Chapter 10

QUANTUM DOTS: BIOLOGICAL AND BIOMEDICAL APPLICATIONS

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ABSTRACT

Current issue solved in the area of quantum dots (QDs) synthesis and application, is to find highly luminescent semiconductor nanocrystals, which are easy to prepare, biocompatible, stable and soluble in aqueous solutions. These are made up of 100–100 000 number of atoms typically range in size from 1 to 10 nm in diameter. The most popular types of QDs include CdTe, CdSe, ZnSe, ZnS, however also other semiconductor metals such as In, Ga, and many others can be used.

QDs play an important role mainly in the imaging and as fluorescent probes for biological sensing (DNA, proteins, peptides, and drugs). QDs designed for biological applications are mainly applied in colloidal form. To date, two original approaches have been reported for the preparation of colloidal QDs: synthesis of hydrophobic QDs and aqueous synthesis routes. Hydrophobic QDs are insoluble in aqueous solution, they cannot be directly employed in bioapplications and require further surface modification to achieve water solubility, biocompatibility and stability. On the other hand, the aqueous synthesis route produces QDs with excellent water solubility, biological compatibility, and stability. One of the most widespread approaches to creating water-soluble QDs is ligand exchange with thioalkyl acids such as mercaptoacetic, mercaptopropionic and

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mercaptoundecanoic acid or reduced glutathione. These QDs exhibit lower quantum yields than the above mentioned ones (up to 30%) without any following treatment.

Besides the applications as simple sensors, the main function of the QDs based on their exceptional fluorescent properties in the biochemical and biomedical research area is their use as unique fluorescent labels. Various specific labeling strategies are known and most of these approaches are based on bioconjugation with other biomolecule exhibiting some specific affinity to the target compound. One of these strategies utilizes the biotin-avidin (respectively streptavidin and neutravidin) interaction known for its very high specificity. Modification of QDs by the streptavidin proved to be a very successful method evaluated in numerous publications and due to this success, streptavidin-QDs are nowadays also commercially available. Also biotin-functionalized QDs were developed to exploit the same interaction. Moreover, so called multicolor QDs, which means particles modified by several different molecules, are nowadays of a great interest. Finally, QDs may be employed for labeling of nucleic acids and subsequently used in microarray technology.

INTRODUCTION

Typically, QDs are represented by nanocrystallites or atomic clusters consisted of few hundreds to few millions of atoms, but only a small number of electrons (≤ 100) are free [1]. QDs can be based on metallic (e.g. Ni, Co, Pt, Au) [2] or mostly on broad scale of semiconductor materials, particularly from periodic group II-VI (CdTe, CdSe, CdS, ZnSe, ZnS, PbS, PbSe, PbTe, SnTe). Moreover other semiconductor elements from III-V group such as In, Ga, and many others can be used for QDs fabrication (e.g. InP) [3].

Because of their reduced size, QDs behave differently from bulk solids due to the quantum-confinement effects that are responsible for their remarkably attractive properties intermediate between compounds and single molecules, namely intensive photoluminescence. The QDs fluorescent properties arise from the fact, that their excitation states/band gaps are spatially confined. The quantum confinement effects occur, when the nanoparticle radius a is lower than one of this magnitudes: a_e, a_h and a_{exc} (Bohr radius of electron, hole and exciton, respectively). In other words, the physical size of the band gap determines the photon's emission wavelength, which can vary from UV to NIR wavelengths (400–1350 nm). For example, larger QDs having smaller band gaps emit red light, while smaller QDs emit blue light of higher energy [4]. These effects depend not only on the core size, but also on the chemical composition of the core. Therefore there is not a clear line to say that a nanoparticle is a QD or not if one regards only its size (e.g. Bohr radius for InAs is 36 nm, while for CuCl only 0.7 nm).

QDs play an important role mainly in the imaging and as highly fluorescent probes for biological sensing that have better sensitivity, longer stability, good biocompatibility, and minimum invasiveness. The long lifetime in the order of 10–40 ns increases the probability of absorption at shorter wavelengths and produces a broad absorption spectrum [5]. Most interest in QDs is focused on the core-shell structure rather than on the core structure [6]. Regardless of a core/shell structures, it is the size of the core nanocrystal which determines the emission wavelength of the QDs. For example, CdS QDs is used to generate ultraviolet and blue emission, CdSe is used to span most of the visible spectrum, CdTe is well suited for

the red and near infrared region and PbS and PbSe have been used to create cores that emit in the near infrared. The shell greatly improves the QY and stability of the core nanocrystals.

Majority of sensing techniques employing QDs in biological systems are applied in solution (colloidal form). Up to present days, the most frequently used approaches have been reported on the preparation of colloidal QDs: synthesis of hydrophobic QDs with subsequent solubilization step, direct aqueous synthesis or two-phase synthesis. Compared to hydrophobic or two-phase approaches, aqueous synthesis is reagent-effective, less toxic and more reproducible. Furthermore, the products often show improved water-stability and biological compatibility.

The current issue solved in the area of QDs synthesis is to find highly luminescent semiconducting nanocrystals, which are easy to prepare, biocompatible, stable and soluble in aqueous solutions. Thus, the semiconductor core material must be protected from degradation and oxidation to optimize QDs performance. The other problematic issue is high reactivity of QDs cores, which suffer from very strong unspecific interactions with macromolecules leading to the particle aggregation and fluorescence variation. Shell growth and surface modification enhance the stability and increase photoluminescence of the core. And the last, but probably most important is the QDs toxicity (mainly because of their toxic heavy metal composition), which can be greatly reduce by surface modification as well. The protecting layer should also hinder the creation of reactive oxygen species (ROS) such as free radicals (hydroxyl radical: $\cdot OH$ and superoxide: $O_2\cdot-$) and singlet oxygen ($^1O_2$), which are known to cause irreversible damage to nucleic acids, enzymes, and cellular components such as mitochondria and both plasma and nuclear membranes [3].

As results from above mentioned, the key step in QDs preparation ensuring the achievement of above mentioned required properties is QDs functionalization. Coating of inorganic surface of QDs should provide two functions, a chemical and physical stabilization of the QDs as well as the ability to modify them for a wide range of applications by attaching certain surface groups [7]. Most of these approaches are based on bioconjugation with some biomolecule [8]. Many biocompatible molecules can be used for this purpose, especially those compounds possessing the surface amino and carboxyl functional groups. Glutathione (GSH), member of thiol compounds family, as the only one carries both these functional groups. GSH coated QDS can be further modified, for example with biotin giving biotinylated-GSH QDs which can be employed in specific labeling strategies [9]. Namely, these biotin functionalized GSH coated QDs has high specific affinity to avidin (respectively streptavidin and neutravidin) [10].

