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Review article

Quantum dots and their immunochemical applications

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Abstract: *Quantum dots (QDs) are nanometre size semiconductor crystals which possess unique physical and chemical properties. In recent years they were widely used as signal enhancers in biological analysis, mainly because of their high quantum yield, photostability and long-lasting photoluminescence. Compared to common organic fluorophores QDs exhibit wider absorption spectra (QDs absorb photons when excitation energy exceeds the bandgap), narrow emission wavelengths and high Stoke's shift which allow usage of several different-coloured QDs in single multiplex assays. QDs' synthesis can be conducted by top-down or bottom-up approach. Both methods of synthesis may lead to surface imperfections which may negatively affect QDs' optical properties. To avoid this problem surface passivation is required. The most widely used passivation method is to cover the QD's core with material having larger band gap (ZnS). QDs can be widely used in different applications due to the ease of surface functionalization by means of organic and inorganic molecules (polymers, dendrimers, proteins, antibodies and etc.) by many different approaches like ligand-exchange, silanization, amphiphilic combination and other mechanisms. Functionalized QDs have been used for various purposes in in-vitro and in-vivo imaging, drug delivery, therapeutics and other. However this review is mainly focused on immunochemical applications of QDs such as immunohistochemistry, FLISA, FRET, immunosensors etc. QD-based immunological assays are being used for detection of pathogens, toxins, proteins, metal ions (Hg^{2+}) and allergens. Based on growing rate of QDs' applications it can be concluded that in the coming years their number is going to increase.*

Keywords: *antibody, quantum dots, fluorophores, immunochemistry, immunoassay.*

Introduction

In recent years, development of high sensitivity analytical methods is observed. Fluorescent methods are becoming more common not only in medical applications. Currently used organic fluorophores are not suitable for ultrasensitive bioassays, mainly because of poor stability and broad emission spectra. Advances in nanotechnology have allowed development of novel

materials which may meet the requirements of modern bioanalysis [1]. Quantum dots (QDs) belong to the popular group of nanoparticles which are used in different bio-detection assays. QDs are semiconductor nanocrystal made of a few hundred to thousands of II/VI or III/V elements arranged in binary (e.g., CdS, CdTe, GaS) or ternary (i.e., InGaN, InGaP) structures with uncommon chemical and physical properties [2-4]. In comparison to widely used organic fluorophores (e.g., Cy3, TAMRA, Nile Red) QDs possess several advantages. Their optical properties strictly depend on the parameters like particle dimensions, size dispersion, chemical composition and surface chemistry. The wavelength of the emitted radiation is associated with the size of quantum crystals. QDs have narrow emission wavelength and broad absorption spectra, therefore it is possible to use different kinds of QDs in multiplex imaging with the use of one excitation wavelength. High quantum yields and big Stokes shifts are also typical for these nanoparticles [1, 5, 6]. Compared to organic fluorophores QDs are stable on long term exposure to light and resistant to photo-bleaching, which make them more suitable for long-term measurements and storage [6, 7].

The aim of this review is to present recent advances in QDs' functionalization techniques and their applications with particular emphasis on immunoassays. We begin with a brief description of QDs and their synthesis methods. Then different approaches of particles functionalization are described. Finally, potential applications of modified QDs in various bioassays are presented.

Quantum dots characterization and synthesis

Quantum dots photoluminescence effect is caused by excitation of the electron through the absorption of incident electromagnetic radiation – QDs absorb photons when excitation energy exceeds the bandgap. It is important to mention that particles' excitation by shorter wavelength is possible because multiple electronic states are present at higher energy levels [8]. When the amount of energy delivered to the QD reaches the required level, electrons will be promoted from the valence band to the conduction band and form an exciton (pair of hole and electron). A recombination of hole-electron pairs leads to luminescence [6, 9]. Radiation wavelength is regulated by quantum confinement of electron and hole. This phenomenon leads to an increase in the effective band gap of the material with decreasing size of the crystal. As a consequence of the reduction in the particles size, absorption and emission of quantum dots spectra shift to the blue [8].

