Fluorescence properties of quantum dots used in the study of microorganisms

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Semiconductor quantum dots (QDs) are robust and bright light emitters that have captured special interest in biology and medicine due to their superior optical features. This paper is a study of the fluorescent properties of QDs and their role as fluorescent probes for various biological applications. The bioimaging applications, with reference to the study of microorganisms, are discussed. QDs are rapidly being employed for a series of existing and emerging technological applications, but our study deals with their capacity of microorganism labeling.

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1. Introduction

Nanotechnology is an area of research comprising multidisciplinary studies (including chemistry, physics, engineering and biotechnology). It is based on the fact that physical and chemical properties of matter change dramatically at nanoscale, in contrast with those that they exhibit at the macroscale. Among many different nanotechnology products, semiconductor nanocrystals or quantum dots (QDs) have a potential to lead to major progress detection and imaging as a new class of fluorescent probes in biological field.

Semiconductor QDs are highly molecular sized semiconductor nanocrystals, in the range of about 10 nm and consist of hundreds to hundreds of thousands atoms. They are generally composed of atoms from groups II and VI (e.g., CdSe and CdTe) or groups III and V (e.g., InP and InAs) of the periodic table.

Many of the traditional fluorophores (organic dyes and protein - based fluorophores) suffer from chemical and photophysical limitations, which narrow their effectiveness in long-term imaging and multiplexing. Some of these limitations are pH dependence, susceptibility to photo-bleaching, narrow absorption windows, broad red-tailed emission spectra, small Stokes shifts, and short excited state fluorescent lifetimes [1].

In contrast with the traditional fluorophores, QDs have unique optical and electronic properties, which include: high quantum yield, high molar extinction coefficients, broad absorption, large effective Stokes shifts, high resistance against photo-bleaching and against photo -and chemical degradation [2]. The researchers mentioned two QD properties of interest to biologists: their ability to size-tune fluorescent emission as a function of core size, and their broad excitation spectra, which allow excitation of mixed QDs at a single wavelength [2]. Due to their large surface area-to-volume ratio, a single QD can be conjugated with various molecules, thus making them ideal candidates for the use in designing complex multifunctional nanodevices.

These properties give QDs tremendous advantages over the traditional fluorophores and open the doors for QDs to an increasing variety of biological applications.

In this paper, we focus on the photophysical properties of QDs and discuss their role as fluorescent probes for the study of microorganisms.

2. Photophysical aspects of QDs

QDs are fluorophore nanocrystals whose excitation and emission are fundamentally different than those of the traditional organic fluorophores. By optical excitation, the exciting of the electrons from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) occurs in organic fluorophores. Instead of these types of transitions, QD fluorescence implies the exciting of an electron from the valence to the conduction band, making it a conduction electron and leaving behind a hole. Thus the so-called "excitons", electron-hole pairs, are generated in semiconductor QDs upon excitation. The eventual recombination of electron-hole pair, i.e. the relaxation of the excited state, leads to the emission of a characteristic photon with an energy that matches the energy of the bandgap. This emission is known as fluorescence.

The QDs discrete size-dependent energy levels are the result of the confinement of the charge carriers (electrons, holes) in three dimensions [3-7]. As a consequence, the

energy difference between excited and ground state (i.e., the gap energy) of a QD strongly depends on its size [8].

The wavelength of fluorescence depends on the bandgap and thus, on the size of the QD [3, 9-11]. Therefore, QDs of the same material, but with different sizes, can emit light of different colors. The larger the dot is, the redder (of lower energy) its fluorescence spectrum. When the semiconductor QDs get smaller, the spacing between energy levels increases, so that the radiation from QDs suffers a blue-shift (to higher energy), which is similar with the "particle-in-a-box" model from quantum theory.

