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QUANTUM DOTS FOR BIOIMAGING APPLICATIONS: PRESENT STATUS AND PROSPECTS

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Abstract. This review considers the present status and the prospects for future development of the bioimaging applications of quantum dots. The preparation and optical characteristics of quantum dots are discussed, as well as the general strategies for their post-synthetic chemical modifications, which are required in order to obtain biocompatible fluorescent probes. Special attention is given to the problem with the cytotoxicity of quantum dots, which is the major limitation toward their utilization in biological research. Recently developed applications of quantum dots as fluorescent markers for colloidal drug carriers are reviewed. Despite the currently existing problems, it is expected that quantum dots will reveal many important details about the mechanisms of interaction between biological cells and nanosized materials.

Key words: quantum dots, semiconductors, nanoparticles, nanocrystals, fluorescence, bioimaging.

1. INTRODUCTION

The quantum dots (QDs) are spherical nanocrystals (2–10 nm in diameter) made of semiconductor materials. A nanocrystal consists of about few hundreds to few thousands of atoms and is thus an intermediate between a molecule and the bulk. Since their first discovery in 1981 [1], the QDs and their chemical synthesis is a rapidly expanding area of research in materials science [2-4]. These nanocrystals represent a novel type of inorganic fluorophores. QDs were first utilized as fluorescent biolabels in 1998 [5,6], which promoted further extensive research in this area. Bioimaging applications of QDs also represent a rapidly developing field and up to date there are many reviews concerning different aspects of the problem [7-18]. In this report we review the influence of synthesis methods on the optical characteristics of quantum dots, their subsequent processing and suitability for different bioimaging applications. The general principles and ideas in bioimaging with QD-probes are illustrated. The barriers restricting further progress are discussed taking into account the significant potential for cytotoxicity of QDs both *in vitro* and *in vivo*. The potential of QD-probes for revealing the mechanisms of

interactions between biological cells and nanosized materials is discussed from the viewpoints of recent advancements in the fields of cancer cell biology and drug delivery research.

2. QUANTUM DOTS FOR BIOIMAGING

2.1. Optical properties

Quantum dots absorb and emit visible light at room temperature due to a quantum effect, known as the size-confinement of the exciton [14,19-24]. The most impressive for QDs are their size-dependent optical properties. Their absorbance and emission wavelengths depend on the nanocrystals diameter [25]. After an electron is excited, some of its energy is lost to atomic vibrations. This energy is typically converted to heat. When the electron decays into the ground state, it emits light at longer wavelength, because of its energy loss – this is the so-called normal band-edge emission [26]. Typical absorbance and fluorescence spectra of CdSe QDs prepared by us using the hot-matrix method are shown in Fig. 1. Due to the thermal energy loss, the fluorescence spectrum is red-shifted with respect to the absorbance spectrum (so-called Stokes shift). The nanoparticles can exhibit also a unique type of fluorescence resulting from a trapping of an electron at the crystal surface [26,27]. When a defect is entrapped into the crystal, it introduces a potential energy state in the band gap. Electrons are trapped at this state. The emission from this state leads to an electron decay to the ground state and is thus called trap-state emission. The respective fluorescence band is broader than the band-edge emission and located at a longer wavelength, respectively. A QD can exhibit both band-edge and trap-state emission simultaneously.

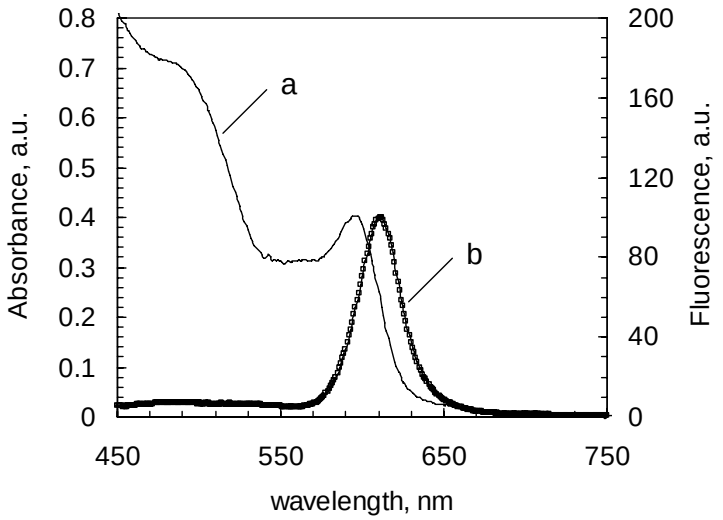

Fig. 1. Typical absorbance (a) and fluorescence (b) spectra of CdSe QDs with average size of 4.5 nm. The QDs are prepared by hot-matrix method in liquid paraffin at 250 °C.

The quantum confinement effect results in unique optical and electronic properties of QDs, giving them numerous advantages over the current fluorophores. The conventional dyes have narrow excitation spectra. It requires an excitation by light of a specific wavelength, corresponding to the maximum in the absorbance spectrum. Also, the conventional organic fluorophores have broad emission spectra, meaning that the spectra of different dyes may overlap thus limiting the possibilities for multicolor labelling. Furthermore, most of the organic fluorophores have a poor photostability and usually photobleach after only a few minutes of

exposure to UV-light. Also, the organic dyes have a fast fluorescence emission (~5 ns), which is similar to the fluorescence lifetime of the background from many naturally occurring substances, which leads to reduction of the signal-to-noise ratio in fluorescent bioimaging.

Now, let us summarize the advantages of QDs [16]. They have broad absorption spectra, allowing excitation by light of a wide range of wavelengths. This may be used to excite simultaneously multiple coloured QDs using a single wavelength of light. The QDs have narrow emission spectra, which can be easily controlled by varying the core size and composition, and also by variation of the surface coatings. Furthermore, the QDs are extremely stable against photobleaching and can remain fluorescent for hours under UV-light illumination. Finally, the QDs have a long fluorescent lifetime after excitation, which can be advantageous in time-gated imaging [14]. In the time-gated analysis, the photons hitting the detector in the first few nanoseconds are disregarded to decrease the background noise and increase sensitivity.

