as drug delivery system to form a theranostic (drug therapy + fluorescence diagnostics) agent. Savla et al. demonstrated a QD-based drug delivery system for specific targeting of ovarian cancer cells and simultaneous delivery of an anti-cancer drug. The overexpression of mutated Mucin 1 was targeted with aptamer-conjugated QDs, to which Dox was bound via a pH-sensitive hydrazone. This bond is stable at neutral and slightly basic pH but undergoes a rapid hydrolysis in mildly acidic pH. Thus the theranostic QDs were stable during blood circulation but could efficiently release Dox inside the cancer cells. In vivo imaging studies showed a higher toxicity for cancer cells using the QD-conjugates compared to free Dox. The theranostic potential of liposomes for simultaneous bioimaging and drug delivery was investigated for the delivery of camptothecin for the treatment of melanoma. Cationic liposomes together with carboxylated QDs showed a high accumulation in solid tumours and the drug entrapment was nearly complete (>99%),

Another cancer theranostic in vivo concept was developed by Shao et al., who used NIR-QDs conjugated with a DNA-encoded suicide gene and could monitor the therapeutic efficiency in mice. Transfection of tumour cells with suicide genes can lead to inhibition of the DNA polymerase and/or incorporation into DNA, which finally leads to DNA chain termination and tumour cell death. The authors could observe a tumour regression indicated by a significant decrease of the PL intensity at day 14 after administration of the QD-complexes.

6.2 QD in the lymphatic system

The lymphatic system plays a crucial role as drainage system of the body. Its main functions are transportation of the lymph and filtration of prospective immunogens from the extracellular space. It consists of capillaries, vessels, and nodes, is spanned over the whole body, and is also correlating with the blood stream. If tumour cells invade a regional lymph node the large lymphatic network enables a fast spreading of the tumour cells over the whole body and is therefore playing an active role in metastasis. So-called sentinel lymph nodes (SLNs) are found...
in close distance to tumours and thus represent a gate for metastasis.\textsuperscript{427} Mapping of SLNs is important for assessing the stage of cancer and for planning an efficient treatment. Different studies showed the possibility of SLN mapping using NIR-QDs\textsuperscript{428–432} and demonstrated the possibility for an image-guided surgery for the resection of SLNs.\textsuperscript{433–435} Hama et al. demonstrated for the first time a simultaneous imaging of two different lymphatic drainages \textit{in vivo} and their trafficking to lymph nodes.\textsuperscript{416} In the same year Kobayashi et al. used five different NIR-QDs for spectral fluorescence lymphangiography (Fig. 15). Their study demonstrated simultaneous visualization of five separate lymphatic flows \textit{in vivo} and their trafficking to distinct lymph nodes, which had not been possible using other imaging techniques. The QDs were injected into five different sites and monitored the lymphatic drainages in the neck and the upper trunk. \textit{Ex vivo} fluorescence imaging of the draining lymph nodes confirmed the \textit{in vivo} results. This study evidenced the important advantages of QDs for multiplexed diagnostics by demonstrating the possibility of investigating separated drainage patterns and mixing of five adjacent lymphatic basins \textit{in vivo}.\textsuperscript{437} A profitable application for this would be multiplexed \textit{in vivo} monitoring of cellular trafficking inside the lymphatic system.

Noh et al. performed non-invasive tracking of dendritic cells \textit{via} NIR-QDs during their migration into the lymph nodes. The monitoring of injected cells to their target tissue is very important for improving the performance of immunotherapeutic cells.\textsuperscript{438} Pic et al. studied the kinetics of NIR-QD accumulation in specific regional lymph nodes. \textit{In vivo} fluorescence images could be confirmed by \textit{ex vivo} elemental analysis (ICP-MS). QD accumulation was detectable 5 min after injection, reached a maximum after 4 h, and then decreased over a 10 days period. The overall level for the uptake of QDs by other organs remained rather lower.\textsuperscript{439} Kosaka and coworkers presented a method of real-time tracking of the lymph flow. The lymph flow is difficult to track in real-time because of the lack of appropriate imaging methods. The authors combined macro-zoom fluorescence microscopy with interstitial injected QDs that allowed tracking lymph through lymphatic channels into the lymph nodes. Further they used multiplexed two-colour real-time tracking with two QDs.\textsuperscript{440} Mathieu et al. used the combination of QDs and hyperspectral imaging to follow cerebrospinal fluid (CSF) drainage into the lymphatics. This is important to decipher central nervous system disorders, which are characterized by disturbances of CSF drainage. The authors could successfully monitor the QD signal in submandibular lymph nodes after 20 minutes. Compared to the monitoring using a surgically approach, this method allowed the imaging at multiple time points after injection in an intact organism without the need of surgery.\textsuperscript{441}