Quantum dots can be classified into different types based on their composition and structure. First division is based on the differences in the composition of nanoparticles. We can distinguish quantum dots which are made of heavy metal elements (CdSe, CdTe, PbSe, GaAs) and non-heavy metals (InP, ZnS, Ag_2S, Si, C etc.) [9, 10]. Second division is based on differences in the structure of the nanoparticles. We can distinguish three basic types of QDs' architecture: core, core-shell and alloyed quantum dots (Fig. 1). First type is made of single component materials with uniform internal composition, core-shell structures consist of bare core material covered with other structurally similar

semiconductor with wider band gap (ZnS, ZnTe, CdS) [8, 11, 12]. Such additional layer provides better surface passivation, which results in significant improvement of the fluorescence quantum yield [13]. Alloyed QDs have more complex internal composition (CdSeTe) in which the proportions of the components allow to tune the quantum coefficient without changing the particles diameter [14].

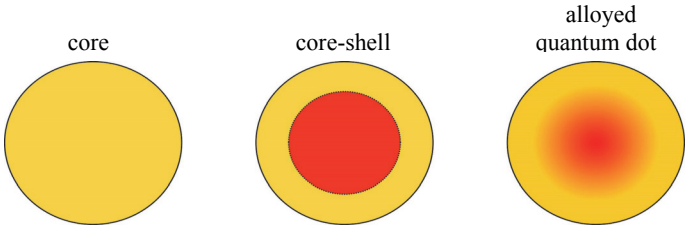

Figure 1. Types of quantum dots divided by differences in the structure of nanoparticles

In recent years many methods have been developed for QDs' synthesis. All techniques used for QDs' creation can be divided into two types. The most popular approach, which is commonly used for bio-applications, is bottom-up colloidal synthesis. It can be divided into organometallic approach and aqueous-colloidal synthesis [15, 16] and be carried out using microemulsion, sol-gel [17, 18], competitive reaction chemistry, hot-solution decomposition, microwaves, electrochemistry [19] or sonic waves [20] techniques. For example, reaction mechanism developed in the previous century relies on an organometallic route in the presence of organic solvents under high temperature (270-320°C) and inert environment [2, 15, 21] (Fig. 2). This procedure yielded high-quality nearly monodisperse hydrophobic QDs with high fluorescence quantum yield around 60-85%. Application in immunochemical methods requires the QDs to be water-soluble. The QDs obtained in organic solvents require subsequent modifications in order to maintain solubility and stability in water solutions [22]. To obtain straight off hydrophilic nanoparticles, the aqueous synthesis approach was used. The first QDs synthetized in aqueous solution by Rogach et al. in 1996 were thiol-stabilized CdTe QDs [15]. Properties of synthetized particles could be adjusted by changing reaction conditions [23].

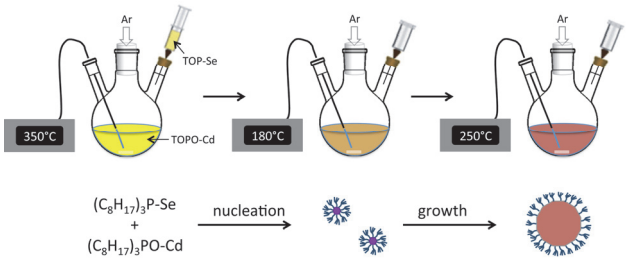

Figure 2. Example of quantum dots bottom-up synthesis [24]

In a top-down approach, a bulk semiconductor is thinned. To achieve this goal techniques like molecular beam epitaxy (MBE), lithography, ion implementation, chemical vapour deposition (CVD) were used [19, 25, 26]. However, methods used in top-down approach may cause the formation of structural imperfections [19].

It should be mentioned that bare core QDs are rarely used in various applications because of the crystalline structure defects, like vacancies, which results in lower quantum yield, fluorescence blinking, leaching of metal ions surface oxidation etc. The formation of mentioned imperfections is connected with high QDs surface energy. This phenomenon is a result of high surface to volume ratio. Decrease of particles size increases the number of atoms which are exposed to the environment compared to the overall volume of the QDs. It results in high surface energy and susceptibility to the formation of surface defects [6, 10-13]. To reduce this energy, atoms undergo a redistribution and reconstruction or creation of surface defects mentioned before [6]. To overcome this problem and protect particle's core against oxidation or other chemical reactions with surrounding environment QDs' surface should be passivated. Surface passivation can be obtained by forming a shell composed of a few atomic layers of a material with larger band gap (e.g. ZnS) on the QDs' cores [10, 13].