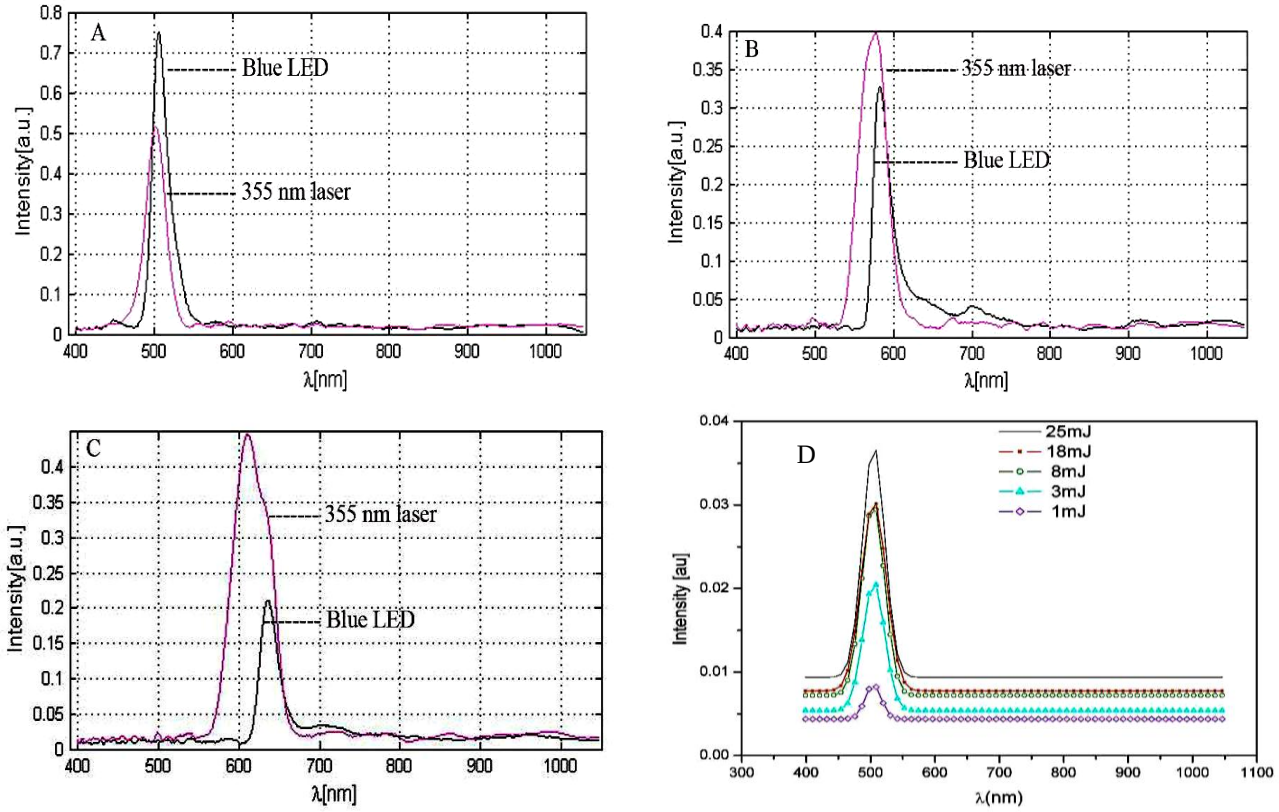

Fig. 1 The fluorescence spectra of CdSe/ZnS core-shell QDs of different sizes, recorded with an ARCspectro HT-HR (ARCOPTIX S.A. Switzerland) Fourier Transform Spectrometer (FTS). A. Fourier Transform spectra of the 0490 QDs fluid for two excitation sources: a laser (NdYAG@355 nm) and a blue LED; B. Simultaneously presented spectra of the 0560 QDs fluid for the excitation sources: a laser (NdYAG@355 nm) and a blue LED; C. A superposition of two spectra for the 0600 QDs fluid in the case of those two excitation sources that were used; D. Quintet Fourier transform spectra at increasing energy of the laser. When the energy of the laser grows, the height of the main peaks also increases and the main fluorescence band broadens.

In order to reduce the process of recombination of electron-hole pairs without emitting fluorescence light (i.e., by the non-radiative relaxation on the QD surface) and to result in brighter emission, QDs can be synthesized with a second shell of a semiconductor material, which has a higher bandgap. This shell can be grown around the semiconductor core [12]. For QDs to be useful probes for the study of biological specimens, the surface must be hydrophilic. Therefore typical water soluble nanocrystals consist of [3]:

a) a semiconductor core (e.g. CdSe, CdS, CdTe), whose diameter decides the wavelength of fluorescence,

b) an (optimal) shell of a semiconductor material with higher bandgap than the material of the core (e.g. a ZnS shell around CdSe core) in order to increase the quantum yield,

c) a hydrophilic coating which provides a surface for conjugation to biological and nonbiological species, warranting their water solubility.