### 6.3 QDs and tumours

The eminent relevance of cancer in the life sciences has made investigations of location and distribution of tumours and tumour cells to a high priority research field for \textit{in vivo} imaging. To get an insight in the trafficking of cancer cells, it is possible to label such cells with QDs and to inject them subcutaneously or to use systemic application mostly in rodents.\textsuperscript{64,442} Antibodies are commonly applied to target tumours.\textsuperscript{216,443,444} Tada et al. used a dorsal skinfold chamber for single-particle tracking of QDs inside living mice. They were interested in the pathway of QDs labelled with antibodies and could decipher six steps of delivery, namely (i) circulation within a blood vessel, (ii) extravasation, (iii) access to the extracellular region, (iv) binding to a receptor on the cell membrane, (v) movement from the cell membrane to the perinuclear region, and (vi) access to the perinuclear region. Knowing antibody delivery pathways can provide helpful information for improving therapeutic efficacy.\textsuperscript{445} Diagaradjane and coworkers investigated the interaction of NIR-QD-labelled EGF with EGFR in xenographed tumours in mice. They were able to distinguish three phases of the nanoprobe: tumour influx within ca. 3 min, clearance within ca. 60 min, and enhanced accumulation within ca. 1–6 h. Although unconjugated QDs also showed tumour influx, the concentration gradually decreased over time. After 24 h the tumour fluorescence decreased to near-base-line levels for both pure QDs and EGF-QDs.\textsuperscript{446} Not only trafficking is important to understand the circulation of tumours cells. Also the interactions with membranes play a significant role in metastasis. Gonda et al. studied the membrane dynamics in metastatic tumour cells using QDs labelled with antibodies against a metastasis-promoting factor. Changes in membrane morphology and membrane protein dynamics based on its fluidity are critical for cancer metastasis. The authors were able to image the membrane dynamics with a spatial precision of 7 to 9 nm.\textsuperscript{447}

Yang et al. investigated the sensitivity of NIR-QDs for \textit{in vivo} imaging. They used commercial Qdot 800 QDs (Life Technologies)
conjugated with CPPs for endocytosis into oral carcinoma cells. The QD-labelled cells were inoculated in the dorsum, back muscle, and under the oral mucosa of nude mice in different amounts. The results revealed that at least 10^6 QD-labelled cells are necessary to obtain a sufficient signal. The highest cell amount used (10^6 cells) could be visually observed over 16 days.448 Similarly, Yukawa et al. investigated the intensity of NIR-QD-labelled Adipose tissue derived stem cells, which were transplanted subcutaneously into the backs of mice.449 Stroh et al. explored the multiplexing capability of QDs for in vivo imaging. They used multi-photon microscopy techniques and transgenic mice that expressed GFP and combined them with QDs. This allowed them to differentiate tumour vessels from both perivascular cells and the matrix.450 Lim et al. monitored natural killer cells that were used for immunotherapeutic cell-based cancer therapy via labelling with QDs. Qdot 705 QDs (Life Technologies) were attached to the killer cells using antibodies and a similar therapeutic effect was found compared to unlabelled killer cells. The conjugates were intratumourally injected and imaged using NIR fluorescence. Immunotherapeutic cells labelled with QDs can be a versatile platform for the effective tracking of injected therapeutic cell, which is very important in cell-based cancer therapies.451

Similar to the resection of lymph nodes Li et al. demonstrated a QD-based imaging-guided surgery (IGS) of U87 MG tumour xenografted mice (Fig. 16).452 NIR-QDs (728 nm PL maximum) were conjugated with tumour-specific peptides (cRGD) and injected into the tail veins of anesthetised mice. 48 h after injection the NIR-QDs were accumulated in the tumour, which could be used to distinguish it from other tissue and to resect it. Active targeting with cRGD–QD conjugates led to approximately five times stronger PL intensity in the tumour compared to passive targeting using QDs without cRGD.

