Passive nonspecific labeling of cyanobacteria in natural samples using quantum dots

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The aim of this paper is to investigate the nonspecific labeling of cyanobacteria in biological samples with quantum dots (QDs). We focused on some interesting features of interaction between CdSe/ZnS QDs and filamentous and unicellular cyanobacteria from marine and sulfurous mesothermal springs. The fluorescence microscope images indicated that the labeling of the unicellular cyanobacteria with QDs did not take place, but in exchange, these QDs migrated in the direction of filamentous cyanobacteria and remained fixed on their sheaths. Based on digital epifluorescence image analysis, we tried to explain the color changes of cyanobacterial filament labeled with QDs, by adding supplementary QD quantities to the cyanobacteria cultures.

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1. Introduction

Cyanobacteria are the largest and most diversified, ecologically most successful and evolutionarily most important group of prokaryotes [1]. They are clearly defined by the ability to carry out both oxygenic photosynthesis in their thylakoid membranes and respiration in both cell membranes and thylakoid membranes [2,3] with the ability to live in very different conditions [4].

Biological samples containing bacteria are generally studied in order to evidence different bacteria species and to analyze their evolution characteristics. In this respect, the use of epifluorescence microscopy to study different aspects of prokaryotes, both at population and single cell level, significantly improved the knowledge concerning species present in a given sample, the cell density and the metabolic status of the population as a whole or of single prokaryote cells [5-8]. In the last decades there is also an increase in the development and use of different softwares for automated analysis of the digital images [9-15] in order to understand the final epifluorescence image.

The difficulties in evidencing the small bacteria, their number and activity urge to use new methods for their labeling and detection, including the use of quantum dots (QDs). The QDs are semiconductor nanocrystals known to show important optical characteristics like photostability, fluorescence yield, broad absorption spectra and size-dependent narrow emission spectra, leading to major advances in medical diagnostics, targeted therapeutics, microbiology, molecular and cell biology [16-20]. The properties of QDs, especially broad absorption spectra, size-dependent narrow emission spectra and high fluorescence yield could significantly improve the investigation of the cells, including small ones like bacteria [21-23]. Generally two main tasks are targeted in using QDs in microbiology: the labeling of microbial cells [22, 24-26] and the study of cytotoxic effects of QDs [27-29].

In this paper we are studying the QD attachment on biological samples of cyanobacteria, the interaction of the QDs with the bacteria cultures and the effect of CdSe/ZnS QDs upon the fluorescence color of the QDs bound to cyanobacteria.

2. Materials and methods

In order to analyze the QD interaction with bacteria we have used both enriched natural samples and microcosms (*vide infra*).

Natural samples were prepared on the basis of cyanobacterial samples with contaminants collected in October 2008 and May 2009 from sulfurous mesothermal spring (Obanul Mare) placed near Mangalia City (43˚49’53.6’’N; 28˚34’05.3’’E). Samples containing bacteria are studied in order to control the safety of the local specificity of a mesothermal and sulfurous ecosystem in the natural environment from Obanul Mare. The specificity of this site related with the moderate temperature all over the year and the presence of rather high content of hydrogen sulfide are important for the

specific microbiological group of organisms but also for their influence on the macro-systems.

We used also natural samples of cyanobacteria isolated from Black Sea because these bacteria have often been recorded as the dominant phototrophs in marine ecosystems [30], cyanobacteria being the most important primary producers of organic matter in aquatic ecosystems.

Natural samples from sulfurous mesothermal spring (Obanul Mare) inoculated in either BG11 (pH = 9.6 and pH = 8.6) or BG 0 media, were incubated in culture chambers at 25 ± 1 ºC and illuminated, for 10 weeks, with fluorescent tubes with a photon rate of 50 μmol m^{-2} s^{-1} at surface of the culture vessels. The bacteria culture contains unidentified filamentous cyanobacteria and also unicellular spherical cyanobacteria.