QDs display several favorable features compared to organic fluorophores, one of which is their unique optical spectra. Traditional fluorophores typically have narrow absorption spectra, i.e. they can only be optically excited within a narrow window of wavelengths. Additionally, they have asymmetric fluorescence spectra broadened by the so-called "red tail", i.e. a tail to longer wavelengths. On the other hand, QDs have broad absorption spectra, enabling excitation by a wide range of wavelengths. Fluorescence can be excited with any wavelength shorter than the wavelength of fluorescence [1, 13]. This favorable spectral property allows excitation of multicolor nanocrystals of various sizes using one single wavelength (light source), making them excellent for practical multiplex assays. The possibility to select any wavelength shorter than the wavelength of fluorescence is a useful property for the reduction of the auto-fluorescence of biological probes [3]. This feature of QDs can be achieved

by choosing the most appropriate excitation wavelength for which auto-fluorescence is minimized.

The fluorescence emission spectra of QDs are relatively narrow (these spectra are about twofold more narrow than traditional fluorophores [1, 13]), roughly symmetrical Gaussian-shaped and do not exhibit a red tail. The red shift of the emission spectra, with respect to absorption spectra, known as Stokes shift, is one of the most important quantities that determine the optical properties of the QDs. Stokes shifts of the semiconductor QDs can be as large as (300-400) nm (very large Stokes spectral shifts [14]) and can be used to further improve detection sensitivity. These characteristics allow different QDs with various emission spectra wavelengths to emit different colors when excited by a monochromatic light. As a consequence, the QDs are the best candidates for optical bar codes for multiplexed assays [15, 16]. The emission spectrum can be tailored by controlling the QD geometrical size, shape and strength of the confinement potential.

These qualities of QDs captivated not only researchers from different areas of biology, but also physicists and chemists to biologists. Among various QDs, CdSe/ZnS core-shell are widely applied in biology due to their narrow emission bands that span the whole optical spectrum.

We have analyzed and evaluated the emission properties of various QDs produced by Evident Technologies by using Fourier transform visible spectroscopy [17]. We have used two excitations sources: a UV laser (NdYAG@355 nm) and a blue LED (λ=480 nm). The QDs have the dimensions in the domain of (3÷5) nm, and according with that, their emission is situated in the range (490÷600) nm.

In the figure 1A we obtained Fourier Transform spectra of the 0490 Evidot suspension, for two cases. For the first case, when we use for excitation the laser light from a NdYAG@355 nm, in order to irradiate the sample, we observed that the maximum of the peak intensity corresponds to an average wavelength calculated at 500 nm, with a root mean square error of 15 nm, for a value of integration time of 80 ms. In the second case, when the excitation source is a luminescent diode (λ=480 nm), we observed that the fluorescent wavelength which corresponds to a maximal value of the intensity rises to 511 nm with a root mean square error of 15 nm. In this case, the integration time was adjusted to a value of 40 ms.

We also examined the Fourier Transform spectra of the 0560 Evidot (Fig.1B) and found, for the first case, that the corresponding average wavelength is 572 nm and the root mean square error is 19 nm, whereas for the second situation, the fluorescence wavelength is found at (588 ± 18) nm.

A superposition of two spectra for the 0600 QDs fluid is depicted in the figure 1C. When we irradiated the probe with the laser light, the corresponding average is (14 ± 22) nm and in the case with the blue LED, the average wavelength is (634 ± 21) nm. The integration time was set at 80 ms.

In the Fig. 1D one can see five Fourier Transform spectra for the following values of the laser energy: 1 mJ, 3 mJ, 8 mJ, 18 mJ and 25 mJ, respectively. For this last case we have used as a probe a 0520 Evidot suspension with the concentration 23 μl QD in 3 ml toluene in a quartz container. The value of the integration time was fixed at 150 ms.

The Gauss fitted curves represents experimental data taken at room temperature and independent normalized, obtained in our group [17]. By irradiating QDs with the laser or luminescent diode light, a clear spectral shift of QDs fluorescence spectra correlated to exciting emission wavelengths is observed.

The QDs also have molar extinction coefficients (ε) of the order of 5.5×10^6 $M^{-1}cm^{-1}$ [18]. Due to their large ε values, combined with a high quantum yield (typically of 20-70%), QDs exhibit excellent brightness. It has been estimated that QDs are 10-20 times brighter than organic dyes [14].