Apart from the tumour itself another important target is the vascular of the tumour. Angiogenesis is the process by which new vasculature establishes a blood supply to a growing tumour. Receptors, such as integrins, are highly expressed in tumour cells during angiogenesis and diagnostic targeting of such receptors can provide insight into the type and extent of diseases, including cancer.389 Integrins can be efficiently targeted by the peptide RGD.453-455 Chen and coworkers reported the development of vascular cell adhesion molecule-1 binding peptide (VCAM-1 binding peptide) functionalized QDs (VQDs). The authors observed an increase of the fluorescence intensity for in vivo and ex vivo experiments in inflamed endothelium as well as blue-shifted emission. Their investigations revealed that this shift was related to the binding of VQDs to VCAM-1, which could be a useful tool for VCAM-1 detection in vivo.456 Antibody conjugates can also be used to image the vasculature. Jayagopal et al. used QD-antibody conjugates for noninvasively assessing the retinal vasculature and to be able to monitor continuous vascular events using fluorescence angiography. They were able to image three cell adhesion molecules and one leukocyte subset within the same animal and without spectral overlap. This demonstrated the possibility of detailed multiplexed studies of diseases and biological processes.457 A study of Kwon et al. supported the usefulness of QD-antibody conjugates as a promising imaging tool for angiogenesis of cancer by labelling anti-vascular endothelial growth factor receptor 2 (VEGFR2) antibodies to QDs. For their xenograft models they injected human prostate cancer cells (PC3) to nude mice on hind limbs. The imaging results after QD-conjugate injection revealed an increased fluorescence at the tumour localization after 12 h.458

### 6.4 Multimodal imaging

For a more comprehensive view on a disease (or other biological systems of interest) it may be desirable to use a contrast agent that can be applied to several imaging techniques. Apart from the development and production of only one single imaging agent with equal biological properties for different imaging techniques, another advantage for in vivo use can be the avoidance of multiple administrations. Fluorescence imaging provides high spatial resolution and sensitivity but low tissue penetration depth. MRI, CT, and PET provide high penetration depths and are non-invasive. On the other hand they have a relatively low spatial resolution (for MRI and PET) and low target sensitivity (for MRI and CT). Multimodal probes allow the overall optimization of space, time, sensitivity, selectivity, and dose properties for gaining information about uptake efficiency, distribution in organs and tissue, and activities on the cellular and subcellular level.459 It should be noted that in proof-of-principle studies the demonstration of combining any multiple imaging techniques in a single nanoparticle could have higher priority than the usefulness of this combination for biosensing.

Cai et al. developed a tumour targeting NIR-QD-based multimodal probe for PET and NIR imaging. They functionalized an NIR-QD with cyclic RGD-peptides for targeting the tumour vasculature and immobilized ^{64}Cu–DOTA complexes on the QD surface to allow PET imaging. The combination of the mainly
7. Toxicology

Despite the superior photophysical properties of QDs compared to other fluorophores, there are certainly a couple of drawbacks, some of which we want to discuss in this section. One of the major reasons, why QDs are not implemented in clinical imaging and drug delivery applications on humans, is the concern about their toxic effects. The literature contains many reviews with a general overview about toxic effects of nanoparticles as well as reviews focused on QD toxicity. Because of the tremendous amount of related articles, we can only give an overview of the main reasons for toxic effects of QDs in vitro and in vivo as well as the related consequences. It should be mentioned that general toxicity properties for all QDs do not exist. Toxicity parameters must be determined for each QD–cell system in order to be sure about the toxic effects, which can be dependent on concentration, time, material, size, shape, coating, environment, and experimental conditions. In vitro cytotoxicity tests revealed that the release of Cd ions as well as the triggered formation of reactive oxygen intermediates (ROI) are responsible for induced toxic effects. Additionally, the intracellular distribution of QDs can enhance associated nanoscale effects. Cytotoxicity can be judged on many different parameters, such as cell number, cell growth, apoptosis, and cellular morphology of metabolic activity. The following examples from the literature give an outline of possible effects and their origins. We will start with in vitro experiments and then describe some in vivo systems.

7.1 In vitro investigations

Shortly after the first applications of QDs in a biological context, first concerns were raised over the novel imaging agents. These concerns were mainly related to the chemical composition (heavy-metal ions), their size (nanomaterials), and the interference of these properties within cellular components and processes. In 2004 Derfus et al. investigated the effect of CdSe core materials on primary hepatocytes cells, which were used as a liver model. The toxic effect of CdSe was evaluated by measuring the mitochondrial activity using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) viability test. Exposure of the QDs with UV-light in the presence of air and subsequent incubation with cells led to a strong decrease of cell viability (97%). The authors suspected that the oxidation of the particles resulted in reduced Cd on the QD surface and that the release of Cd ions caused the cell damage.