Many researchers have reported that the structure of core/shell QDs ensures higher quantum yield, more stable luminescence and chemical stability. Additionally, this type of QDs is relatively easy to functionalize with the use of different chemical or biological ligands. Therefore, core/shell quantum dots are widely used in biological applications [6, 14]. Second QDs' type which can be used in biomedical application is alloyed QDs. As mentioned before, properties of this type of QDs can be tuned (e.g. color change (Fig. 3)) without changing their dimensions – this is very useful especially in bioconjugation. Due to small size alloyed QDs do not affect the activity or structure of attached biomolecules [15].

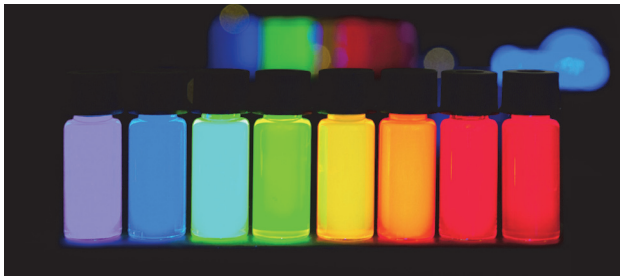

Figure 3. Example of QDs tuned in order to obtain different colour fluorescence [27]

Quantum dots can be also obtained using various biological systems. This approach has become more popular in recent years because it is more environmentally friendly and allows to synthesise hydrophilic particles which can be used in biological applications. QDs' formation process involves specific biomolecules (peptides, protein, nucleic acids) that take part in particles

formation as templates to direct and promote the growth of QDs [16, 17]. Biosynthesis can be carried out with the use of bacteria [18-20], yeast [21], viruses [16] and fungi [22].

Quantum dots surface functionalization

To use QDs for biological applications they have to be soluble in aqueous media. Commonly used shell materials (ZnS) improve the stability and quantum yield of QDs but they do not affect the particles' solubility [6]. In order to increase particles' compatibility with water solutions QDs can be modified with hydrophilic ligands [+]. Several modification strategies exist, and one of the most popular is ligand exchange. Bifunctional groups of ligands such as thiol, carboxyl, amine or hydroxyl substitute native hydrophobic ligands that are attached to the QDs' surface or interact with core material improving particles' solubility. In fact, it was found that ligand exchange approach adversely affects photochemical stability and fluorescence intensity of QDs [16, 23, 25]. Dendrimers like polyamidoamine (PAMAM) were also used in QDs' solubilisation with the use of ligand exchange approach [26]. Surface silanization is another method to obtain water soluble particles. It is a process of capping the surface of QDs with a thin layer of silanes that can be crosslinked. Newly synthetized silicon layer has numerous functional groups that can be used for conjugation of biomolecules [28]. Third method is called amphiphilic combination. In this approach native hydrophobic ligands are preserved and water solubility is ensured by hydrophilic groups of di- or triblock copolymers attached to QDs' surface by hydrophobic attraction between particle surface and groups present in copolymer chain [6, 10].

The conjugation of QDs with specific biomolecules is another way to obtain water-dispersible particles which can be widely used for numerous biological applications [29]. Ligand binding to a QDs' surface can be achieved by electrostatic or ligand-receptor interactions (biotin-avidin/streptavidine or antigen-antibody), covalent cross-linking (carbodiimide chemistry or by mercaptoacetic acid as linking molecule), metal-affinity coordination (histidine rich proteins), click chemistry reaction [30] or even by virus encapsulation technique (nanocages) [5, 16, 31]. Biofunctionalized QDs can then be used in biosensors, immunology, cell and tissue imaging, drug delivery mechanisms *etc*. [16].

Application of QDs in immunochemistry

Usage of quantum dots for biological applications was first reported by Chan and Nie in 1998 [32]. Since then QDs have be used for many different purposes like in vitro and in vivo imaging [33], drug delivery and therapeutics [34], biosensing or single-quantum dot tracking of extra- and intracellular targets [35].