The second type of natural cultures (i.e., the microcosms) consists in natural samples harvested from the Black Sea. The microcosms construction was realized on the basis of the water samples collected from the Black Sea (0.5 m depth) in polyethylene transparent bottles. In our experiments, the microcosms (in volume of 1.5 L) of natural sea water were kept at ambient temperature and natural illumination for two months (from March 29 to May 30). They also contain unidentified filamentous cyanobacteria, and unicellular spherical cyanobacteria. The cells were subjected to different chemical and physical treatments to selectively eliminate different extraparietal constituents, in order to better understand the mechanism(s) by which QDs can label cyanobacterial cells.

However, due to the unicellular status, this kind of cells are more difficult to be seen by conventional epifluorescence microscopy as compared with filamentous cyanobacteria. This is why the following measurements were done on filamentous cyanobacteria present in sea water and sulfurous mesothermal springs.

In this work we have studied the interaction between QDs and different bacteria from the natural samples both by the QD fluorescence and natural fluorescence of cyanobacteria, in order to put in evidence the movement of the QDs and their attachments to these bacteria.

Table 1. General characteristics of the QD 0560.

Specifications	**Evidot 0560 nm**
Colour	Hops yellow
Emission	560 nm
Form	Solution
Crystal diameter	3.8 nm
Molar extinction coefficient	9.0E+04
Molecular weight	140 μg/nmol
Quantum yield	50 %
1st Exciton peak	540 nm
Emission peak tolerance	+/-10 nm

In order to label these bacteria with QDs we have used CdSe/ZnS core-shell QDs produced by Evident Technologies. For these QDs, all the specifications are given in Table 1.

The CdSe/ZnS QD interaction with bacterial cultures was studied both by optical and fluorescence microscopy. In fluorescence microscopy the cell specimens were illuminated with a large band light source. By this technique we can observe the bacteria autofluorescence and also the QD fluorescence. We have used a Zeiss Optical Microscope (Imager. Z1m). Images were captured with an AxioCam MRc5_digital camera. The images (2584 × 1936 pixels) were acquired at 500× magnification and stored as 376 KB, 224 KB, 385KB, 400 KB JPEG files.

For image processing we used ImageJ software (a public domain Java image processing and analysis program inspired by NIH Image for the Macintosh - http://rsbweb.nih.gov/ij/). The software was used to display, analyze, process and save 8–bit, 16–bit and 32–bit epifluorescence digital images, image format including JPEG, supporting "stacks" and hyperstacks, a series of images that share a single window.

In order to study cyanobacteria from marine samples, we created color histograms for captured microphotographs. Each picture was analyzed in three channels: red, green, blue and the mean intensity value of pixels were automatically calculated for any picture in the case of every red/green/blue channel, according to the instruction manual (ImageJ 1.44 user guide).

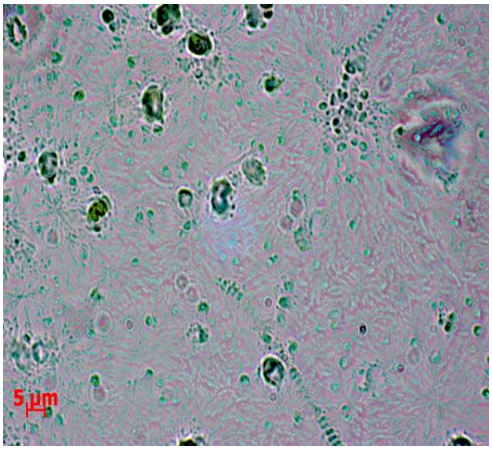
3. Results and discussions

3.1 Visible and fluorescence microscopy of the labeled cyanobacteria

Natural samples from Obanul Mare containing both filamentous and unicellular cyanobacteria were visualized and studied both in visible and fluorescence microscopy (Fig. 1).

Thye cell cultures were fixed on the microscope slides by evaporation to dryness. After visualization by visible and fluorescence microscopy, the samples were treated with known volumes of QD suspension. QD application was realized *in situ*, with the sample positioned under the microscope. In this way we have the possibility to visually inspect the QD movement and also to follow the same bacteria during a sequence of QD treatments.