1. SYNTHESIS OF HYDROPHOBIC QDS

The synthesis of the most frequently used semiconducting colloidal QDs, consisted of metal chalcogenides (sulphides, selenides and tellurides), is based either on usage of organometallic precursors (e.g. dimethylcadmium [11], diethylzinc [12]), metallic oxide (e.g. CdO [13]) or metallic salts of inorganic and organic acids (e.g. stearate [14,15], acetate [16,17], nitrate [18]). The sources of chalcogenide anion are usually pure chalcogen elements (e.g. S, Se, Te). Whatever precursor is used, the resulted QDs are hydrophobic, but their quantum yields (QY) are higher (in the range of 20–60 %) compared to QDs prepared by

aqueous synthesis route (below 30 %). However, the trend is to avoid the usage of organometallic precursors, because they are less environmentally benign compared to other ones, which are more preferable [19].

The most common approach to synthesis of colloidal hydrophobic QDs is the controlled nucleation and growth of particles in a solution of organometallic/chalcogen precursors containing the metal and the anion sources. The method lies in rapid injection of a solution of chemical reagents into a hot and vigorously stirred coordinating organic solvent (typically trioctylphosphine oxide (TOPO), trioctylphosphine (TOP) or hexadecylamine (HDA) [20]) that can coordinate with the surface of the precipitated QDs particles [21]. Consequently, a large number of nucleation centers are initially formed at about 300 °C. The coordinating ligands in the hot solvents prevent or limit subsequent crystal growth (aggregation) via Ostwald ripening process (small crystals, which are more soluble than large ones, dissolve and reprecipitate onto larger particles), which typically occurs at temperatures in the range of 250–300 °C [22]. Further improvement of the resulting size distribution of the QDs particles can be achieved through selective preparation [23].

Because these QDs are insoluble in aqueous solution and soluble in nonpolar solvents only, further functionalization with various organic molecules possessing thiol, carboxy or amino groups is required to achieve their solubilization. However, this inconveniency is compensated with higher QY of these QDs as mentioned previously.

1.1. Solubilization of Hydrophobic QDs

Solubilization of QDs is inevitable for many biological and biomedical applications. The process of transformation from hydrophobic to hydrophilic QDs is difficult and demanding multiple steps. It requires sophisticated surface chemistry alteration. Current solubilization strategies without affecting key properties are mostly based on exchange of the original hydrophobic surfactant layer (TOP/TOPO) capping the QDs with hydrophilic one or the addition of a second layer [24]. However, in most cases, the surface exchange results in not only broadening of the size distribution but also reductions in QY from 80 % in the organic phase to about 40 % in aqueous solution [25].

The first technique involves ligand exchange (sometimes called cap exchange). The native hydrophobic ligands are replaced by water-soluble bifunctional molecules in which one end connects to QDs surface and the other end is hydrophilic and may also be reactive to biomolecules. This may be achieved using surface anchoring thiol-containing molecules (e.g. sodium thioglycolate, glutathione [6], etc.) or more sophisticated ones (e.g. based on carboxylic or amino groups such as oligomeric phosphines, dendrons and peptides) to bind to the QDs surface and hydrophilic end groups (e.g. hydroxyl and carboxyl). Nevertheless, this approach can negatively alter the chemical and physical states of the QDs surface and cause a dramatic decrease in the quantum efficiency. For example, Jin et al. modified the surface of hydrophobic CdSeTe/CdS QDs with GSH in tetrahydrofuran-water solution and these QDs exhibited the QY only of 22 % [26]. Moreover, thiol-based molecules (e.g. mercaptopropionic acids [27]) may form disulphides over time and come off from the QDs surface and finally QDs aggregate and precipitate out of water. And next, most of water-soluble bifunctional molecules are expensive and instable.

The second strategy employs polymerized silica shells with polar groups using a silica precursor (functional organosilane molecules containing –NH2 or –SH, e.g. 3-aminopropyltriethoxysilane [3]) during the polycondensation to insulate the hydrophobic QDs. Silica coating enhances the mechanical stability of colloidal QDs and protects them against oxidation and agglomeration. The other advantage of silica encapsulation is QDs chemical stability over a much broader pH range compared to carboxy-terminated ligands, which limit the QDs dispersion to basic pH. The procedures creating a controllable silica layer (coating) around hydrophobic QDs core are relatively complicated regarding to the other strategies. The simple way to encapsulate QDs with silica is reverse microemulsion method, which is described in details below.
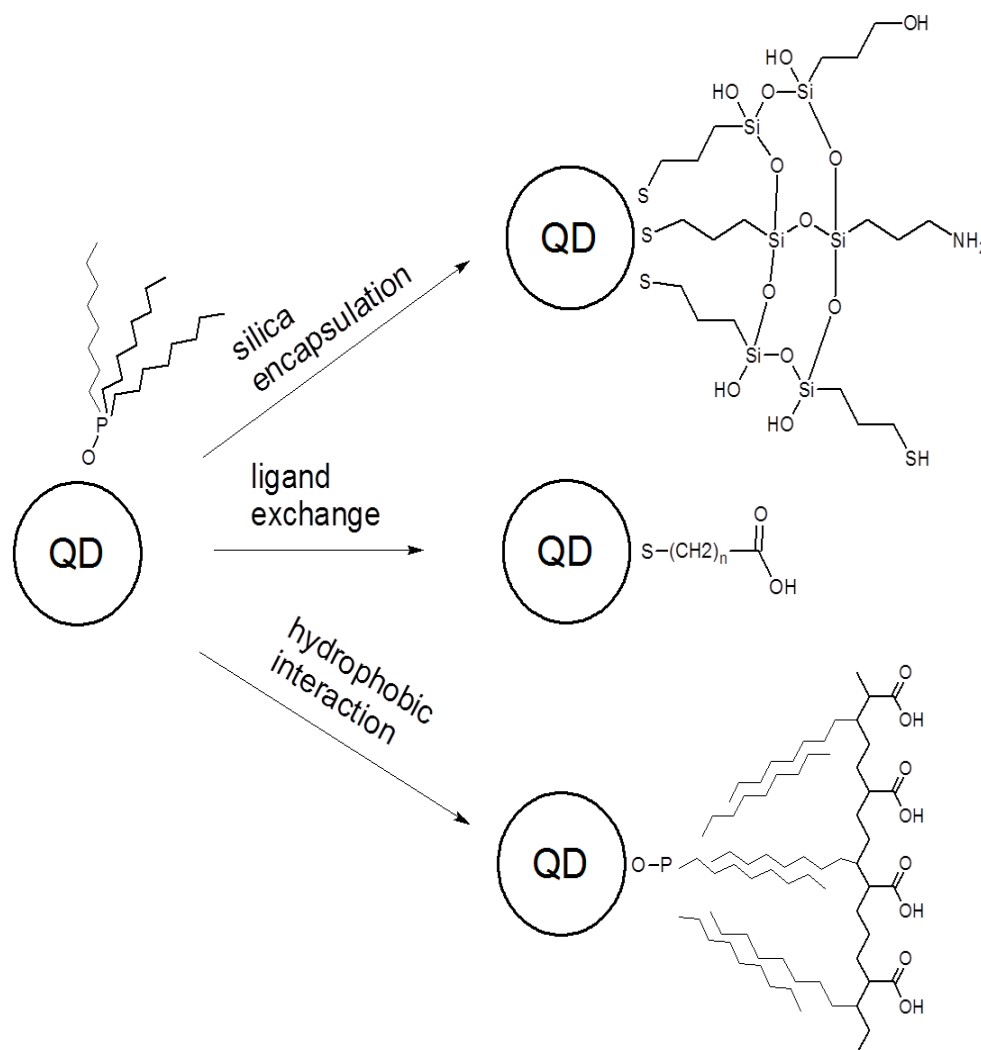

Figure 1. Schematic representation of different QDs solubilization routes including silica encapsulation, ligand exchange using water-soluble bifunctional molecules or polymer phospholipid encapsulation through hydrophobic interaction.