This review focuses on examples of how QDs are being used in immuno-histochemistry and different immunoassays. All cases described below are gathered in Table 1.

Immunohistochemistry

Immunohistochemistry (IHC) refers to a process during which antigens in the cells of tissue sections are detected. It is done using the principle of antibodies binding specifically to antigens.

In 2003 Kaul et al. [36] used Qdots™ 605 Streptavidine conjugates to stain mortalin in normal and transformed cells. Mortalin is a member of heat shock protein-70 (Hsp70) family of proteins. It shows different staining patterns for normal and transformed cells. Therefore, it can be a reliable marker for such cells. After staining the cells with QDs immunoconjugates Kaul proved that this method can be used for visualization of proteins. What is more, stability of quantum dot staining was compared with the stability of fluorescence staining. Both normal and cancer derived human cells were stained for mortalin and visualized by QDs and Alexa Flour® 488. The test showed that fluorescence staining with Alexa started to fade away after 3-4 mins and was lost after 8 minutes, whereas mortalin stained with QDs did not weaken. It proved that QD-staining of proteins has a practical value for such studies as structural, temporal or interactive ones.

Qu et al. [37] developed QD-based immunofluorescent approach for detection of a mutation in the epidermal growth factor receptors (EGFR). EGFR mutations status has significant role in therapeutic decision making for non-small cell lung cancer (NSCLC) patients. QDs conjugated with three primary antibodies (total EGFR monoclonal antibody [D38B1], EGFR del E746-A750 mutation-specific monoclonal antibody [6B6], and L858R mutation-specific monoclonal antibody [43B2]) were used for detection of mutations in NSCLC specimens from patients with lung adenocarcinoma. The study showed that QDs-IHC was able to accurately detect EGFR mutation protein localization in NSCLC, proving that this technique is suitable for detection of EGFR mutation signals.

In 2010 Gonda et al. [38] visualised the movements of protease-activated receptor 1 (PAR1) expressed by cells during metastasis *in vivo* by tracing cancer cells marked with anti-PAR1 antibody conjugated QDs (anti-PAR1-QDs). In 2015 Gonda used QDs to show that the migration and invasion by PAR1-expressing cancer cells were blocked by an administration of an anti-PAR1 antibody and to determine expression levels of PAR1 in HER2 (human epidermal growth factor 2)-negative patient tissues [39]. During the test normal breast tissue samples from breast cancer patients, samples from HER2-negative breast cancer patients that remained non-metastatic for more than 5 years after surgery, and samples from HER2-negative patients with metastasis within 3 years after surgery were stained with anti-PAR1-QDs. Gonda calculated the number of QD particles in a cell by single-QD imaging. Results showed that the number of QDs in a cancer cell was greatly related to the relapse-free survival time of HER2-negative breast cancer patients with metastasis within 3 years after surgery. The time of return became gradually shorter in proportion to the number of anti-PAR1-QDs in the cancer tissue.

Immunoassays

The luminescence of quantum dots can be also used to detect analytes by using various assays. To improve selectivity of those assays conjugation of QDs with antibodies was employed.

Fluorescence-linked immunosorbent assay

Fluorescence-linked immunosorbent assay (FLISA) is a technique that was developed in order to overcome disadvantages of enzyme-linked immunosorbent assay (ELISA) such as time-consuming incubation step or instability.

Zhang et al. [40] developed a direct competitive fluorescence-linked immunosorbent assay (cFLISA) to detect aflatoxin B_1 (AFB_1) in peanuts. AFB_1 is one of the most toxic aflatoxins. It was identified as a key factor that causes liver cancer[41]. A lot of different food products can be contaminated by AFB_1. Thus, there has been raising interest to develop an advanced assay of aflatoxins. Zhang used CdTe QDs conjugated with monoclonal antibodies (MAb) for cFLISA. The developed test is a rapid, sensitive and simple analytical method with high performance that can be potentially used to detect other mycotoxins.