The most pronounced advantage of QDs over the organic fluorophores is their superior photostability, demonstrated in a number of reports [5,6,28]. This may be exploited in situations where a long-term monitoring of labelled substances is required, and is an area in which QDs may find use. For example, the comparison of the photostability of silica-coated QDs and Rhodamine 6G at excitation wavelength of 488 nm shows that the QDs exhibit a stable emission for at least 4 h, while the Rhodamine dye bleaches after 10 min [8]. This difference in the photostability is clearly illustrated by photographs comparing organic fluorophore vs. QDs in fluorescent tracking of cells in the course of embryogenesis [28] and in fluorescent labelling of cell structures [29]. Another example represents dihydrolipoic acid-capped CdSe-ZnS QDs, which show no loss in intensity after 14 h, and are nearly 100-fold as stable as, and also 20-fold as bright as, Rhodamine 6G [6].

2.2. Synthesis of core-only quantum dots

Most of the classical biological applications of QDs involve Cd-based nanoparticles. The CdSe QDs are produced in the 90s by the organometallic synthesis using dimethylcadmium as the Cd-precursor and toxic organic solvents, such as trioctylphosphine oxide (TOPO) and aliphatic amines [2,30-32]. In the 2000s, the organometallics are replaced with CdO [3,4]. We have developed a new approach using liquid paraffin as a solvent for the QDs synthesis [33], which allows the systematic investigation of nanocrystal growth and studies of the effects of various factors on this process [34-38]. The liquid paraffin solvent possesses a number of advantages in comparison with the classical solvents used in nanocrystal synthesis. The liquid paraffin is cheap, natural, non-toxic, chemically inert, and has a high boiling temperature (>320°C). The synthesis of QDs in liquid paraffin leads to the formation of relatively monodisperse in size nanocrystals of high fluorescence quantum yield (~25 %) and well-controllable size. The size of QDs, respectively their optical properties, can be controlled by various factors, such as the synthesis temperature [2-4,35-37,39], the precursor molar ratio [36, 39-41], and the composition of reaction medium (matrix) [38,39,42,43]. Different in size QDs can be obtained by simply taking aliquots from the matrix at different times of their growth, or by rapidly cooling down the reaction mixture. After the synthesis, the QDs can be easily purified and isolated applying simple extraction procedures [4,44]. A synthesis of QDs, suitable for biological applications, has been proposed directly in water dispersions [45-48]. In this case, the obtained QDs carry functionalities (like thioglycolic acid) on their surface, which can serve as linkers with biomolecules.

Some commercially available QDs for bioimaging applications are also non-cadmium. Recent report deals with the potential bioapplication of Zn-based QDs [49]. Particular interest represents the nanoparticle synthesis of $A^{III}B^{V}$ semiconductors. The colloidal synthesis of various $A^{III}B^{V}$ QDs is less studied and needs more effort in order to obtain high-quality nanocrystals for

extended bioimaging applications [50]. For example, colloidal QDs of InAs (diameters ~2.5-6 nm) are synthesized in a hot matrix of trioctylphosphine (TOP) [51] serving as both the solvent and capping agent. The colloidal InAs QDs show absorbance and fluorescence affected by the quantum confinement. $InGaP_2$ based QDs are commercial products for applications as markers in infrared light, where the biological tissues are more transparent.

2.3. Synthesis of core-shell quantum dots

A core-shell quantum dot consists of a semiconductor nanocrystal core, coated with a shell of another semiconductor material. The core-shell QDs have a core of a narrow band-gap semiconductor like CdSe and a shell of a wide band-gap semiconductor like ZnS or CdS [52]. The core-shell nanocrystals typically have brighter fluorescence [52-59] and are more stable against photodegradation [54] than the core-only QDs. The fluorescence quantum yield of core-shell nanocrystals can be 50-80 %, however a fluorescence quantum yield of up to 40 % is usually achieved [5, 60-63].

Core-shell CdSe/ZnS QDs have been first synthesized in a hot matrix of TOPO [52]. A wide spectral range of bright fluorescence from different in size samples of CdSe/ZnS can be obtained (the fluorescence peaks occur from 470 to 620 nm). The position of maximum in the fluorescence spectrum shifts to red with increasing of the shell thickness. The overcoating of CdSe QDs with either ZnS [52,53] or CdS [54,64] shell has become routine and usually results in almost an order-of-magnitude enhancement in the fluorescence quantum yield compared to the initial core-only QDs. Recently, we prepared CdSe/CdS QDs by a novel approach using the hot-matrix method in liquid paraffin [65]. In this procedure, the sulfur precursor is injected at once to a dispersion of CdSe cores and cadmium stearate in liquid paraffin at ~100 °C. Then, the temperature is gradually raised up to 250 °C, resulting in CdS shell growth. The gradual heating, allows the successful preparation of highly fluorescent (florescence quantum yield ~65 %) core-shell QDs relatively fast, at the same time avoiding dissolution and size defocusing of the CdSe cores.

2.4. Hydrophilization of quantum dots

The QDs, synthesized in a hot organic matrix, have the disadvantage of being capped with hydrophobic organics, which do not allow their dispersion in aqueous medium for the biological applications. Further special treatment is necessary to replace the hydrophobic organic layer with a hydrophilic one [8]. Water-insoluble QDs can be grown easily in hydrophobic solvents, but the solubilization in water requires sophisticated surface chemistry alterations and presents a significant challenge. There are three general strategies for water solubilization of QDs: (i) ligand exchange, (ii) micelle formation through hydrophobic interaction, and (iii) silica encapsulation. One should take into account that some types of QDs, such as CdTe suitable for biological applications, can be synthesized directly in water dispersions, however they are less stable and easily undergo aggregation [45-48].

2.4.1. Ligand exchange

Usually, the QDs synthesized in organic solvents have hydrophobic surface ligands, such as trioctylphosphine oxide (TOPO), trioctylphosphine (TOP) [2,43,66], tetradecylphosphonic acid (TDPA) [3] or various long-chain fatty acids (lauric, stearic, oleic, etc.) [4,33,43,65,67]. These hydrophobic ligands could be replaced by some water-soluble bifunctional molecules, in which the one end is connected to the nanocrystal surface and the other end is hydrophilic and may also be reactive to biomolecules. Examples of some water-soluble bifunctional molecules

used are mercaptocarboxylic acids (HS-(CH_2)n-COOH, n=1–15) [6,8,48,68,69], 2-aminoethanethiol [68], dithiothreitol [70], dihydrolipoic acid [61], hydrophilic phosphines [71], peptides [72], neoglycoconjugates with a reactive thiol group [73], etc. (Fig. 2). The thioglycolic acid (TGA) was used in the first bioapplication of QDs [6], while recently dihydrolipoic acid (DHLA) is more used, because of its biocompatibility, lower toxicity and higher stability of the obtained QD-dispersions in water [61]. However, TGA is widely used to obtain hydrophilic QDs (Fig. 3). Our experience shows that such TGA-capped QDs can be prepared by extraction of stearate-coated QDs (synthesized by the hot-matrix method in liquid paraffin, purified and dispersed in chloroform) with water solution of sodium thioglycolate. However, the ligand exchange in most cases dramatically decreases the fluorescence quantum yield of QDs. Also, the thiol-based molecules (e.g. mercaptocarboxylic acids) may form disulfides over time and come off from the quantum dot surface and finally the QDs aggregate and precipitate out of water; the other water-soluble bifunctional molecules are expensive and instable, either.