The third method maintains the native ligands on the QDs and uses various amphiphilic diblock and triblock copolymers and phospholipids to tightly interleave the alkylphosphine ligands through hydrophobic interactions [28,29]. Aside from rendering water solubility, these surface ligands play a critical role in insulating, passivating and protecting the QD surface from deterioration in biological media [8]. Phospholipids can encapsulate QDs by forming oil-in-water micelles through interaction between their hydrophobic ends and the surface ligands of QDs and provide water-solubility via hydrophilic exterior ends. A more promising approach is to use long chain-length amphiphilic polymers to form micelle-like structures and hence to transfer the hydrophobic QDs into water. For example, Tortiglione et al. transferred hydrophobic TOP/TOPO-capped CdSe/ZnS QDs into aqueous solution by wrapping them up in an amphiphilic polymer shell (diamino-PEG 897) [30]. Das and colleagues employed block copolymer spheres for encapsulation of CdS QDs in an aqueous emulsion polymerization process. First, stable dispersions of CdS QDs in water were prepared using a polymer dispersant, either poly(acrylic acid) or a random copolymer having an average of ten acrylic acid and five butyl acrylate units. These polymer dispersants were prepared by reversible addition-fragmentation chain transfer polymerization. Then, the CdS QDs dispersed in water were encapsulated in a polystyrene shell using an emulsion polymerization process [31]. This strategy is generally better than the ligand exchange because there is no direct interaction with the QDs surface atoms, which can preserve the original quantum efficiency to a highest extent. Moreover, the polymer's large number of hydrophobic side chains strengthens the hydrophobic interaction to form more steady structures and consequently more stable water-soluble QDs. And finally, these amphiphilic polymers are commercially available and cheap that make them better materials than other molecules such as peptides and phospholipids in large-scale preparation.

2. AQUEOUS SYNTHESIS OF QDS

The second and more utilized way is the aqueous synthesis producing directly water soluble QDs with excellent biological compatibility and stability (usually more than two months). Compared with organic phase synthesis, QDs synthesized in aqueous way exhibit good reproducibility, low toxicity, and are less expensive. Basically, the fabrication process of water-soluble QDs takes place in reflux condenser (usually in a three-necked flask equipped with this reflux condenser, see Figure 2).

This synthesis route usually consists in reaction of heavy metal (Zn, Cd, etc.) precursor with chalcogen precursors. Ordinary used precursors of heavy metals easily dissolving in water are acetates, nitrates or chlorides. The chalcogen precursors can be either commercial solid powders (e.g. Na_2TeO_3 in the case of CdTe QDs) or freshly prepared before using in reaction procedure, e.g. H_2Te (preparation by adding sulphuric acid dropwise to the aluminium telluride (Al_2Te_3) [32]) or NaHTe (forming by reaction of sodium borohydride ($NaBH_4$) with Te powder [33,34]) in the case of CdTe QDs. However, NaHTe and H_2Te are unstable compounds under ambient conditions; therefore the synthesis of CdTe QDs generally has to be performed in inert reaction systems. Since Na_2TeO_3 is air-stable, all of operations can performed in air, avoiding the need for an inert atmosphere. The synthetic pathway is thus free of complicated vacuum manipulations and environmentally friendly.
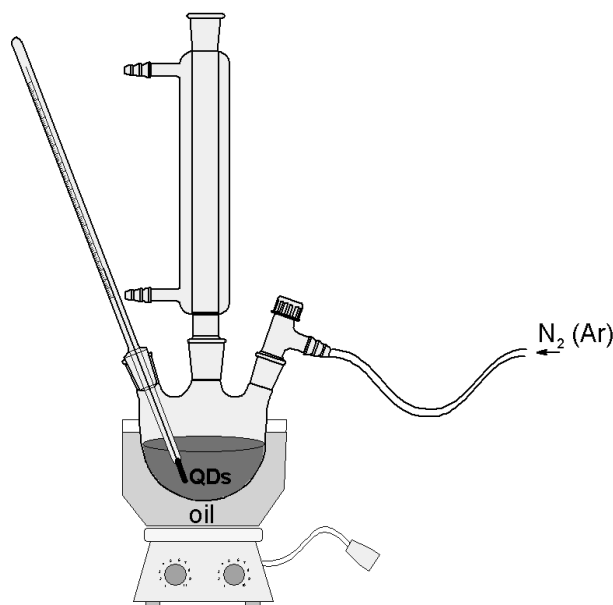
Figure 2. Schema of apparatus for water soluble QDs preparation in reflux condenser.

Nevertheless the procedure in water phase needs very long reaction time ranging from several hours to several days. Recently, new strategies employing microwave-assisted (MW) synthesis, which seems to be faster compared to the reflux one, were published as well (see below). The other disadvantages of QDs synthesized through aqueous route are the wider FWHM (the full width at half maximum) and lower QY which can be attributed to defects and traps on the surface of nanocrystals [35]. These defects can be eliminated by the selection of capping agents. The process of functionalization involves ligand exchange with thioalkyl acids such as thioglycolic acid (TGA) [36], mercaptoacetic acid (MAA) [37], mercaptopropionic acid (MPA) [38,39], mercaptoundecanoic acid (MUA) [40], mercaptosuccinic acid (MSA) [41] or reduced GSH [42,43]. From these ligands, GSH seems to be very perspective molecule, since it provides an additional functionality to the QDs due to its key function in detoxification of heavy metals (cadmium, lead) in organism [44]. Thus, GSH QDs as biological probe should be more biocompatible than other thiol-capping ligands.

2.1. Microwave Irradiation Synthesis

Reflux methods for QDs synthesis require long reaction times and often result in a large number of surface defects on synthesized QDs with low photoluminescence QY, therefore some other more sophisticated approaches were investigated. Microwave synthesis was found to be very effective since it provides high-quality QDs in one-pot and in shorter time [45,46]. The process is based on rapid homogeneous heating realized through the penetration of microwaves. Compared to conventional thermal treatment, this way of heating allows the elimination of defects on QDs surface and produces uniform products with higher QY [47]. The sizes of QDs can be easily tuned by varying the heating times. The QDs growth stops when the MW irradiation system is off and product is cooled down.