The tunable frequencies of QDs make them more resistant to photobleaching, a problem of particular importance for experiments which involve long term imaging, such as fluorescence labeling of transport processes in cells or tracking the path of single membrane bound molecules [3, 19, 20]. Whereas organic fluorophores can undergo irreversible light induced reactions, upon optical excitation, resulting in loss of fluorescence and limit their imaging capabilities, QDs used as imaging agents do not erode. This reduced tendency to photobleach increases QDs photostability, which facilitates the construction of 3D cell images and the quantification of fluorescent signal intensity. Some studies have been reported the so-called phenomenon of "photo-brightening" which adverts to the initial increasing of the QDs fluorescence intensity upon optical excitation [3, 21, 22].

There are also some photophysical properties of QDs that can, in some cases, be disadvantageous. One of these is the property called "blinking" or fluorescence intermittency, that is, QDs switching between two states: an emitting state-on and a nonemitting state-off [23, 24]. Blinking frequency is dependent on the excitation intensity. The reason of this behavior is not well understood even today. However, in a recent study it was suggesting that this problem can be solved by bathing the dots in a chemical solution used as a food additive [25]. In this way, the research team from JLA/NIST increased the photon emission rate of the quantum dots, which is generally considered an immutable property of the dot. The scientists reduced the average delay time between excitation of a QD and the photon emission by four- to fivefold (from 21 ns to 4 ns), while reducing the probability of blinking up to 100 fold.

Another interesting fact is that QD remains fluorescent long time after the external stimulus has stopped. Colloidal QDs have fluorescence lifetimes of 10 to 40 ns [20, 26] which are superior those of the traditional organic dyes. If the time-gated detection is combined with the longer fluorescence lifetime of QDs, then the separating the QDs fluorescence from background

fluorescence (a technique known as time-domain imaging) could be achieved. Thus, the signal-to-noise ratio can be significantly improved. For this argument, the technique mentioned above has been used for colloidal CdSe/ZnS QDs as contrast agents, inside the fibroblast [27].

Additionally, the enormous two-photon absorption cross-section of colloidal QDs is up to two to three orders of magnitude higher than that of organic dyes [28], making them ideal candidates for two-photon excitation. Due to this property, typically all CdSe/ZnS QDs are quite well suited for cellular imaging.

3. Applications of QD in the study of microorganisms

The unique optical properties of QDs such as size tunable light emission, improved signal brightness, excellent photostability, broad absorption and narrow emission bands and the longer excited state lifetime, make QDs exceptional substitutes for traditional organic dyes in a variety of biological applications.

QDs have been found to be useful in morphological study of microorganisms in order to evidence their form, position, evolution, number, and so on. Regarding the processes of labeling with QDs, we must mention a major branch of applications of QDs which rely on the various methods for rapid and sensitive detection of different bacteria. These techniques are extremely important in biotechnology, medical diagnosis, and food safety. Many papers reported immunoassays with QDs-labeled antibodies and enzymes, in order to rapidly detect and identify various microorganisms [29], while limited used CdSe/ZnS core-shell QDs, as microbial labeling, both for pure cultures of cyanobacteria (*Synechocystis* PCC 6803) and for mixed cultures of phototrophic and heterotrophic microorganisms, thus opening the possibility to check the QDs usefulness as tools for total counting of microorganisms, instead of classical fluorochromes [30, 31, 32]. Our group obtained results concerning labeling of the biological samples containing cultures of the microorganism mentioned above, with 0520 Evidot suspension, which were incubated in darkness at room temperature.

As one can observe in Fig. 2 and 3, the natural samples containing filamentous cyanobacterial cells can be easily seen and microscopically (OPTIKA B-352 LD2) examined by transmission in white light or by epifluorescence, using the blue and green filters [17, 30].

As one can see in the figures 2 and 3, the individual filamentous cyanobacteria cells, in the natural samples evidenced in visible microscopy Fig. 2, 3 (left), can be easily seen due to the QD attached on them: QD green fluorescence (Fig. 2) which in Fig. 3 (middle) is overlapping with the natural red fluorescence of cyanobacteria.