We can see that the natural culture is forme of red fluorescent unicellular cyanobacteria (which appear as red points) and red fluorescent spiral filamentous cyanobacteria (Fig. 1B). The proper fluorescence of cyanobacteria is red, due to the chlorophyll content in their thylakoids.



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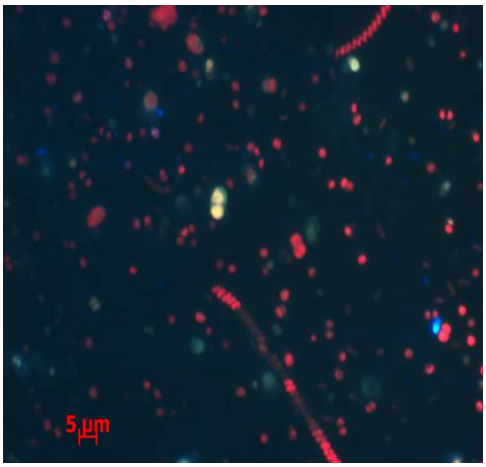
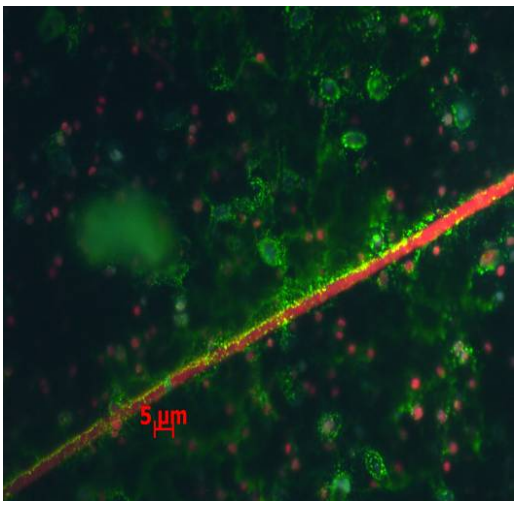


b

Fig. 1. Natural sample (from Obanul Mare) enriched in unicellular and filamentous bacteria: visible microscopy view (A) and fluorescence microscopy view (B).

In order to analyze the QD labeling characteristics of the natural samples, 2 µL suspension of QD 0560 (2 µg/mL working solution in toluene) was added to the already prepared cell smears. The visual inspection of the fluorescence under the microscope after the QD addition permitted us to notice the movement of the QDs (with green fluorescence) along the direction of the filamentous cyanobacteria up to a steady state image.

The fluorescence microscopy analysis of the stationary image have put in evidence that the green fluorescent QD 0560 are fixed onto the external sheaths of the filamentous cyanobacteria (Fig. 2A). It seems that the filamentous cyanobacteria do not take up, by endocytosis, the QDs. The red points represent the unicellular fluorescent cyanobacteria. They appear free of QDs because no green fluorescence can be seen around them. This means that no QD are fixed on the unicellular cyanobateria. However, some green spots are observed in fluorescence microscopy images in the presence of 0560 QDs. We suppose that these green spots are QDs fixed on surfaces of the existing of some eukaryote microorganisms which do not present a proper fluorescence (Fig. 2A). In order to remove this supplement of QDs, the samples were washed with distilled water and dried. Fluorescence microscope image (Fig. 2B) indicates that, indeed, these spurious QDs were removed from the external part of the filamentous bacteria, but by this operation, the fluorescence color of the filamentous bacteria was changed and the fluorescence intensity have became lower.



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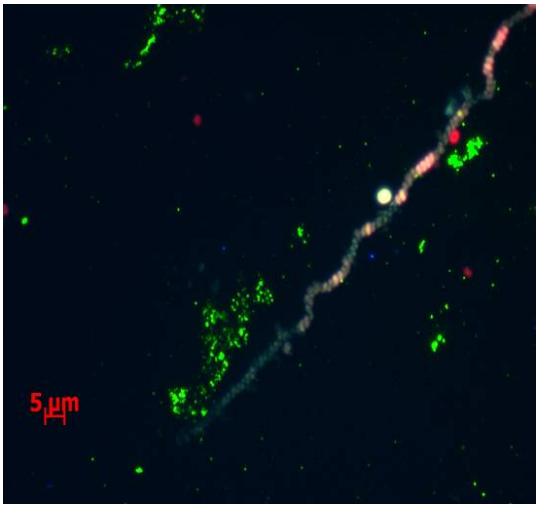