From chemical point of view, the most frequent types of QDs synthesized using microwave irradiation are CdTe, CdSe, CdS, $Zn_{1-x}Cd_xSe$ and ZnSe. As usual, these QDs can be functionalized with various thiol ligands such as MPA, MSA [48], TGA, 1-butanethiol, 2-mercaptoethanol [49] or GSH [50]. However, thiol ligands can be also used as sulphur source in one-step MW synthesis of QDs. Qian et al. reported on a seed-mediated and rapid synthesis of CdSe/CdS QDs using MPA, which was decomposed during MW irradiation releasing S^{2-} anions at temperature of 100 °C [51]. In this step, only CdSe monomers were nucleated and grown by the reaction of NaHSe and cadmium chloride. The initial core was rich in Se due to the faster reaction of Se with Cd^{2+} compared to S. The amount of released S^{2-} anions increased, when the temperature rose to 140 °C which resulted in formation of alloyed CdSeS shell on the surface of CdSe nanocrystals. As prepared QDs showed the QY up to 25 %.

2.2. Microemulsion Synthesis

The microemulsion synthesis belongs to other non-refluxed method for fabrication of water-soluble QDs, which is simple, inexpensive and highly reproducible method enabling excellent control of QDs size and shape [52]. This control of particle size is achieved simply by varying water-to-surfactant molar ratio. Nevertheless, the microemulsion synthesis gives relatively low yield of product; even large amounts of surfactant and organic solvent are used compared to bulk aqueous precipitation. The key point of this procedure is extraction of the nanoparticles from microemulsion into aqueous phase and to maintain their structural and surface features. In order to reach feasible yields of nanoparticles, the higher concentration of precursors in microemulsion should be used, which leads to much larger particle density inside the reverse micelles.
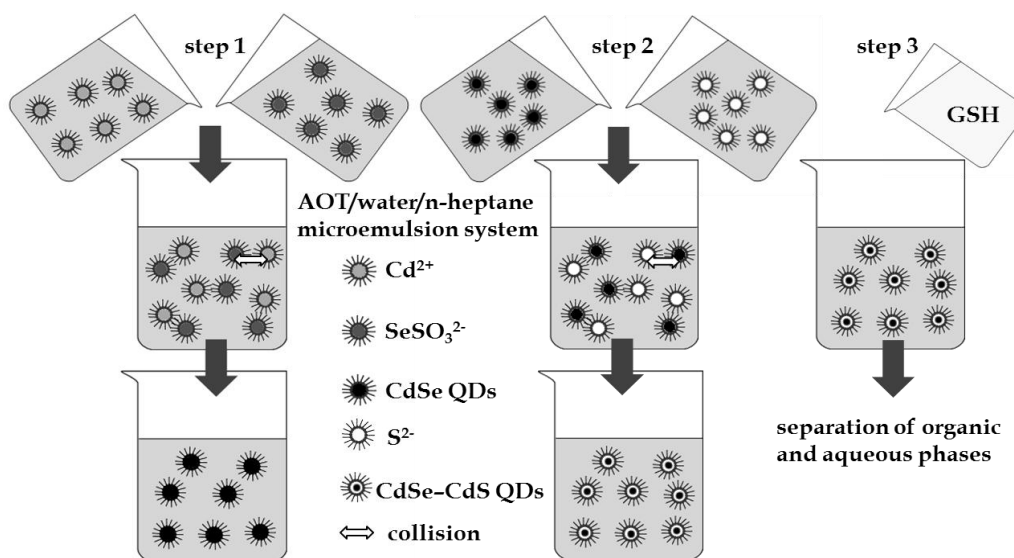

Figure 3. Schematic representation of QDs synthesis in microemulsion approach: step 1 involves the preparation of particular microemulsion containing only core QDs, which serves as starting material in step 2, where microemulsion of core-shell nanoparticles is produced and finally, these core-shell QDs are stabilized with GSH in step 3.

In this synthesis way, various thiol ligands are employed for QDs coating, for example mercaptoacetic acid, mercaptopropionic acid and GSH [42]. Briefly, the typical microemulsion synthesis of CdSe QDs can be described as follows: Se powder is added to Na_2SO_3 solution under continuous nitrogen bubbling at higher temperature forming Na_2SeSO_3 (sodium selenosulfate). Subsequently, this precursor was mixed with reverse micelle system prepared by dissolving AOT (sodium bis (2-ethylhexyl) sulfosuccinate) in n-heptane. Similar microemulsion was prepared with $Cd(NO_3)_2$. Finally, these two microemulsions were vortex-mixed which leaded to formation of CdSe QDs inside the reverse micelles. In the second step, a shell of CdS was created by addition of $(NH_4)_2S$ microemulsion under vortex-stirring. The last step consisted in core-shell QDs stabilization using thiol ligands aqueous solution, which is added to solution of QDs. The process is accompanied with color change of organic phase (initially orange–red) to translucent. This color change indicated the complete transfer of thiol-capped QDs into the aqueous phase (Figure 3.).

The CTAB is another molecule which can be used to cap QDs forming a micelle. This surfactant is known to create spherical micelle in the aqueous medium, where the nucleation and growth of the QDs takes place in the cavity of the CTAB micelle [53].

3. CHARACTERIZATION OF QDS

The most important characterization of QDs dealing with their optical properties is usually provided by UV-VIS and photoluminescence spectroscopy, which offer fast, non-destructive and contactless option. Besides monitoring the excitation, absorption and emission spectra, which are usually applied for calculation of quantum yield, band gap studies can be also carried out by optical diffuse reflectance spectra measurement. Size of QDs can be calculated from absorption edges using Henglein empirical curve, which relates the wavelength of the absorption threshold to the diameter of QDs [31]. In particular cases, the Fourier transformed infrared (FTIR) spectroscopic measurements are also necessary [54]. Raman spectroscopy, as one of the best non-destructive techniques, can be employed for QDs characterization as well, since it allows to probe the active optical phonon modes and to explore the confined electronic structure of QDs [53,55].

As mentioned before, the optical properties (fluorescence emission) of QDs can be fine-tuned by the QDs size, which is a key parameter that determines the spectral position and purity of photoluminescence. QDs size and morphology (shape and structure) are generally calculated using conventional techniques like scanning electron microscopy (SEM), transmission electron microscopy (TEM), and dynamic light scattering (DLS) studies. For these measurements, QDs are usually transformed to powder form either by simple drying of QDs solution or by precipitation (for example with ethanol), centrifugation and final drying. Besides these techniques, field flow fractionation, which belongs to high resolution liquid chromatography-like elution methods for separating and sizing, can be also successfully employed for QDs size distribution analysis [56].