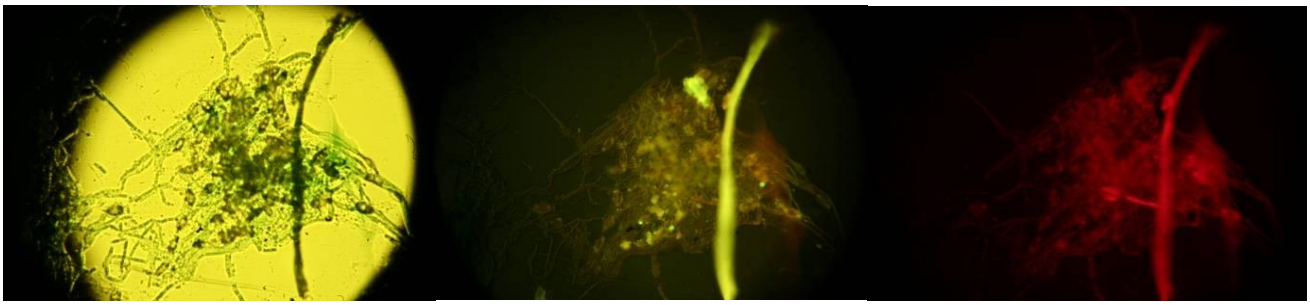

Fig. 2. Microscopic appearance of QD - labeled cyanobacterial cells from natural samples (sample 1): Left - transmission in white light; Middle - epifluorescence using the blue filter; Right - epifluorescence was using the green filter (for natural chlorophyll fluorescence).

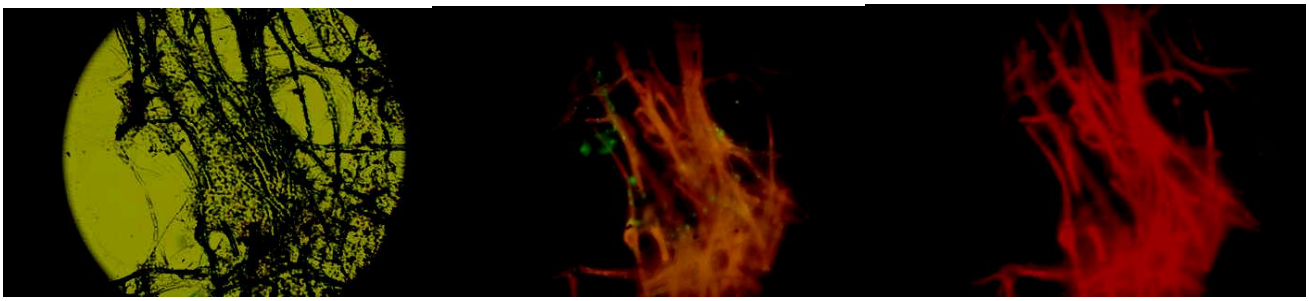

Fig. 3. Microscopic appearance of QD- labeled cyanobacterial cells from natural samples (sample 2): Left - transmission in white light; Middle - epifluorescence using the blue filter; Right - epifluorescence was using the green filter (for natural chlorophyll fluorescence).

As one can see in the figures 2 and 3, the individual filamentous cyanobacteria cells, in the natural samples evidenced in visible microscopy Fig. 2, 3 (left), can be easily seen due to the QD attached on them: QD green fluorescence (Fig. 2) which in Fig. 3 (middle) is overlapping with the natural red fluorescence of cyanobacteria. At this time we cannot, however, distinguish between the following three theoretical possibilities:

a) QDs are attached only at the exterior of the microbial cell,

b) QDs are only inside the microbial cells,

c) QDs are attached both at the exterior and inside the microbial cells.

It is interesting to see also the fact that unicellular heterotrophic bacteria which are live together with cyanobacteria in natural environments are labeled by the green QDs, and can be viewed accordingly. These heterotrophic bacteria have no natural fluorescence to be seen with a microscope, so the light emitted by the QD is not mixed with other fluorescent light, as is the case with the filamentous cyanobacteria which naturally emit a red fluorescence. In Fig. 2 (Middle) and 3 (Middle) can be observed green points which have not correspondence in the visible microscopy images and in epifluorescence with green filter images. We suppose that in this case we have heterotrophic bacteria labeled with quantum dots.

4. Conclusions

By exploring and understanding of the QDs optical properties at ensemble and single – particle level, this study seeks to provide insight into their applicability for biomedical detection and optical imaging applications in a biological milieu. If the issues related to the potential toxicity of QDs will be solved, they will certainly be one of the valuable constituents of the envisioned multifunctional nanodevices enable to detect, treat and monitor the diseased tissues, in real time.

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