In vitro PET and NIR organ imaging and indicated that the assumed co-localization of $^{64}$Cu and QDs is true for different organs and tissue except for the liver. The $^{64}$Cu–DOTA complexes dissociated in the liver and led to an overestimation of QD-PET probe concentrations by PET. Instead of a peptide Chen and coworkers used the vascular endothelial growth factor protein (VEGF) for their multimodal probe. VEGF and its receptor are major angiogenic regulators. The results revealed good correlation between in vivo PET and ex vivo NIR imaging results. Duconge et al. were the first who reported of a fluorine-18-labeling and quantitative whole body imaging study of PEG-phospholipid QD micelles with PET. The long circulation in the blood stream (up to 2 h) and the slow uptake of the reticulo-endothelial system led to a distribution throughout the body. Using fibered confocal fluorescence imaging, they were also able to access the kinetics of cellular uptake. Oostendorp et al. circumvented the inherently low sensitivity of MRI using a commercially available PEGylated QD as scaffold for Gd-bearing complexes and tumour targeting Asn–Gly–Arg (εNGR)-peptides. This allowed non-invasive and selective detection of tumour neovascularization using quantitative molecular MRI. The MRI results were confirmed using two-photon laser scanning microscopy and it could be shown that the QDs provide a MRI contrast enhancement. Preliminary in vivo experiments indicated that these particles provided a MRI contrast enhancement. Ding et al. combined the high spatial resolution of CT with the high sensitivity and specificity of fluorescence imaging. Because CT contrast agents are non-specific and eliminate rapidly from blood vessels after intravenous injection, encapsulation via nanoemulsions or liposomes can be used to slow down the diffusion and to enable accumulation in lesions. Despite this increased blood circulation time, CT still suffers from low target sensitivity and large doses need to be applied, which can lead to adverse health effects. The authors developed a QD-iodinated oil nanoemulsion probe to target macrophages, which are key targets for atherosclerosis imaging. Different to other approaches they used hydrophobic QDs, embedded them in iodinated oil, and subsequently dispersed them in water to form an oil–water nanoemulsion. After thorough characterization of the samples, they extensively studied them in terms of qualitative information of QD fluorescence imaging and highly quantitative tomographic imaging of PET permitted a sensitive and precise assessment of the pharmacokinetics and tumour-targeting efficacy. The authors could show an excellent linear correlation between in vivo PET and ex vivo NIR imaging results. Instead of a peptide Chen and coworkers used the vascular endothelial growth factor protein (VEGF) for their multimodal probe. VEGF and its receptor are major angiogenic regulators. The results revealed good correlation between ex vivo PET and NIR organ imaging and indicated that the assumed co-localization of $^{64}$Cu and QDs is true for different organs and tissue except for the liver. The $^{64}$Cu–DOTA complexes dissociated in the liver and led to an overestimation of QD-PET probe concentrations by PET. Duconge et al. were the first who reported of a fluorine-18-labeling and quantitative whole body imaging study of PEG-phospholipid QD micelles with PET. The long circulation in the blood stream (up to 2 h) and the slow uptake of the reticulo-endothelial system led to a distribution throughout the body. Using fibered confocal fluorescence imaging, they were also able to access the kinetics of cellular uptake. Oostendorp et al. circumvented the inherently low sensitivity of MRI using a commercially available PEGylated QD as scaffold for Gd-bearing complexes and tumour targeting Asn–Gly–Arg (εNGR)-peptides. This allowed non-invasive and selective detection of tumour neovascularization using quantitative molecular MRI. The MRI results were confirmed using two-photon laser scanning microscopy and it could be shown that the QDs were primarily located on the surface of tumour endothelial cells and to a lesser extent in the vessel lumen. Immobilization of Gd complexes on the QD surface can have significant effects, as was shown by Gerion and coworkers, who embedded QDs into a thin silica shell doped with paramagnetic Gd–DOTA complexes. Relaxivity measurements revealed a five to ten-fold enhancement depending on the type of relaxivity. The increase was not related to the nature of the core but rather to the immobilization on the silica shell, which reduced the rotational motion of the Gd complexes and to the hydrophilic nature of the silica surface, which allowed an efficient interaction with water molecules. Preliminary in vivo experiments indicated that these particles provided a MRI contrast enhancement. Ding et al. combined the high spatial resolution of CT with the high sensitivity and specificity of fluorescence imaging. Because CT contrast agents are non-specific and eliminate rapidly from blood vessels after intravenous injection, encapsulation via nanoemulsions or liposomes can be used to slow down the diffusion and to enable accumulation in lesions. Despite this increased blood circulation time, CT still suffers from low target sensitivity and large doses need to be applied, which can lead to adverse health effects. The authors developed a QD-iodinated oil nanoemulsion probe to target macrophages, which are key targets for atherosclerosis imaging. Different to other approaches they used hydrophobic QDs, embedded them in iodinated oil, and subsequently dispersed them in water to form an oil–water nanoemulsion. After thorough characterization of the samples, they extensively studied them in terms of cytotoxicity and affinity to three different cells in vitro. Micro-CT and in vitro cell confocal microscopy confirmed the staining of murine macrophages. The contrast agent was then used in atherosclerotic rabbits and showed in vivo accumulation in macrophages, which could be measured with clinical-CT and fluorescence imaging (Fig. 17). This investigation demonstrated the high potential of QD-based dual contrast agents also in larger animals.
They also suggested that appropriate coating of the core would reduce the negative effects on cell viability.