The structure of QDs is usually analysed by X-ray diffraction (XRD) [57] and the elemental composition of QDs can be studied by energy dispersive X-ray analysis (known as EDS, EDX or EDAX) [54]. Other techniques, like inductively coupled plasma atomic

emission spectrometry (ICP-AES), was used for analysis of QDs as well, namely for the metal ions content in final QDs [58]. Recently, also electrochemical methods were applied to the study of QDs behaviour [59-61].

4. BIOLOGICAL AND BIOMEDICAL APPLICATIONS OF QDS

In the 1970s and early 1980s, understanding the photophysical properties of semiconductor structures was important for a broad range of computer and electronic applications. It was hypothesized that the physical properties of structures in an intermediate size range (between single atoms and bulk) could be tuned by alteration of size and shape. For electronics and computer applications, such a system allows an engineer to synthesize a large set of nanometer-sized building blocks for constructing faster and smaller computer chips or more efficient light-emitting devices. Very soon it was realized that these nanometer size structures can have significantly much more applications. Nowadays a wide range of QDs utilizations are found in the biological, biochemical and biomedical areas. Besides the applications as simple sensors [62,63], the main function of the QDs based on their exceptional fluorescent properties in the biochemical and biomedical research area is their use as unique fluorescent labels [64-66].

4.1. QDs Toxicity

To use QDs in biology, it is extremely important to deal with the biocompatibility and toxicity. However, very few studies have examined the toxicity of these nanomaterials [67-69]. Moreover, the toxicity of nanomaterials is highly complicated, due to the diversity of materials. In sharp contrast to conventional hazardous materials, the attention has to be paid to the nanoparticle-specific problems; including the fact that surface of nanomaterials is highly active due to the large surface area, and surface to volume ratio. In addition, it is necessary to exclude the effect of solubility and possible contamination, which also would decrease the validity of any toxicity testing [68]. In 2006, Hardman reviewed the toxicity of QDs as a function of physicochemical and environmental factors [70]. QDs size, charge, concentration, outer coating bioactivity (capping material, functional groups), as well as oxidative, photolytic, and mechanical stability have each been shown to be determining factors in QDs toxicity. *In vitro* studies suggest certain QDs types may be cytotoxic.

4.2. QDs Biocompatibility

To improve the biocompatibility, it is needed to passivate or cap the QDs with a layer of ZnS or CdS. The ZnS or CdS improve the fluorescence QY of the QDs and protect them against photo-oxidation (which is important for minimizing cytotoxicity and for enhancing photostability). The ZnS shell has larger band gap energy than CdSe, eliminating the core's surface defect states. Also, the ZnS shell has a similar bond length to the CdSe, minimizing crystal-lattice strain and allowing epitaxial growth. Even with advances in synthesis,

obtaining biomedically useful QDs is still problematic due to differences in optical properties from batch to batch. From one synthesis to the next, QDs with different QYs and fluorescence spectra may be produced. Moreover, further functionalization is needed for incorporation of required chemistry.

Various surface modification techniques were developed to ensure the specific bioconjugation. This is usually achieved by decorating QDs with proteins, peptides, nucleic acids, or other biomolecules that mediate specific interactions with living systems. Surface engineering is thus crucial not only for tuning the fundamental properties of nanomaterials and for rendering them stable and soluble in different environments, but also for creating nanoparticle–biomolecule hybrids capable of participating in biological processes. Such hybrids should combine useful properties of both materials involved, i.e. optical properties of the nanocrystals and biological functions of ligands attached [64]. One of these strategies utilizes the biotin-avidin (respectively streptavidin and neutravidin) interaction known for its very high specificity. Modification of QDs by the streptavidin proved a very successful method evaluated in numerous publications [9,64,71-73] and due to this success, streptavidin-QDs are nowadays also commercially available. In addition, biotin-functionalized QDs were developed to exploit the same interaction.

Recent achievements in merging nanoparticle encapsulation and bioconjugation steps and design of pre-functionalized surface coatings promise to provide more compact, stable, and biocompatible nanoparticles with controlled density and orientation of ligands attached. Amphiphilic polymers with a maleic anhydride backbone are being actively explored for this purpose. In organic anhydrous solvents, such polymers encapsulate TOPO-coated QDs and introduce reactive anhydride groups on the surface. In basic aqueous buffers, anhydride rings are quickly hydrolyzed, yielding negatively charged carboxylic acid groups and rendering QDs water soluble [74]. More importantly, anhydride groups are highly reactive towards amine-containing molecules, thus allowing covalent conjugation of a variety of biomolecules to the polymer chains without the need for post-encapsulation modification [75,76].
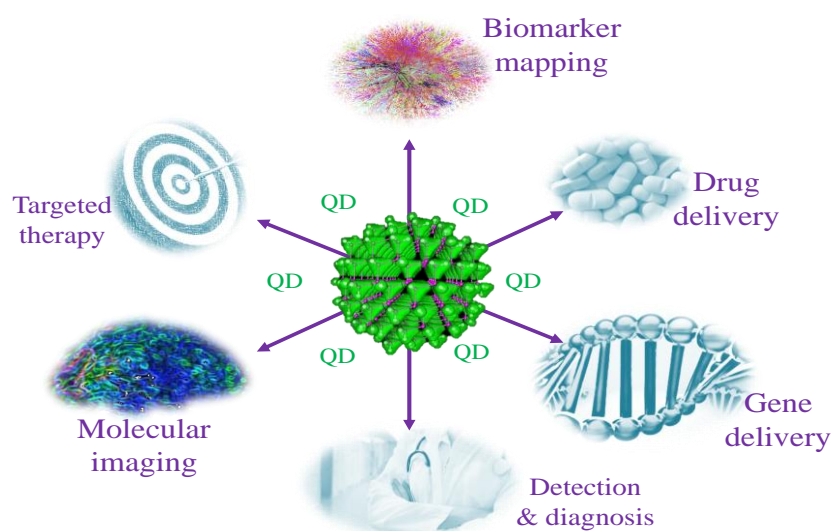