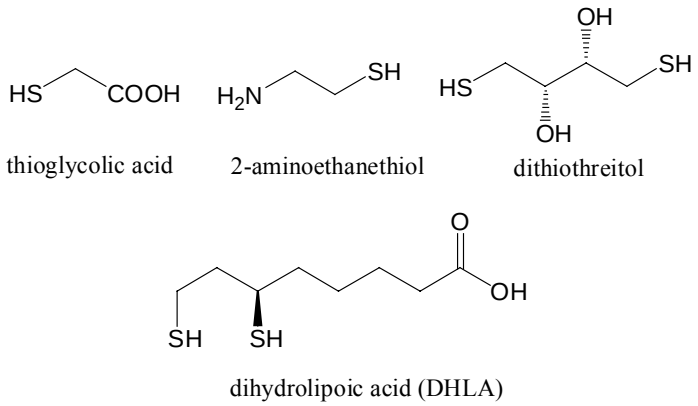

Fig. 2. Chemical structures of various bifunctional ligands used for hydrophilization of QDs.

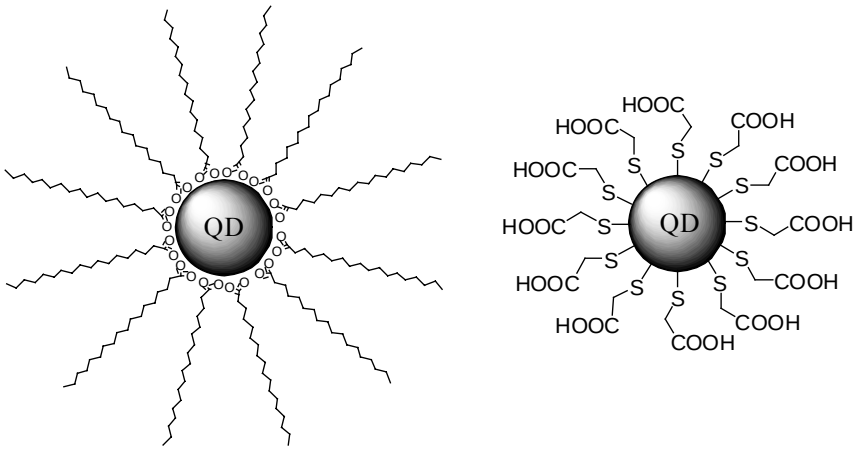

Fig. 3. Schematic representation of a hydrophobic stearate-coated QD (left) and a hydrophilic QD, coated with thioglycolic acid (right) obtained after ligand exchange reaction. Stearate-coated QDs can be prepared by hot-matrix synthesis in liquid paraffin (see the text for details).

2.4.2. Encapsulation in micelles

Phospholipids, such as 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine have both hydrophobic and hydrophilic ends. They could encapsulate QDs in the core by forming oil-in-

water micelles through hydrophobic interaction between their hydrophobic ends and the surface ligands of QDs thus providing water-dispersibility [28]. Recent work reports on the preparation of water-dispersible QDs, coated with Gemini surfactants [74]. These QDs are biocompatible, photostable, and suitable for live cell imaging.

A more promising approach is to use amphiphilic polymers to form micelle-like structures for transferring the hydrophobic QDs into water [75,76]. A triblock polymer, containing segments of poly(butylacrylate), poly(ethylacrylate) and poly(methacrylic acid), is used to transfer hydrophobic TOPO-coated QDs into water, in which the methacrylic acid segments were also partially derivatized with octylamine and PEG-NH_2 through a two-step EDC-coupling [76]. The hydrophobic side chain is directly attached to the hydrophilic acrylic acid segment and interacts strongly with the hydrophobic tails of TOPO. The strategy of using amphiphilic polymers is generally superior to the ligand exchange, because:

(i) There is no direct interaction with the atoms on the nanocrystal surface and therefore can preserve the original fluorescence quantum yield to the highest extent.

(ii) The large number of hydrophobic side chains on the polymer strengthens the hydrophobic interaction to form more steadily structures and stable water-dispersible QDs.

(iii)The amphiphilic polymers can be carboxylate or amine functionalized in order to attach various ligands or biomolecules [77].

Other relatively large organic molecules, such as amphiphilic dendrimers [78] and amphiphilic hyperbranched polyethylenimine [79], are also used for hydrophilization and stabilization of QDs in water dispersions.

2.4.3. Encapsulation in silica

A layer of silica can also encapsulate the fluorescent QDs in order to make them biocompatible [5,80]. Functional organosilicone molecules, containing $–NH_2$ or –SH, can be incorporated into the silica shell thus providing surface functionalities for biomedical applications. The silanization method includes replacement of the hydrophobic organics on the nanocrystal surface with mercaptopropyl-tris(methyloxy)silane (MPS). The methoxysilane groups (Si-OCH_3) hydrolyze into silanol groups (Si-OH), which form siloxane bonds upon heating, thus releasing water molecules. Then, fresh silane precursors, containing a functional group (F) -SH, -NH_2; -PO- (O-CH_3), are incorporated into the shell. The remaining –OH groups are converted in $–OCH_3$ groups; this last step blocks further the silica growth. This method seems to stand in between the above two strategies, but is much closer to the ligand exchange. The quantum dot surface changes once introducing the organosilicone molecules and usually results in a decrease of the fluorescence quantum yield. The procedures to make a controllable silica coating around the hydrophobic QDs are complicated. The silica coating needs to be carried out at dilute conditions, which is a limitation for large quantity production. However, the fluorescence of silanized QDs is much more stable in comparison with the organic fluorophores [80].