These early-observed toxicity results were supported in the following years by several studies, which showed that the surface modification had a tremendous influence on the cytotoxicity of QDs. Cell toxicity of QDs has been mainly attributed to reduction of the QD surfaces, leading to free Cd ions and generation of ROI. Because QDs are fluorophores that are usually excited by light, the photoactivated QDs can non-radiatively transfer their energy to proximal molecular oxygen to produce singlet oxygen, which will then produce ROI species like superoxide anions, hydroxyl radicals, and hydrogen peroxides. The molecular mechanisms of Cd ion-induced cell death were studied by many groups. Cd-ion leaching and ROI induce apoptotic biochemical changes, which create a loss of the mitochondrial membrane potential, mitochondrial release of cytochrome c, activation of caspases, and finally death. Brunetti et al. presented an alternative to the standard Cd based QDs. They compared the toxic effects of InP and CdSe QDs. Interestingly both materials were prone to ion leaching but In ions showed a lower intrinsic toxicity. Cho et al. measured the intracellular concentration of Cd when cells were exposed to CdCl₂ and CdTe QDs. The Cd salt showed a linear behaviour of decreasing metabolic activity and intracellular Cd ion concentration. QDs also showed a dose-dependent correlation, but not linear as in the case of the Cd salt. The authors concluded that intracellular Cd ions and triggered ROI formation are not solely responsible for the toxic effect but that also lysosomal enlargement and intracellular distribution of QDs need to be taken into account. This was supported by a study of Stern et al., who investigated the toxic effects of CdSe and InGaP core materials. Both QDs led to toxic effects without a detectable elevation of the caspase activity or the upregulation of genes that were responsible for both the reaction with intracellular Cd ions and oxidative stress. This pointed out that the whole nanoparticle should be taken into account in terms of toxic behaviour. Domingos et al. investigated the consequences of QDs on the algae Chlamydomonas reinhardtii and they could show that free Cd ions and Cd-based QDs have distinctly different biological effects.

Stern and coworkers also presented evidence for an induction of autophagy in QD-treated cells. Autophagy plays an important role in sensing oxidative stress, removing damaged proteins and organelles, and eliminating the damaged machinery that is responsible for excessive ROI production. This is an important defence/survival mechanism of the cell and can be activated by nanoparticles. In this regard Chen et al. investigated the effect of QDs on neurons. They found an enhanced autophagy formation due to an increased creation of ROI by QDs, which led to synaptic dysfunctions. By using an autophagy blocking agent the activity of the autophagosomes could be suppressed and the synaptic impairment restored. Luo et al. tried to decode the mechanism and functions of QD-induced autophagy in RAG (recombination-activation gene) cells. Experiments revealed that using an antioxidant to decrease QD-induced ROI creation led to a reduced autophagy activity and enhanced cell death. Similarly, the direct use of an autophagy inhibitor also led to increased cell mortality. These results supported the hypothesis that autophagy is a cellular protective mechanism in response to QD treatment.

Cell compartments have different physiological properties that can have different impacts on QDs. For instance, the cytosol...
has a neutral pH whereas endosomes and lysosomes have an acidic pH, which will increase within the endocytosis process. The stability of QD coatings is a highly important property for imaging applications because degradation of the shell inside the cell would most probably lead to toxic effects. Corazzari et al. investigated ion release in artificial media representing the cytosolic and lysosomal cellular compartments. Their QDs showed a significant release of Cd and Zn ions in acidic pH. Because the endocytic pathway is one of the most common uptake mechanisms for QDs, pH-induced instabilities are a significant drawback and require profound investigation, which can allow to quantitatively and qualitatively assessing the toxic potential of QDs before their use in vitro or in vivo.