Yang et al. [42] established a competitive fluorescence-linked immunosorbent assay (FLISA) for specific quantification of bovine α-lactalbumin (α-La) in dairy products. α-La is one of the most common cow's milk allergens recognized nowadays. To develop a new assay detecting α-La Yang used QDs conjugated with MAbs against α-La. FLISA method provided more sensitive detection compared to ELISA method.

Le et al. [43] used an indirect competitive fluorescence-linked immunosorbent assay (ic-FLISA) and quantum dots for direct screening of tilmicosin and tylosin in edible animal tissues. Tilmicosin and tylosin are the most commonly used pre-mix macrolide antibiotics. They were widely used in animal production to prevent and treat enteric and respiratory infections in domestic animals. However, due to the incorrect use of these antibiotics, they might leave residues in edible animal tissues, European Union banned them [44]. To detect those antibiotics Le used QDs 655 Goat anti-Mouse IgG Conjugate (GaMIgG-QD). To validate ic-FLISA results ic-ELISA and HPLC methods were used.

Fluorescence (or Förster) resonance energy transfer

The nonradioactive transfer of resonant fluorescence energy from an excited donor to a ground-state acceptor fluorophore is used in a technique called FRET. Quantum dots can be used as resonant energy donors because of (i) their wide absorption bands that allow an excitation wavelength far enough from the signal; (ii) their narrow and size-tunable fluorescence spectra that helps to control the spectral overlap; (iii) their high quantum yield; (iv) their photostability; and (v) the ability to be conjugated with different proteins and molecules.

Wang et al. [45] detected octachlorostyrene (OCS), a persistent and bioaccumulative toxicant, using FRET between CdTe quantum dots and rhodamine B-labeled OCS (RB-OCS). RB-OCS and OCS competed for extremely

specific immunoreaction with an anti-OCS antibodies. After mixing with CdTe QDs, that are used as fluorescent donors, emission of RB-OCS was excited through FRET. The electrostatic interaction that was created between RB-OCS and CdTe QDs simplified he conventional joint between donor and acceptor. Thus, Wang provided a feasible sensing platform for detection of OCS.

Xu et al. [46] used different approach. To detect AFB_1 in rice grains Xu constructed a FRET based immunosensor using two different-sized QDs. Monovalent monoclonal antibody(mAb)-labeled red QDs were used as acceptors and multivalent hapten-labeled green QDs were designed as donors in order to avoid irregular aggregation between two kinds of QDs. The energy transfer from the green QDs to the red QDs was a result of interactions between anti-AFB_1, mAbs and AFB_1 that promoted binding of acceptors with multivalent AFB_1-labeled donor. In this way Xu created the FRET immunosensor for the sensitive and specific quantitative detection of AFB_1.

Imunnosensors

Immunosensors may be defined as devices that are self-contained and can provide specific analytical information. They use antibodies as recognition elements.

Cai et al. [47] designed electrochemiluminescence (ECL) immunoassay that uses specially constructed immunosensor and gold nanoparticles (AuNPs) as a marker to detect mercuric ions (Hg^{2+}). Detection of these ions is important because they can cause brain damage and kidney failure. Cai constructed the immunosensor by immobilization of coating antigen on poly-(diallyldimethylammonium chloride)-graphene-CdSe composites (PDDA-GN-CdSe). PDDA was used to functionalize graphene and CdSe QDs were used to enhance the basal signal.

Liu et al. [48] created immunosensor by combining protein A/G agarose beads with QDs conjugated with anti-GP73 antibody (GP73 Ab). This immunosensor was used to detect Golgi protein-73 (GP73), which is considered to be a promising marker for monitoring liver tumour.

Other immunoassays

Cabral Filho et al. [49] applied dual-colour CdTe quantum dots to quantify red blood cell (RBC) antigen expression by flow cytometry. QDs were conjugated with anti-A antibodies, ant-B antibodies and anti-H lectin (*Ulex europaeus* I, UEA I). It allowed quantitative evaluation of antigens on RBC membranes in A_1, B, A_1B, A2, O and A_{weak} groups.