Figure 4. Main QD´s application areas. QDs can be applied as drug carriers in targeted therapy, as detection and diagnostic components, in gene delivery as well as in molecular imaging.
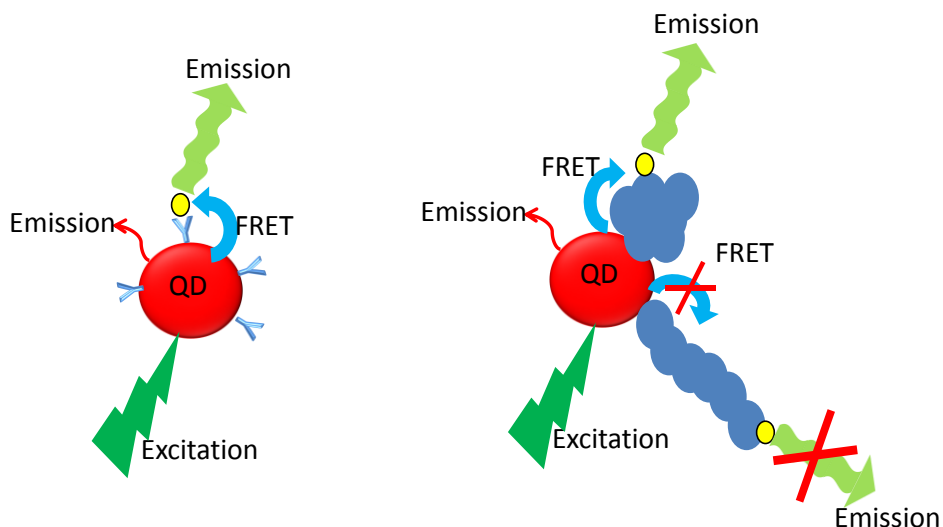
Figure 5. Scheme of FRET method. A) The energy delivered to the QD from external light source is transferred to the analyte and emission specific to this analyte is monitored. B) If the distance between the QD and the analyte exceeds certain distance (Förster radius), the transfer of energy between QD and analyte is impossible and no analyte-specific emission is monitored.

Choice of the bioconjugation approach depends on availability of ligands with suitable functional groups and on specific application requirements. However, common design criteria involve preserved QDs photo-physical properties and ligand biofunctionality, controlled ligand orientation and binding stoichiometry, compact probe size, and good stability in physiological environment. As these criteria can be satisfied in only few specific cases, improvement of existing bioconjugation techniques and design of novel application specific water-solubilization and bioconjugation approaches remains an active area of research. With the development of stable and biofunctional QDs probes, these materials will become nanoscience building blocks with flexible properties that could be further optimized for specific applications including biomedical imaging, detection, and nano-therapeutics [77].

4.3. *In Vitro* Applications

In vitro literally means "in glass". The conditions of the experiment are artificial and simulate what might happen in vivo. In the last decade, surface engineering and bio-functionalization techniques have transformed semiconductor nanocrystals into complex cellular probes capable of interaction with biomolecules and direct participation in biological processes. In 1998, two seminal science papers first demonstrated that semiconductor nanoparticles could be made water-soluble and used as biological imaging probes [78,79]. One approach utilized silica shell encapsulation chemistry in order to produce QDs for a single-excitation dual-color cell staining [78]. When derivatized with trimethoxysilylpropyl urea and acetate groups, green QDs preferentially labeled the cell nucleus, and when derivatized with biotin, red QDs labeled F-actin filaments pre-treated with phalloidin-biotin and streptavidin. The second paper was the first to demonstrate the ligand-exchange approach to QDs water solubilization [79]. Subsequent conjugation of transferrin produced QDs probes

that were endocytosed by live HeLa cells resulting in punctate cell staining, while IgG bioconjugates were used in an aggregation-based immunoassay. Since then, a multitude of surface engineering techniques for QDs solubilization and biofunctionalization have been developed, enabling application specific design of QDs probes. Such probes have found their use in a variety of in vitro applications, such as histological evaluation of cells and tissue specimens, single molecule detection and real-time tracking, long-term live-cell imaging, and study of intracellular processes. Moreover, QDs can be employed as optical labels that probe dynamic biological processes, such as biocatalysed reactions or structurally induced biomolecular transformations using fluorescence resonance energy transfer (FRET) or electron-transfer quenching as photophysical probing mechanisms.

4.3.1. Fluorescence Resonance Energy Transfer (FRET)

FRET involves the transfer of fluorescence energy from a donor particle to an acceptor particle whenever the distance between the donor and the acceptor is smaller than a critical radius, known as the Förster radius (Figure 5). This leads to a reduction in the donor's emission and excited state lifetime, and an increase in the acceptor's emission intensity.

FRET is suited to measuring changes in distance, rather than absolute distances, making it appropriate for measuring protein conformational changes, monitoring protein interactions and assaying of enzyme activity. Several groups have attempted to use QDs in FRET technologies, particularly when conjugated to biological molecules, including antibodies, for use in immunoassays. The sensitivity of these photophysical processes to the distance separating the donor–acceptor or chromophore–quencher pairs prevents, however, the use of the passivated fluorescent QDs as optical probes for dynamic bioprocesses because of the thick stabilizing capping layers associated with the nanoparticles. Thus, a delicate balance between the nanostructure of the modifying capping layer associated with the QDs and its effect on the photophysical features of the particles must be maintained. Some of the first-reported biosensor systems involved FRET, where QDs acted as donors and organic fluorophores acted as acceptors [80,81].

QDs have demonstrated to be applicable for cell labeling, tracking cell migration, flow cytometry, fluorescence *in situ* hybridization, whole-animal contrast agents, pathogen detection, genomic and proteomic detection, FRET sensors, and high-throughput screening of biomolecules. Conventional DNA fluorescent microarrays are based on the sandwich hybridization of target DNA between a capture probe attached to a surface and a fluorophore-modified signaling probe. Recently, the use of DNA-functionalized QDs as signaling probes for DNA microarrays was demonstrated. Fluorescent imaging *in vitro* mainly belongs to three categories, cellular imaging, biomolecular tracking in cells, and tissue staining. Moreover, so called multicolor QDs, which means particles modified by several different molecules, are nowadays of a great interest. Moreover, QDs may be employed for labeling of nucleic acids and subsequently used in microarray technology.

4.4. *In Vivo* Applications

The characterization and analysis of biomolecules and biological systems in the context of intact organisms is known as *in vivo* research. The *in vivo* approach involves experiments performed in the large system of the body of an experimental animal. Compared with the

imaging *in vitro*, QDs imaging *in vivo* faces different challenges caused by the increase in complexity in going to a multicellular organism, and with the accompanying increase in size. There are four main kinds of *in vivo* imaging applications with QDs: biodistribution of QDs *in vivo*, *in viv*o vascular imaging, *in vivo* tracking of QDs, and *in vivo* tumor imaging.

Utilization of nanomaterials, particularly nanoparticles for the *in vivo* monitoring of cell transplantation is one of near future appealing application. Currently MRI provides low resolution and no difference between original and transplanted cells is registered. Any cells can be labeled – stem cells, Langerhans cells, tumor cells, etc. Incorporation of the contrast agents into the cells can be done by either phagocytosis or conjugation of the contrast agent to the cell surface via antibody interaction with the receptor. Labeled cells can be transplanted into the animal as well as bacteria. This method is available especially for bacterial screening avoiding time-consuming procedures and it is advantageous in clinical diagnosis and environmental monitoring.