Apart from toxicity due to instable QDs, other aspects of QD–cell interactions are often equally important. A living organism provides many possible interactions with QDs (e.g., with proteins and ions) and therefore synergistic effects need to be scrutinized for future in vivo applications. For example, Zhao et al. could show the dramatic effect of QDs in presence of copper ions in human liver cells. Their investigations revealed a strong synergistic effect, leading to an 8-fold decrease of cell viability and a 3-fold increase of intracellular ROI levels. Nagy et al. reported about controversial behaviour of cytotoxicity and genotoxicity when using differently charged QDs. Negatively charged QDs with MPA (methoxy propyl acetate) capping ligands showed to be relatively noncytotoxic compared to positively charged, cystamine capped, QDs. In contrast, genetical analysis revealed that MPA-QDs induced a high number of DNA-strand breaks due to the upregulation of metallothioneins, which bind the Cd ions that leached from the core and thus reduce the ability for DNA repair. ROI can also induce nucleobase damage of DNA, which is attributed to the abstraction of hydrogen atoms from nucleobases and ribosyl groups. These results led to the conclusion that cytotoxicity assays must be complemented with genotoxic analysis in order to get the full picture of cellular damage.

Stem cell research is of high interest for clinical applications. Mesenchymal stem cells (MSC) are multipotent cells with the potential to differentiate into bone, cartilage, fat, and muscle cells. In clinical applications they are used in cell-based transplantation therapy and tracking of stem cells after implantation could be highly beneficial for evaluating the therapeutic outcome. Hsieh et al. investigated the effect of liposome transfected QDs into MSCs on cell proliferation for chondrogenic and osteogenic lineage. In both cases there was no effect on cell proliferation and the QDs were clearly visible after several cell passages. However, initiation of MSC differentiation was not successful as the internalized QDs prevented the full response to the induced differentiation. Muller-Borer et al. could show that cytotoxicity of QDs was dose-dependent and suggested to limit the QD exposure to MSC. An adjusted concentration of QDs led to minimal toxic effects and the QDs could be applied as useful fluorescent labels.

Even if QDs induce a certain amount of toxicity, this toxicity needs to be compared to other existing and frequently used fluorescent imaging agents, such as organic fluorophores, under equal (or at least comparable) experimental conditions. Many imaging fluorophores, such as Hoechst dyes and BODIPY, are so commonly used that their cytotoxic effects are often completely disregarded. Bradburne et al. performed a direct cytotoxicity comparison of QDs versus organic dyes in cellular proliferation. The authors could identify small but significant effects on cellular viability when the dyes (acridine orange, Hoechst 33342) were used in a concentration range suggested by the suppliers. Using an iterative process to identify dosing conditions, surface ligands, and delivery modality, it was found that the applied type of QDs showed similar toxicity effects as the commonly used cell-labelling fluorophores. This study demonstrated the importance of the physical and chemical stability provided by appropriately selected surface ligands. Another comparative study using some of the most common imaging nanoparticles, namely CdTe QDs, Au NPs, and carbon dots, revealed that QDs had the strongest toxic effect on a variety of cell lines and plant growth. Uptake of QDs led to decreased cell metabolic activity, shrinkage of cells, breakage of mitochondria, and finally cell death. The toxicity of QDs was time and dose dependent. In comparison, Au NPs led to decreased metabolic activity but no further effects of cellular/subcellular structures and carbon dots did not show any obvious toxic impact. It should be pointed out that the QDs used in the different studies discussed above were of different origins and that the results do not allow to draw a general conclusion about QD cytotoxicity.

7.2 In vivo investigations

In most cases in vitro cell culture investigations are not able to fully represent in vivo tissue systems, in which cell–cell and cell–matrix interactions as well as different diffusion/transport conditions play important roles. Lee et al. developed a 3D spheroid-interaction-based nanoparticle toxicology testing system. This consisted of hydrogel inverted colloidal crystal scaffolds that could be used to create a physiological relevant 3D-liver tissue. A comparison of toxicity of CdTe QDs in cell culture and in the 3D model revealed a lower toxicity in the latter, which correlated well with animal data. Although such model systems may be useful as intermediates between in vitro and in vivo measurements, it is still extremely difficult to make reliable predictions for in vivo effects based on results from in vitro investigations. The exact mechanisms of how QDs interact with the body and especially distribution, metabolism, and excretion are not well understood. In contrast to cell culture studies, the nanoparticles are not directly exposed to the target cell but have a rather long trip through different body transport systems. One of the major application methods for QD-based in vivo measurements is injection. The exogenous particles are then carried via the blood that contains various serum proteins. QD–protein interactions can lead to the formation of protein layers on the nanoparticle surface, known as “protein corona”. This protein corona influences the biodistribution and biocompatibility. With the bloodstream the nanoparticles will be delivered to the different organs and tissues, where their
distribution is dependent on both the physio-anatomical features of the vasculature and the physicochemical properties of the nanoparticles. After successful distribution the QDs will undergo more or less metabolic processing and will be excreted from the body via the kidneys and/or in the feces. Retained nanoparticles can disturb over long-term periods the normal functions of organs or tissue and induce chronic organ toxicity, metabolic toxicity, immunotoxicity, or even genotoxicity. The immense surface of vascular endothelial cells provides plenty of space for interactions with QDs, in particular with negatively charged ones. Therefore, anionic QDs have a relatively low residence time in the blood stream and preferably accumulate in organs. On the other hand it was also shown that high doses of QDs could cause pulmonary vascular thrombosis by triggering the coagulation cascade via contact activation.