A simple aqueous synthesis of silica-capped, highly fluorescent CdTe quantum dots is developed [81]. The synthesis of silica shell in this case is carried out through a modified Stöber method. The photoluminescence studies show that the silica shell results in greatly increased photostability in tris-borate-ethylenediaminetetraacetate and phosphate-saline buffers. To further improve their biocompatibility, the silica-capped QDs are functionalized with poly(ethylene glycol) and thiol-terminated biolinkers. Through the use of these linkers, antibody proteins are successfully conjugated.

Another general method for silica encapsulation in toluene is also reported [82]. The biocompatible modification and multi-functionalization of QDs has been carried out through direct reaction of organic silanes on the surface of QDs. Functionalized QDs, including

CdSe/ZnS, CdSe/CdS core/shell and PbS QDs, have been prepared at a high concentration up to 10^{-4} M in toluene. Different organic silanes can be used to prepare various organosiloxane shells.

2.5. Bioconjugation of functionalized quantum dots

Various water-dispersible functionalized QDs have been used in both *in vitro* and *in vivo* bioimaging and detection. A number of published review articles provide excellent overviews about a variety of biomedical applications of QDs [7,8,14,76,83]. Here, we will summarize the bioconjugation reactions used for the preparation of functionalized QDs.

2.5.1. Bioconjugation of carboxyl-functionalized QDs

The bioconjugation reactions involving amino-functionalized QDs are summarized and represented schematically in Fig. 4. The carboxyl-functionalized QDs are usually conjugated to biological molecules by using the reaction with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), known as EDC-coupling. EDC is a water-soluble derivative of carbodiimide. Carbodiimide catalyzes the formation of amide bonds between carboxylic acids or phosphates and amines by activating carboxyl or phosphate to form an O-urea derivative. This derivative reacts readily with nucleophiles (usually amine groups). Historically, EDC-coupling was first used to conjugate TGA-coated QDs with transferrin and IgG [6]. Now EDC-coupling is among the most used techniques for direct conjugation of QDs with various proteins, including antibodies [6,48,72,77,84], or small biomolecules, such as γ-aminobutyric acid [85].

Antibodies can be conjugated to carboxyl-functionalized QDs by three different ways (Fig. 4): (i) directly, by covalent attachment via EDC-coupling [6]; (ii) indirectly, by using streptavidin (or avidin)-coated QDs and biotinylated antibodies [9,53]; (iii) indirectly, by using a special adapter protein, which is electrostatically adsorbed on the carboxyl-functionalized QDs (see for more details section 4.4.).

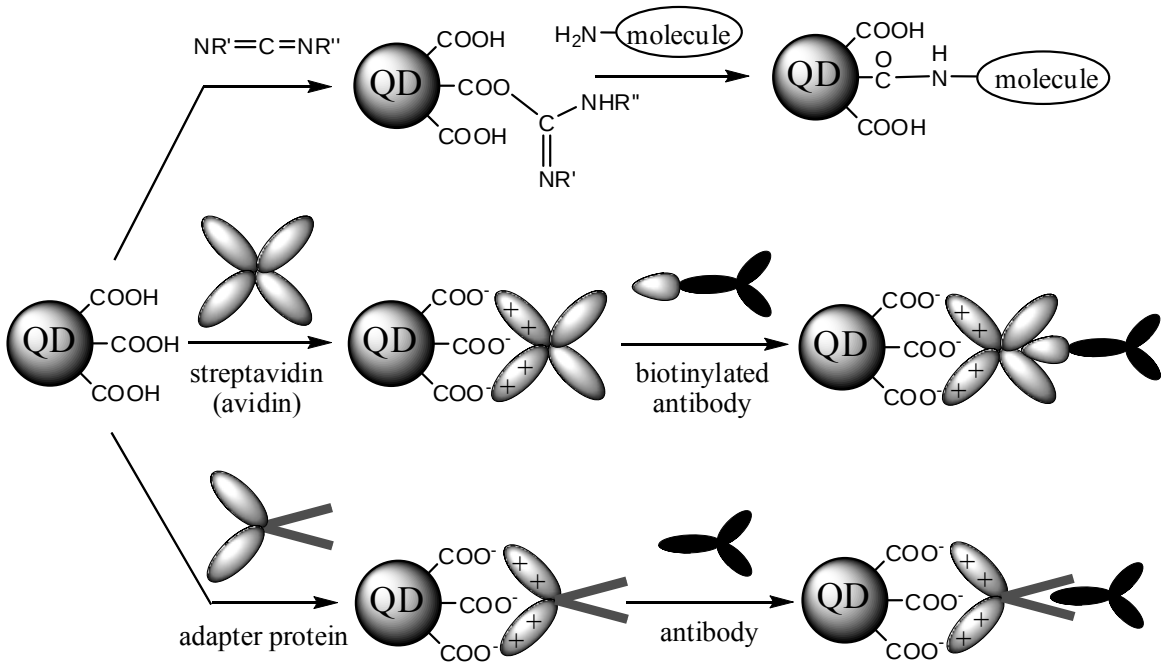

Fig. 4. Schematic view of some bioconjugation reactions involving carboxyl-functionalized QDs (see the text for details).

The avidin-biotin and streptavidin-biotin interaction is widely used for conjugation of QDs to various biomolecules and ligands [72]. Most of the QDs, currently used in immunofluorescent assays, are streptavidin-coated for interaction with biotinylated ligands and antibodies. The avidin-biotin and streptavidin-biotin interaction is among the strongest known non-covalent, specific interaction between protein and ligand. The bond formation between biotin and avidin is very rapid and, once formed, is unaffected by wide extremes of pH, temperature, organic solvents and other denaturating agents. The complex can withstand incubation in 2 M urea and is not significantly affected by pH values between 2 and 13. Streptavidin is a biotin-binding protein, which has a molecular weight of ~60,000 Da and consists of 4 subunits. Each subunit is capable of binding of one biotin molecule. The streptavidin protein has 32 lysine residues and can be conjugated to various molecules, as well as with QDs, using EDC-coupling. Biotin, a naturally occurring vitamin with a molecular weight of 244 Da, can be conjugated to proteins using hydrophobic or hydrophilic linkers with different lengths. Usually biotinylated antibodies are used as primary antibodies in a sandwich immunoassay for binding with the streptavidin-QD conjugates. Various applications of QDs-biomolecule conjugates via biotin-streptavidin interaction are discussed in the next chapters.