A new and exciting direction of research for QDs is their application as a contrast agent for *in vivo* imaging [28,82,83]. Organic fluorophores and chemiluminescence probes are currently the most commonly used optical probes for animal imaging. However, a limitation of optical contrast agents is the lack of available probes that emit in NIR emission range (> 650 nm). The NIR emitting window is appealing for biological optical imaging because of the low tissue absorption and scattering effects in this emission range. The bounds of the NIR optical window for animal imaging are typically set at 650–900 nm. Since the optical properties of QDs can be tuned by size and composition, it should be possible to prepare a series of NIR-emitting QDs for animal imaging. CdTe, CdTeSe, InPAs, PbS, and PbSe have been successfully synthesized with NIR emission [84,85]. For most investigations of *in vivo* imaging, QDs are usually directly injected into the live animal intravenously or subcutaneously and thereby are delivered into the bloodstream. The behavior of QDs *in vivo* is very interesting because they have to interact with the components of plasma, blood cells, and the vascular endothelium.

4.4.1. Vascular Imaging

One of the most common *in vivo* applications of QDs is fluorescence contrast imaging of the blood vasculature and lymphatic drainage system [86]. Intravenously injected QDs can highlight morphological abnormalities in vasculature, model biodistribution of nanoparticle-based drug delivery vehicles, and monitor the blood circulation dynamics, whereas intradermally delivered QDs can map the lymphatic basins along with sentinel lymph nodes (SLN) and uncover disease-related transport mechanisms (e.g. tumor metastasis pathways). Furthermore, the multicolor nature of QD probes makes it possible to investigate separate vascular systems in a multiplexed manner, providing insight into the intricate blood and lymph circulation networks within organs and tissues. In clinical practice, the ability to map tumor vasculature and lymphatic drainage pathways might not only enhance the accuracy of diagnostics, but also provide intraoperative image guidance for more effective and less invasive tumor and lymph node resection. Therefore, real-time vascular imaging with QDs has the potential to improve our understanding of vasculature-related physiological and pathological processes as well as advance clinical diagnostics and therapy [87].

With such a great potential, this application requires virtually no additional surface engineering of QD probes satisfying general requirements for *in vivo* use, as no extravasation, organ selectivity, cellular uptake, and specific target binding are necessary. However,

prolonged circulation and enhanced stability in physiological conditions are often desirable for reliable data collection and long-term monitoring of probe biodistribution. Therefore, the major design in engineering of QDs contrast agents for vascular imaging should be placed on synthesis of non-fouling and possibly biodegradable coatings. These coatings should efficiently protect the QD core, evade reticuloendothelial system (RES) uptake and renal filtration for the duration of experiment, and then enable eventual particle degradation and excretion. Further, engineering of fluorescence imaging systems suitable for deep-tissue *in vivo* imaging will be indispensable for the success of QD-based angiography [87].

Pioneering studies done by Larson et al. have demonstrated the value of QDs probes for the dynamic imaging of the blood vasculature of skin and adipose tissue in live mice [88]. The relatively large size and high stability of polymer-encapsulated QDs have provided bright and persistent fluorescence contrast after intravenous injection. Performing line scans across capillaries and monitoring the propagation of QDs fluorescence, the investigators have been able to measure blood flow velocities. At the same time, the large two-photon excitation cross-section of QDs probes has enabled nearly background-free vasculature imaging at tissue depths of several hundred microns with two-photon fluorescence intravital microscopy. However, the surface coating used in this study was not specifically designed for prolonged QDs blood circulation, and the fate of QDs probes was not investigated.

Ballou et al. have systematically studied the effect of additional PEG coating on the circulation half-life and biodistribution of polymer-coated QDs using whole-animal real-time fluorescence reflectance imaging [89]. QDs decorated with long-chain methoxy-PEG have shown significantly longer circulation half-life compared to non-modified QDs; however, the PEG shell has failed to significantly reduce the RES uptake and sequestration of particles within liver and spleen, thus limiting the blood circulation to only a few hours. Moreover, extravasation of QD-PEG probes into surrounding tissues has been observed even for large particles, which might result from the non-specific interaction between QDs and endothelial cells and cause increased fluorescence background detrimental for dynamic vascular imaging. Yet, even a shorter blood circulation time is often sufficient for detailed vascular imaging. In one example, Stroh et al. have combined two-photon intravital microscopy, blue-emitting QDs encapsulated in PEG-phospholipid micelles, and a transgenic mice model with GFP (green fluorescent protein)-expressing perivascular cells to study the morphology of the tumor vasculature [90]. Following systemic administration, QDs highlight the vessel boundary providing a clear picture of tumor vessel morphology while resisting extravasation for at least 30 min, whereas GFP fluorescence indicates the distribution of perivascular cells. Poor QDs extravasation has been employed by Kim et al. for studying the patho-physiology of viral infection of the central nervous system in mice [91]. Using intravital two-photon microscopy, QDs extravasation from brain microvasculature has been monitored as a measure of disease-associated vascular injury and blood-brain barrier breakdown. Initial studies on QD-based blood vasculature imaging outline the numerous beneficial features of QDs probes for this application as well as emphasize the urge for novel coatings that would efficiently prevent interaction with biomolecules, recognition by the immune system, and extravasation, thus improving the probe circulation half-life and imaging accuracy. In addition, future coatings might feature controlled biodegradation functionality, enabling disintegration of bulky QDs probes into smaller components that could be safely eliminated from the bloodstream via renal filtration. Engineering of QDs contrast agents for lymphatic angiography and lymph node mapping is governed by less strict and somewhat different

design principles. Unlike probes for blood vessel imaging, QDs need to be small enough to be transported from interstitial space into lymph vessels, and yet large enough to be trapped in lymph nodes (in general particles with diameter of 5–50 nm are retained). However, neither the particle size (within 20–50 nm range) nor the surface charge has shown significant effect on the SLN mapping, providing more flexibility for probe design [86]. More importantly, this is probably the only *in vivo* application where QDs long-term toxicity and excretion routes do not present a major concern, as labeled SLNs and tissues are often removed during surgery.

Cardiovascular and lymphatic angiography have been two of the most successful QD-based *in vivo* imaging applications. In combination with fluorescence reflectance imaging, QDs highlight macroscopic structures on a whole-animal or whole-organ scale and serve as visual tags for image-guided surgery. Two-photon intravital microscopy provides high-resolution examination of superficial vessels and their surrounding tissues; and emerging advanced imaging techniques, such as multiphoton microscopy with a needle-like gradient index lens for deep-tissue imaging [92], promise to enable detailed studies of intact vasculature deep within organs. Yet, further translation of this technology into clinical practice will heavily depend on engineering of non-toxic, non-fouling, and biodegradable QDs coatings as well as stable and bright QDs cores.