Understanding the interaction of QDs with blood cells would help to improve QD design for theranostic drug delivery purposes. Fischer et al. characterized the in vivo kinetics, clearance, and metabolism of two different coated QDs in rodents. QDs coated with BSA accumulated to 99% in the liver and the authors were not able to detect them in the urine or feces for up two ten days after intravenous injection. Fitzpatrick et al. supported the hypothesis of a slow metabolic degradation of QDs. Their long-term study over two years using in vivo injected QDs in mice revealed a shift of the QD emission peak from the QDs. Their long-term study over two years using in vivo and can lead to avoid chronic long-term effects of the used imaging agent but also to minimize non-specific background for the imaging application. The authors confirmed their assumptions by receptor-specific imaging of prostate cancer and melanoma, where the fast clearance was very advantageous for the imaging procedure. A drawback of small-sized particles may be their ability to cross important barriers inside the human body, for example the blood–brain-barrier. Therefore it is important to investigate the effects of QDs on the central nervous system. Gao and coworkers investigated the consequences of chronic QD exposure in rats for their synaptic plasticity and spatial memory. The results show enhanced synaptic transmission and an impairment of the synaptic plasticity in the hippocampus that is important for learning and memory formation. Behavioural studies gave further evidence for impairment of the spatial memory. The authors concluded from the rat model that long-term exposure may induce impairments of hippocampal synaptic plasticity and spatial memory. King-Heiden et al. investigated QD toxicity on embryos of Zebrafish. They exposed the embryos to QDs and observed similar effects as in rodent models. In addition to the excellent transparency of Zebrafish embryos compared to rodents this animal model usually causes significantly lower costs for assessing structure–toxicity relationships of QDs.

Similar to in vitro investigations also in vivo studies are controversial mainly due to the differences in QDs, their coatings, and their concentrations. Hauck et al. investigated biodistribution, animal survival, animal mass, haematology, clinical biochemistry, and organ histology for QDs injected in rats. They could not detect significant toxic effects in rats over four weeks of QD administration. Su et al. investigated aqQDs and their toxic effects. After short times (4 h) the aqQDs accumulated in the liver and after long-time blood circulation (80 days) in the kidneys. Larger molecules could be found in the spleen. Histological and biochemical analysis and body weight measurements demonstrated no evidence for overt toxicity even at long-time exposure. In another pilot study, rhesus macaques were injected with phospholipid micelle-encapsulated CdSe/CdS/ZnS QDs. The evaluation of blood and biochemical markers remained within the normal ranges. Also the histology of the major organs after 90 days of QD exposure showed no abnormalities. Using chemical analysis, traces of Cd accumulated in liver, spleen, and kidney were found. This was taken evidence for the slow breakdown and clearance of QDs from the body. These results were supported using similar QDs in mice. After 112 days of exposure there was no noticeable toxicity effect. The main accumulation areas were liver and spleen.

To investigate an alternative body uptake mechanism, Hsieh et al. studied the toxic effects of QDs inhaled by mice. They measured the spatial distribution of QDs in lung tissue slices using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). With this method they were able to measure different elements and could show a localized distribution of Cd and Se ions in the bronchiolar area. The assumption that a close accumulation of both elements reflects the QDs were confirmed by fluorescence imaging. Inhaled QDs appeared in the same locations as the lymphocytes accumulated in the lungs. Over 17 days no degradation of the QDs was detected but they triggered inflammation. The results were supported by traditional H&E-staining. After extensive studies on cells via direct exposition of QDs, Zhang et al. investigated the skin permeability of QDs. QDs were topicaly applied to porcine skin via a flow-through diffusion cell system to assess the penetration effect. They found that QDs could penetrate through the uppermost stratum corneum layers of the epidermis and could be localized in the intracellular bilayer close to follicles.
The penetration effect was enhanced when the skin was exposed to UV radiation.523