2.5.2. Bioconjugation of thiol-functionalized QDs

Thiol-functionalized QDs can be conjugated to proteins (as well as any other molecules containing amine groups), using 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide [81]. The scheme of conjugation reactions is shown in Fig. 5. First, the linker molecule reacts with the amine to form an amide derivative. Then, the obtained derivative reacts with a thiol group from the QD-surface to form the QD-conjugate.

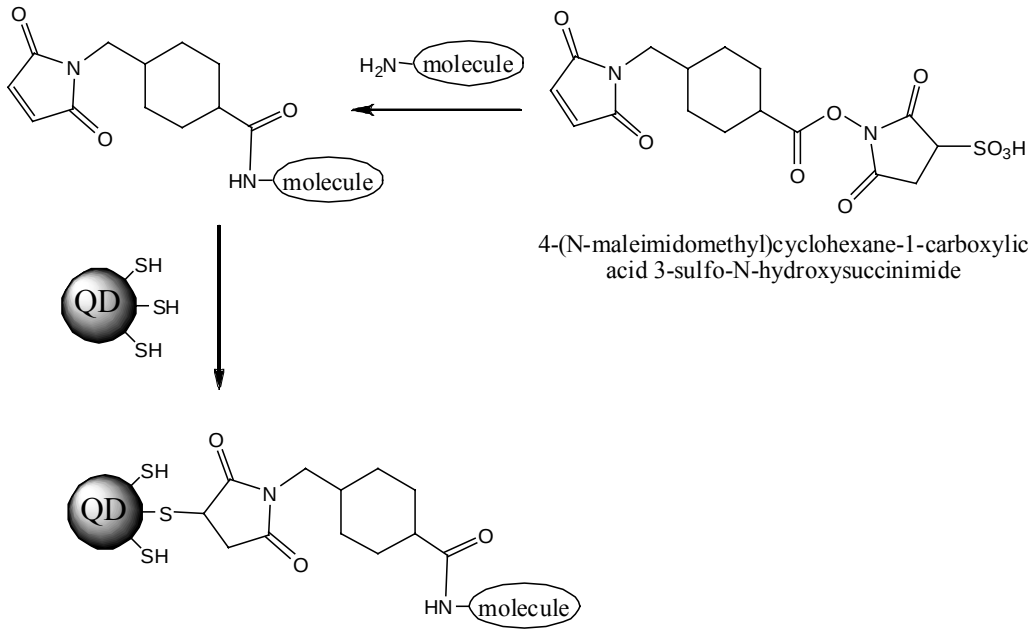

Fig. 5. Schematic representation of a bioconjugation reaction involving thiol-functionalized QDs.

2.5.3. Bioconjugation of amino-functionalized QDs

The bioconjugation reactions involving amino-functionalized QDs are summarized and represented schematically in Fig. 6. The reaction of amine-functionalized QDs with N-(β-maleimidopropyloxyl)succinimide ester is used for the conjugation of QDs to ligands, bearing a

thiol (–SH) group [77]. For example, the ligand Deltorphin-II targeting G-protein coupled receptor is successfully conjugated to amine-functionalized QDs in order to observe the distribution of human δ-opioid receptors expressed in living cells.

A reaction involving the EDC reagent is used also as a first stage in the conjugation of amino-functionalized QDs to oligonucleotides [86]. The 5'-phosphate group of the oligonucleotides is activated with EDC, followed by a reaction with imidazole to obtain a reactive phosphorimidazolide derivative. The obtained phosphorimidazolide derivative reacts with amino-functionalized QDs in order to produce the phosphoramide conjugate.

One of the first bioapplication of amino-functionalized QDs as fluorescent biolabels utilizes biotinylated nanocrystals [5]. Biotinylated QDs may be used as markers attached to different streptavidin (or avidin)-conjugated molecules.