4.4.2. Quantum Dots and Nanocomposites in Cancer Detection and Therapy

Imaging application. It is generally accepted idea that the future treatment for cancer relies on early detection of cancer lesions, as well as efficient and specific delivery of drugs to the cancer cell site [93]. The detection of stage 1 cancer is associated with > 90% 5-year survival rate while conventional anatomic imaging typically cannot detect cancers until they reach > 1 cm diameter. Molecular imaging, especially with QDs covalently linked to biorecognition molecules such as peptides, antibodies, nucleic acids, or small-molecule ligands, is expected to play an important role in future cancer diagnosis. QDs inside cells are particularly useful for cell tracking to study cell division and metastasis. Because of the QDs high stability and multicolor emission, QDs can act as unique markers for tracking cancer cells *in vivo* during metastasis — a critical issue in the development of effective cancer therapies. Whole-body NIR optical imaging is a powerful technique that allows the observation of complex biological phenomena with minimum invasiveness. The absorption of light by tissues in the NIR region is limited as compared with that in the UV and VIS regions. Thus, light can penetrate several centimeters below the body surface, and internal fluorophores can be observed easily. In the NIR region autofluorescence from tissues is also limited, so that the fluorescence of an introduced fluorophore can be more clearly visualized [94,95]. Kim et al. presented a study where NIR QDs emitting at 850 nm were used as markers in sentinel lymph node mapping, a major procedure in cancer surgery, whereby the lymph node closest to the organ affected is monitored for the presence of roaming cancer cells [96]. Also, other authors have reported an elegant study using QDs to simultaneously track different populations of cells in lung tissue [95]. They have used the major features of QDs and the high resolution of fluorescence, combined QDs and emission spectrum scanning multiphoton microscopy to develop a mean to study extravasations *in vivo*. Briefly, to examine the cell metastasis in a natural tissue environment, a mixed population was injected into the tail vein, extracted and fixed lung tissues, and then emission-scanning microscopy was used to distinguish both populations of cells in the whole tissue sample. Thus, there exists a broad range of characterization methods and alternative strategies that researchers

may follow, such as the use of emission spectrum scanning microscopy allowing simultaneously tracking of several different QD-tagged populations of cells in the same living organism. An important aspect to be considered on developing new strategies for cancer detecting and treatment is the QD-target conjugate efficiency on reaching the site *in vivo* assays.

Biomolecule labeling application. Interestingly, some authors have reported on the approach of using engineered-coated QDs that 'disguises' the host system as the surfaces are covered by a synthetic peptide, a tactic inspired nature and found in plants and yeasts. Synthetic phytochelatin-related peptides were used as an organic coat on the surface of colloidal CdSe/ZnS semiconductor nanocrystals synthesized from hydrophobic coordinating TOPO solvents. The peptides are designed to bind to the nanocrystals via a C-terminal adhesive domain [97]. It was demonstrated that through a surface chemistry approach using bioconjugation the process is suitable for targeting and detecting individual protein receptors in living cells. Hence, a picture can be envisioned in which cancer diagnosis and patient management could be considerably improved. For instance, patients at high risk of breast or colorectal cancer would be injected with the color-coded QDs with the engineered antibodies specific for cancer-associated cell surface markers before being submitted to mammography or colonoscopy. In case it is needed, the biopsy would be conducted using the tissue already labeled by the QDs under fluorescence microscopy [98].

Immunofluorescence labeling using QDs showed different staining patterns between normal and cancer cells [99]. Detection of ovarian cancer marker CA 125 in various specimens using streptavidin-conjugated QDs [100]. In addition, a very promising realm of cancer research based on the detection of genetic polymorphisms is discussed. Briefly, QDs can be used for the simultaneous detection of multiple single nucleotide polymorphisms (SNPs). QDs have been used in assays for detection of SNPs of cytochrome p450 and the human p53 tumor suppressor gene; a gene that has been suggested as being involved in over half of the known human cancers. For the reasons expounded above, in the future, a reasonable scenario may be envisaged where polymers, biomolecules, and QDs will be conjugated in a completely integrated hybrid nanostructured composite system aiming to address a large number of problems and challenges faced by the biomedical community.

Therapeutical application. Recent advances in surface modification of QDs have enabled their potential application in cancer imaging. Conjugation of QDs with biomolecules, including peptides and antibodies, could be used to target tumors *in vivo*. QDs with NIR emission could be applied to sentinel lymph node mapping to aid biopsy and surgery [101]. For diagnosis and imaging of breast cancer, an assay that could accurately quantify several cancer-related proteins simultaneously on single tumor sections or small tumor specimens could offer clear advantages over standard immuno-histochemical methods [102]. With this approach, Wu et al. [103] showed specific ERBB2 labelling of fixed ERBB2-positive breast-cancer cells and human ERBB2-positive breast-cancer xenografts. Although this method is easy to use and highly effective for single staining of cell proteins, it is not optimal for multiplex protein detection. Al-Hajj et al. [104] have shown simultaneous multiplex detection of six breast cancer proteins by use of direct conjugation of QDs to antibodies on fixed paraffin-embedded tumor samples. Recently, Hassan and collaborators have published a fine review considering the QDs as a 'nanomedicine toolbox'. With respect to cancer detection and therapy some important examples based or specific targeting ligands were summarized, for instance: detection of Her2 (hairy-related 2) on SK-BR-3 breast-cancer cells by employing

humanized anti-Her2 antibody, a biotinylated goat anti-human IgG, and streptavidin coated QDs [103].

4.5. Coupling of Magnetic Nanoparticles to QDs – Multimodal Nanoparticles

Molecular imaging refers to the characterization and measurement of biological processes at the cellular and/or molecular level, its modalities include optical bioluminescence, optical fluorescence, ultrasound, MRI, magnetic resonance spectroscopy (MRS), single-photon-emission computed tomography (SPECT) and positron emission tomography (PET). Since no single imaging technique can provide complete information about subject´s structure and function, using multiple imaging techniques is required. Nanostructures provide an excellent platform to integrate different functional nanocomponents into one single nanoentity to exhibit multifunctional properties. QDs can be combined with magnetic nanoparticles to exhibit magnetic and fluorescent properties concurrently [105,106].

Unlike MRI, *in vivo* imaging utilizes the incomparable fluorescent properties of QDs such as small size (tens of nm) and unique tunable optical features. QDs are widely being used in place of organic dyes for imaging applications in biological systems due to their much greater temporal stability and resistances to photobleaching than fluorescent dyes do. The combination of superparamagnetism and fluorescence at nanometer scale should help the biological applications of multifunctional nanomaterials. The desirable physical and chemical properties of contrast agents needed for bimodal optical and magnetic imaging can be combined in a single nanoparticle [107-109].

CONCLUSION

QDs, tiny light-emitting nanocrystals, have emerged as a new promising class of fluorescent probes for biomolecular and cellular imaging. In comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties such as size-tunable light emission, improved signal brightness, resistance against photobleaching, and simultaneous excitation of multiple fluorescence colors.

The biomedical applications of nanoparticles are rooted in the advanced functional design, and have been realized in preclinical experimental diagnosis. In the end, they will contribute to personalized clinical treatment based on molecular profiles of each individual patient. The development is rapid and multidirectional, but at present is still in its early stages (Figure 4). The main applications of nanoparticles can be divided into several major directions: diagnostic molecular imaging, delivery of drug and gene, and targeted therapy.

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