In summary, QDs have toxic effects that depend on many parameters, such as their material, composition, concentration, surface coating, and size, as well as the interacting system and the time of interaction. Due to these many factors it is unlikely that a general standard toxicity protocol for QDs will become available in the near future. Many QDs and many biological systems from in vitro cell cultures to several different in vivo animal models have been investigated. Many in vitro and in vivo studies discussed above have shown that the most important aspects of low toxicity are high stability and low concentrations and that advanced QD materials and preparation strategies can result in QDs with relatively low or negligible toxicity in some of the biological model systems. Regarding the almost infinite possibilities of combining different QDs with different biological systems as well as the long durations of representative toxicity studies it is doubtful that QD-based probes will be approved for application in humans in the near future. In cases where toxicity plays an important role for the application, careful and systemspecific long-term investigations are necessary to justify the advantages QDs provide as imaging agents.

8. Conclusion and outlook

To end this review article we allow ourselves a few-sentences philosofic (or rather facetious) conclusion: QDs for imaging-based biosensing are like humans. Different sizes, shapes, compositions, concentrations, colours, time-scales, temperatures, and other properties of the QDs (or humans) and the surrounding lead to a large versatility of interactions with the biological system of interest (or the earth). There are strong (excellent photophysical properties) and weak (bad photophysical properties) QDs, good (efficient and harmless interaction with the biological system and other fluorophores) and evil (toxicity for the biological system and counterproductive interaction with other fluorophores) QDs, smart (multifunctional bioconjugates with highly preserved physicochemical and biological properties) and stupid (non-functional bioconjugates with inefficient photophysical and biological properties) QDs, rich (biosensing of large molecular ensembles) and poor (single-particle tracking) QDs, and maybe even beautiful and ugly QDs (which will strongly depend on the subjective point of view and the opinion of the society or community). However, each QD (even of very similar composition, size, and shape) can have very unique and distinct properties and a categorization is actually almost impossible although too often applied (as for humans).

In a more scientific conclusion the combination of careful material composition and design with the versatile toolbox of biological recognition molecules can lead to very useful and multifunctional QD-based bioconjugates for advanced bioimaging. In this review we have summarized and discussed recent advances of QD-based fluorescence imaging for biosensing. QDs have influenced technologies (e.g., super-resolution microscopy and single-particle tracking, two-photon excitation imaging, multispectral/multicolour imaging, and multimodal imaging) as well as in vitro (e.g., cell delivery, cellular structures, functions, and environment, drug and gene delivery, and tissue diagnostics) and in vivo (e.g., investigations of the lymphatic system, tumours, and metastases) applications. On the other hand their materials (e.g., heavy metals) and their nanometric sizes have also opened an extensive discussion about their toxicity and many studies have reported different toxic effects ranging from completely harmless to very toxic. A careful analysis of QD–biosystem interactions becomes more necessary the more the bioimaging is involved in in vivo environments.

QD-based bioimaging is a relatively young research field and although applications as imaging agents in humans are a rather long-term perspective the future of QDs for more in vitro and in vivo investigations are brilliant just as the QD fluorescence. In particular, the well-studied spectroscopic advantages of using QDs within energy transfer processes such as FRET (QDs as donors and acceptors)524,525 for the detection of diffraction limited distances and the quantification of molecular interactions on the cellular and subcellular level at higher multiplexing dimensions (using PL intensity, lifetime, colour, and polarization) are very promising to further improve spatial and temporal resolution, sensitivity, and multiparameter detection. Advancement of bioimaging technologies and physicochemical and biocompatibility properties together with a more profound understanding of the biological interactions of QDs will pave the way for an even higher impact of QD-bioconjugates on bioimaging applications for the life sciences.

List of abbreviations

2D Two-dimensional
3D Three-dimensional
4D Four-dimensional
AD Alzheimer’s disease
ADM Adriamycin
AFM Atomic force microscopy
AMPA Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP Aminopropanol
aqQDs Aqueous QDs
ATP Adenosine triphosphate
BACE1 Beta-site APP cleaving enzyme 1
BLPs Biotinylated lipid particles
BM Basement membrane
BMPC Human bone marrow derived progenitor cells
BOI Biomolecules of interest
BRET Bioluminescence resonance energy transfer
CC Cervical carcinoma
CB Conduction band
CCD Charge-coupled device
CD4 Cluster of differentiation 4
CPPs Cell-penetrating peptides
CSF Cerebrospinal fluid
CT X-ray computed tomography
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