Song et al. [50] combined array analysis and multicolour QD-based competitive fluorescence immunoassay (mQD-cFIA) to simultaneously detect tetracycline (TC), streptomycin (SM) and penicillin G (PG-G) in milk. To QDs with different emission wavelengths, that function as detection probes, antibodies for TC, SM and PG-G were conjugated. Later a direct competitive fluorescent immunoassay was carried out for simultaneous quantitative and qualitative detection of these antibiotic residues. The assay was based on fluorescence of QD-Ab probes.

Table 1. Summary of the applications of quantum dots

Field of application/method used	Description	Year	Reference
Immunohistochemistry	· Immunofluorescence labelling of mortalin using QDs	2003	[36]
	· Detection of a mutation in EGFR by QD-based immunofluorescent approach	2014	[37]
	· Visualization of the movements of PAR1 expressing cells during metastasis *in vivo*	2010	[38]
	· Determination of expression levels of PAR1 in HER2-negative patient tissue by staining with anti-PAR1-QDs	2015	[39]
cFLISA	· Detection of AFB_1 in peanuts using competitive fluorescence-linked immunosorbent assay	2014	[40]
FLISA	· Specific quantification of α-La in dairy products by competitive fluorescence-linked immunosorbent assay	2014	[42]
ic-FLISA	· Screening of tilmicosin and tylosin in edible animal tissues using indirect competitive fluorescence-linked immunosorbent assay	2015	[43]
FRET	· Detection of OCS	2014	[45]
	· Detection of AFB_1 in rice grains by FRET based immunosensor using two different-sized QDs	2014	[46]
Immunosensors	· Detection of Hg^{2+}	2015	[47]
	· Detection of GP73	2016	[48]
Flow cytometry	· Quantification of RBC antigen expression by flow cytometry based on dual-colour QDs	2015	[49]
mQD-cFIA	· Detection of SM, TC and PG-G in milk using multicolour QD-based competitive fluorescence immunoassay	2015	[50]
QD-LFIAS	· Detection of H5 and H9 subtypes of influenza A virus by QD-based lateral flow immunoassay	2015	[51]
Sandwich immunoassay	· Detection of $A\beta_{1-42}$ by sandwich immunoassay based on quantum dots	2016	[53]

Wu et al. [51] used water-soluble carboxyl-functionalized quantum dots conjugated with specific influenza A virus subtype H5 and H9 antibodies to detect H5 and H9 subtypes of influenza A virus, that causes severe respiratory disease. It was conducted using quantum dot-based lateral flow immunoassay system (QD-LFIAS). Description of the lateral flow assay can be found in article written by Sajid et al. [52].

Pi et al. [53] used quantum dots as nanolabels in a sandwich immunoassay for detection of amyloid-beta peptide 1-42 ($A\beta_{1-42}$), which is considered as a promising biomarker for Alzheimer's disease (AD). In this assay biotinylated C-terminated antibody specific to $A\beta_{1-42}$ (C-Ab) binds to streptavidin-coated magnetic bead (MB). Next, the samples are attached to similarly biotinylated N-terminated antibody (N-Ab). Finally, streptavidin modified QDs are added. Thus, in the presence of $A\beta_{1-42}$ QDs are linked to MBs via formation of immune-sandwich complex. This allows their removal by a magnetic field, resulting in decrease of fluorescence intensity.

Conclusions

Quantum dots possess several advantages over organic fluorophores. Thus, their usage in different fields becomes more and more common. Thanks to their optical properties, that can be tuned, high quantum yields and resistance to photo-bleaching, QDs are the molecules of choice for many researchers. Several different QDs' synthesis methods allow scientists to choose one that is most suitable for them. The biocompatibility of QDs is essential for their biological applications. To achieve that, quantum dots have to be functionalized.

Quantum dots have many different applications. This review focuses on immunochemistry. As shown earlier, QDs are used now in immunohistochemistry for detection of antigens of various proteins. Recently, many new immunoassays were developed based on quantum dots and their properties. Most common are the assays using different versions of fluorescence-linked immunosorbent assay and fluorescence resonance energy transfer. QDs are also used lately as immunosensors. In the last two years assays like multicolour QD-based competitive fluorescent immunoassay [50] and QD-based lateral flow immunoassay system [51] for specific analyses were developed. Based on the current rate of increase in the number of quantum dots' applications it can be concluded that this number will still be growing in the coming years.

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