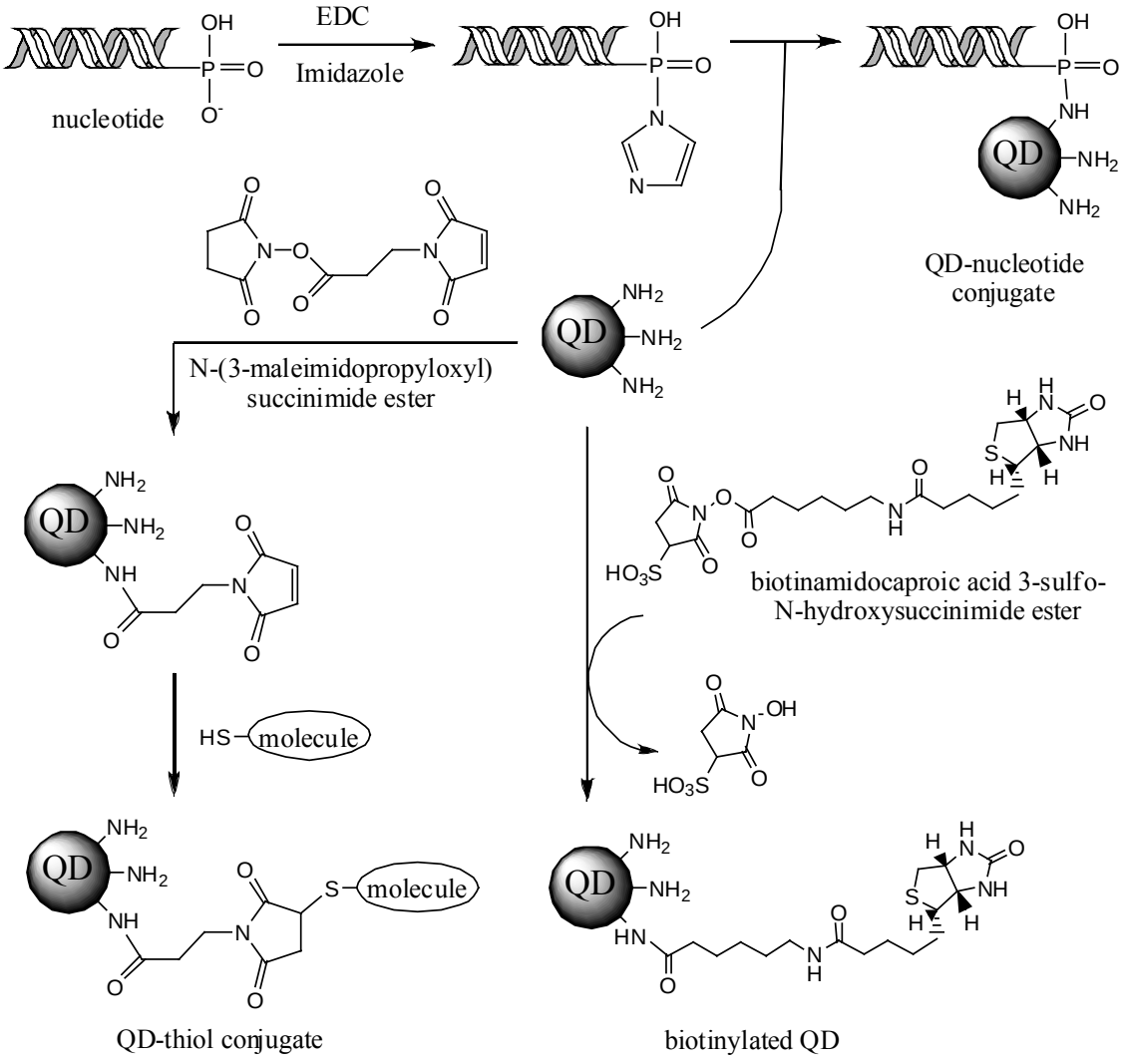

Fig. 6. Schematic view of some bioconjugation reactions involving amino-functionalized QDs (see the text for details).

3. CYTOTOXICITY OF QUANTUM DOTS

As the range of biomedical applications of QDs expands to *in vivo* measurements, questions concerning their short and long-term cytotoxicity are raised. All known studies are focused on the toxic effects of colloidal QDs dispersed in aqueous solution on cells. The extent of cytotoxicity has been found to depend upon a number of factors including size, capping

materials, color, dose of QDs, surface chemistry, coating bioactivity and processing parameters (for detailed review see ref. [87]). Recent reports have indicated that bare CdSe QDs are indeed toxic to cells [88,89,91-99].

A number of mechanisms have been postulated as responsible for QD cytotoxicity [91,100]. These include:

1. Release of free Cd(II) ions (QD core degradation) [88,95]. When appropriately coated, the CdSe-core QDs can be made less toxic and used to track cell migration and reorganization *in vitro*.
2. Free radical formation [95,101]. It is proposed that the mechanism for QD-induced cell death involves the generation of reactive oxygen species (ROS) in the extracellular environment and intracellularly. These ROS can cause plasma membrane damages and intracellular organelle damages.
3. DNA damage caused by CdSe QDs has been observed under exposure to UV-light, most probably due to the production of free radicals and reactive oxygen species [97].
4. QDs, such as CdTe, were found to induce apoptosis in human cells by affecting various biochemical pathways [93,96].
5. Cytotoxicity of QDs can be rendered significantly by due nature of the surface coating material [87, 89]. For example, compounds such as thioglycolic acid are found to be toxic to a number of cells. For that reason the QDs must be purified from any excess of a free surface coating ligand, which may have toxic effects on the living cells.

The reviewed literature suggests that the engineered QDs cannot be considered a uniform group of substances. QD absorption, distribution, metabolism, excretion, and toxicity depend on multiple factors derived from both inherent physicochemical properties and environmental conditions; QD size, charge, concentration, outer coating bioactivity (capping material and functional groups), and oxidative, photolytic, and mechanical stability have been implicated as determining factors in QD toxicity.

4. APPLICATIONS OF QUANTUM DOTS IN BIOIMAGING

4.1. Fluorescence resonance energy transfer (FRET) assays

The fluorescence resonance energy transfer (FRET) represents energy transfer from a donor to an acceptor, which takes place at small enough distance between them [20]. As a result, the donor's fluorescence intensity decreases and that of the acceptor increases. The first investigation, concerning the QD fluorescence quenching by FRET, involves attachment of a chromophore (QSY-7) labeled maltose-binding protein (MBP) and IgG to QDs capped with dihydrolipoic acid [102]. Later research demonstrated that the gold nanoparticles could act as fluorescence quenchers in QDs-based FRET sensors [103]. It must be pointed out that the QD-based FRET-probes are utilized also in gene technology for investigation of the telomerization dynamics and DNA replication [104].

Recently, advanced QD-based FRET systems for investigation of enzymatic activity are developed, providing easily controlled FRET efficiency, as well as data about the enzymatic velocity, Michaelis–Menten kinetic parameters, and the mechanisms of enzymatic inhibition [105,106]. The QDs-FRET-based enzymatic activity probes are used to determine the activity of collagenase in solution [107]. In this study, rhodamine-labeled peptide-coated CdSe/ZnS QDs are synthesized and used as FRET probes to monitor the proteolytic activity of extracellular matrix metalloproteinases (MMPs) in normal and cancerous cell cultures. Taking into account that the MMPs activity in breast cancer cultures is significantly higher compared to normal cells, it is possible to distinguish a normal and cancerous tissue in less than 15 min by using this FRET assay.

4.2. Cell tracking during embryogenesis

The cell tracking during embryogenesis is an extremely important topic in developmental biology, where the QDs have been used as fluorescent labels with superior photostability. In a pioneering study, highly fluorescent QDs have been encapsulated in phospholipid micelles and used to label individual blastomere cells in *Xenopus* embryos [28]. These encapsulated QDs are stable *in vivo*, do not aggregate and are able to label all cell types in the embryo. At the levels required for fluorescence visualization (2×10^9 QDs/cell), the QD-micelles are not toxic to the cells, but concentrations higher than 5×10^9 QDs/cell produce abnormalities. The QDs are confined to the injected cell and the respective daughter cells. Interesting translocation of the QDs to the cell nucleus is observed at a particular stage in the embryo development. Recent studies report the use of QDs on the *Xenopus* embryo development to image mesoderm migration *in vivo* with single cell resolution and provide *in vivo* quantitative data regarding the migration rates [108]. Fluorescent labelling of cells with QDs is also applied in studies of the Zebrafish embryo development [109].

4.3. Labeling of cell surface receptors

Ligand-conjugated QDs are first used to label cell surface receptors in 2002 [110]. It is demonstrated that serotonin-capped QDs interact with the serotonin transporter protein in transfected HeLa cells and oocytes *in vitro*. A serotonin-linker arm ligand is synthesized and used to modify the QDs. It is found that the serotonin-modified QDs inhibit the serotonin transport activity in transfected cells. More recently, new high-affinity ligands for serotonin transporter protein are created, which are then conjugated to QDs (functionalized with -COOH groups) through EDC-coupling [111,112].

QDs are used to track the individual glycine receptors and analyze their lateral dynamics in the neuronal membrane of living cells [113]. This receptor is the main inhibitory neurotransmitter receptor in the adult spinal cord. The issue of lateral mobility of the receptors for neurotransmitters has become central to understand the development and plasticity of synapses. The properties of QDs make it possible to record the mobility of individual molecules at the neuronal surface, even in confined cellular compartments.

A practical method is reported for generating water-soluble QDs and the necessary chemistry for covalently coupling them with ligands targeting G-protein coupled receptors (GPCRs) [77]. GPCRs constitute a large and diverse family of proteins, whose primary function is to transduce extracellular stimuli into intracellular signals. Since 50 % of the pharmaceuticals target GPCRs, new methods for the imaging of GPCRs at the cell surfaces are of interest. As a proof of principle, the QDs are chosen to target the human melanocortin and δ-opioid receptors. It is demonstrated that the QDs could be used for effective imaging of melanocortin receptors. It is also demonstrated that the QDs, conjugated to Deltrophin-II analogs, could be utilized for the selective imaging of δ-opioid receptors on the cell surfaces and for single molecule imaging.

Recent reports describe the development of QD-based probe for fluorescent detection of apoptosis [114]. The QDs are conjugated to Annexin V for specific targeting of apoptotic cells. For that purpose, streptavidin-conjugated QDs are coupled to biotinylated Annexin V, a protein that specifically recognizes and binds to phosphatidylserine moieties present on the outer membrane of apoptotic cells and not on healthy or necrotic cells. This makes the QDs excellent candidates to continuously follow the fast changes occurring at the membrane of apoptotic cells and facilitates the time-lapse imaging as they alleviate any bleaching issue. The investigation of molecular events that take place during apoptosis is extremely important for understanding the programmed cell death, which is usually disrupted in cancer cells and represents a major problem.

4.4. Immunofluorescent bioimaging

The application of QDs in immunofluorescent detection is demonstrated with one of the two first bioimaging applications of QDs in 1998 by conjugating TGA-capped QDs with IgG by EDC-coupling [6]. In this study, antibody-induced agglutination of QDs, conjugated to human IgG, is clearly observed for the first time. This "proof of principle" paved the way for further research and improvements of the QDs applications in immunofluorescent labeling [29,72,115-119]. The reactions for conjugation of QDs to antibodies are discussed in section 2.5. Streptavidin-coated QDs are found as the most suitable ones, because they can be easily conjugated to different commercially available biotinylated antibodies. The QD-antibody conjugate can be used for immunofluorescent bioimaging by two general approaches. In the first approach, a streptavidin-coated QD is conjugated to a biotinylated primary antibody, which recognizes directly the targeted antigen. The second approach for a QD-mediated labeling of antigens involves secondary and primary antibodies. The primary antibody targets the antigen, and is then recognized by a biotinylated secondary antibody, which binds a streptavidin-coated QD (Fig. 7).

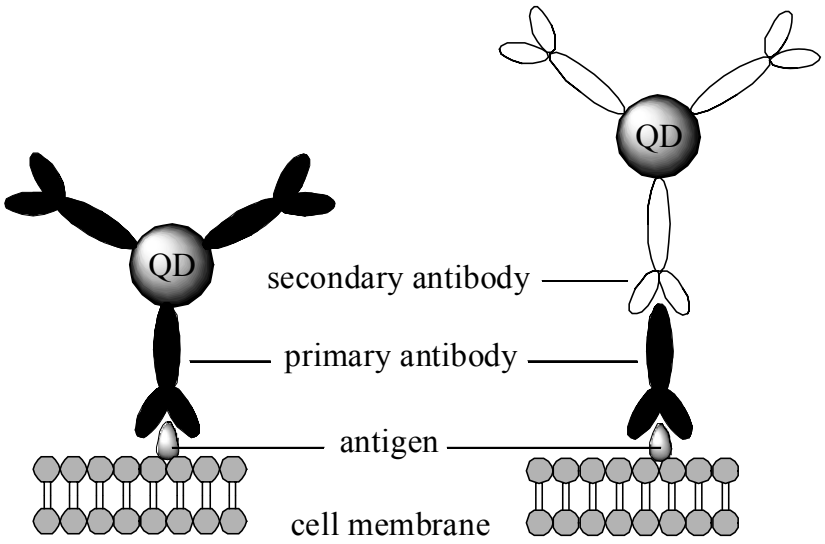

Fig. 7. Schematic representation of the application of QDs for immunofluorescent bioimaging using biotinylated primary antibody (left) or a combination of a primary antibody and a biotinylated secondary antibody (right).

4.5. *In vivo* imaging

Fluorescent QDs are used for whole body imaging, however relatively little work is done due the potential for toxicity of QDs [10,12,76,120-122]. It is worth noting that successful labeling of lymph nodes in pigs is achieved by using NIR-emitting QD-probes [123]. This strategy may be useful for successful surgical resection of lymph nodes containing metastatic cancer cells. A number of complications exist with the QD imaging in animals due to the absorbance and scatter of light by the tissues, as well as observation of autofluorescence upon their excitation. This may be partially overcome by using QDs that emit in the near infrared region (700–1000 nm) [108,120,122]. Another barrier to use *in vivo* QDs is the extensive reticuloendothelial uptake of QDs introduced into the bloodstream [121].

Multifunctional nanoparticle QD-based probes for cancer imaging in living animals are also developed [76]. The structural design involves encapsulation of luminescent QDs with an ABC triblock copolymer and linking of this amphiphilic polymer to tumor-targeting ligands. For

active tumor targeting, antibody-conjugated QDs are used to target a prostate-specific membrane antigen (PSMA). Immunocytochemical studies of QD-PSMA Ab binding activity in cultured prostate cancer cells confirmed PSMA as a cell surface–specific marker for some prostate cancer cell lines, like C4-2 cells. *In vivo* targeting studies of the human prostate cancer growing in nude mice indicate that the QD-probes accumulate at the tumors both by the enhanced permeability and retention of tumor sites and by antibody binding to cancer-specific cell surface biomarkers. Using both subcutaneous injection of QD-tagged cancer cells and systemic injection of multifunctional QD-probes, the authors have achieved sensitive and multicolor fluorescence imaging of cancer cells under *in vivo* conditions. These results raise new possibilities for ultrasensitive and multiplexed imaging of molecular targets *in vivo*.

The tumour vasculature plays an important role in determining the tumour pathophysiology and drug delivery. Angiogenesis, the formation of new blood vessels from preexisting vasculature, is essential for the tumor growth and progression. Integrin αVβ3, which binds to arginine-glycine-aspartic acid (RGD)-containing components of the interstitial matrix, plays a key role in the tumor angiogenesis and metastasis. It is significantly upregulated in invasive cancer cells but not in normal tissues. The *in vivo* targeting and imaging of tumor vasculature using RGD peptide-labeled QDs is reported [122]. Athymic nude mice, bearing subcutaneous U87MG human glioblastoma tumors, are administered QD-RGD intravenously. The tumor fluorescence intensity reaches a maximum at 6 h post injection with a good contrast. The reported results open up new perspectives for integrin-targeted near-infrared optical imaging and may aid in cancer detection and management including imaging-guided surgery.

The *in vivo* applications of QDs are limited from several factors. First, the QDs, including their capping materials, may be immunogenic, which may result in acute immune response as well as massive uptake of QDs by the reticuloendothelial system. Second, the QD core material, as well as the capping organics, may be toxic to the organism or individual cells. Third, the size of QD complexes precludes renal excretion, making clearance from the blood stream unlikely. This may increase the QDs uptake by the liver, which may result in a dangerous hepatic toxicity. Taking into account these serious barriers against the *in vivo* applications of QDs, it is clear that the QD-based fluorescent probes may be more useful for *in vitro* assays.

4.6. Applications of quantum dots in cancer cell research

Attempts for fluorescent *in vivo* imaging of cancer cells are reported [76,90,124]; some of them are described in the previous section. Also, there are many reports concerning the *in vitro* fluorescent labelling of cancer cells by using antibodies and other targeting ligands [125-128]. Here, we shortly describe representative examples of these latter cases.

The epidermal growth factor receptor (EGFR) can be a molecular marker for cancerous cells. It is expressed ubiquitously in cells and is overexpressed in human malignancies including breast cancer, glioma and lung cancer, making it a promising biomarker. A successful direct targeting of EGFR with QDs, conjugated to anti-EGFR antibodies, is carried out and compared to appropriate controls [125]. Also, QD-lectin conjugates are synthesized and applied for identification of leukemia cells from normal lymphocytes using fluorescent confocal microscopy and flow cytometry [126]. The results are compared with commercially available FITC-lectin. Lectins are found to possess a high affinity to several leukemia cell lines, without or with low affinity to normal lymphocytes. The results clearly demonstrate that the QD-lectin conjugates are appropriate fluorescent markers for identification of several leukemia cell lines. It is found that the QD-lectins give higher quality images and possess higher stability against photobleaching in comparison with the commercially available FITC-labeled lectin.

4.7. Fluorescent labeling of colloidal drug carriers

Nanosized drug carrier systems like liposomes, polymer nanoparticles, and solid-lipid nanoparticles have been optimistically considered as “magic bullets”, not only because a wide range of biologically active substances can be encapsulated and targeted to the desired site of action, but also because they can be injected into human or animals without adverse effects [129]. The fluorescent labeling of these nanosized carriers offers a possibility to gain an insight into the mechanisms of their interaction with the biological cells and tissues by visual tracking of the labeled nanoparticles [130,131]. The QDs can serve as highly effective fluorescent markers for such a purpose. For example, a relatively simple approach is reported concerning the efficient encapsulation of CdSe QDs in liposomes, which are stable in biocompatible water buffer [132]. The stable liposome-encapsulated QDs could be used as bright fluorescent labels in biological applications, involving the conjugation of biomolecules such as enzymes, antibodies, and DNA molecules to the liposomes.

Another report considers the preparation of polylactide (PLA) particles, loaded with fluorescent QDs [133,134]. In this study, CdSe QDs are encapsulated in PLA particles and studied *in vitro* and *in vivo*. The PLA-coated QDs are water-dispersible and highly fluorescent - the fluorescence is stable in aqueous solution for more than 30 days. The results obtained in the course of this investigation clearly demonstrate that the PLA-encapsulated CdSe QDs have a high potential for biological labeling and diagnostics.

Poly(alkylcyanoacrylate) (PACA) nanoparticles are considered as ones of the most promising polymer carriers for drug delivery [135]. The poly(alkylcyanoacrylates) are biocompatible, biodegradable and relatively non-toxic. These advantages make them appropriate materials for biocompatibilization of QDs. Highly fluorescent CdSe/CdS core-shell QDs are embedded into poly(butylcyanoacrylate) nanoparticles in order to prepare novel fluorescent nanocomposite particles for bioimaging applications [136]. The investigations with fluorescent microscopy allow the successful visual tracking of the QD-labeled polymer particles in the course of their interaction with biological cells.

A recent report considers the development of a new strategy to prepare folate-decorated nanoparticles of biodegradable polymers for QDs encapsulation, targeted and sustained imaging of cancer cells [137]. Copolymers of poly(lactide)-vitamin E are synthesized, which are then blended at various weight ratio to make QDs-loaded nanoparticles, which are further decorated with folate for targeted and sustained imaging. This study shows that the QDs formulated in folate-decorated nanoparticles are feasible for targeted imaging. These QDs improve imaging specificity and sensitivity with reduced side effects of QDs to normal cells.

Taking into account the above-mentioned examples, one can conclude that the fluorescent labeling of colloidal drug carrier systems with QDs could open a possibility to investigate the mechanisms of carrier-cell interactions, penetration and localization of the colloidal particles in various biological cells.

5. CONCLUSIONS

The applications of quantum dots as a novel generation of fluorescent markers for bioimaging may provide important information about the mechanisms of interactions between the biological cells and nanosized materials. They can be especially important in the cases, where a long-term imaging and a high photostability of the fluorophore are required. The quantum dots may be useful also for imaging in the near infrared region, as well for visual tracking of single molecules and investigation of complicated biological processes, such as embryonic development. However, one should take into account the possible toxic effects of quantum dots or their coating material on the living cells and organisms. The recent advancements in development of non-toxic

and biocompatible quantum dots reveal a great possibility for extension of the exciting biological applications of these nanoparticles. It is expected that the quantum dots will increase our knowledge about the molecular mechanisms of many biological processes and will be important tools in fluorescent diagnosis of cancer and other severe diseases.

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7. REFERENCES

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