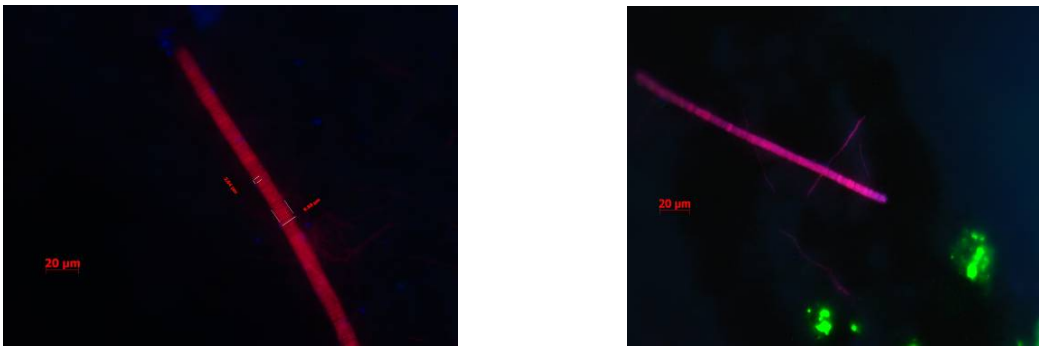
b

Fig. 2. Fluorescence image of natural sample (from Obanul Mare) with QD 0560 in solution added (A) and washed after QD 0560 addition (B).

The marine cultures containing unidentified filamentous cyanobacteria and unicellular spherical cyanobacteria were also microscopically analyzed in visible and in fluorescence, without and with QD 0560 added. In this case the effect of the amount of CdSe/ZnS QDs upon the fluorescence color of the QDs bound on cyanobacteria was analyzed. The fluorescence color evolution of the culture, after step by step addition of fixed

quantities of QD 0560, put in evidence the change of the cyanobateria fluorescence color. In Fig. 3, a sequence of fluorescence images of the marine sample smear in which QD 0560 were added, is presented. The fluorescence color of the filamentous cyanobacteria (Fig. 3A) is red which represents the proper color of the fluorescence of the cyanobacteria. After QD insertion in the marine culture, we have also observed that QDs have preferentially migrated towards filamentous cyanobacteria remaining fixed on them. After the green fluorescent QDs are fixed on the filamentous cyanobacteria, in a short time, a shift of fluorescence color from red to magenta is taking place (Fig. 3B). After 15 minutes, a new quantity of 2 µL of QD suspension was added and the change in color of the fluorescence image continues, the color becoming mauve (Fig. 3C). After other 15 minutes, 2µLof QD suspension was further added and the mauve color of fluorescence became more intensive (Fig. 3D). As it can be observed in this sequence of images, the fluorescence of eukaryotic microorganisms remains constant during the QD supplementary treatment after the first QD addition.

We have tried to explain the change of color from the red fluorescence to magenta as a superposition of colors, namely, the red fluorescence of chlorophyll with the green fluorescence from the QDs, but magenta represents a superposition of red color with blue one. Therefore in order to explain this color change, we made the digital analysis of the fluorescence microscopy images.



A. Cyanobateria natural fluorescence

B. At less than 1 min after QD addition

C. After a second quantity of QD was added

D. After a third quantity of QD was added

Fig. 3. Evolution of a filamentous cyanobacteria epifluorescence after QD 0560 addition in the marine sample smear.

3.2 Digital color analyses of the epifluorescence images

In order to explain the epifluorescence color changes effect of QDs added to the cell cultures on the fluorescence color cyanobacteria, digital image analyses were performed. Up to our knowledge this is the first report showing the change in the color of epifluorescence images of cyanobacteria, resulted from the deposition, step by step, of QDs on cyanobacterial filaments.

We studied digital images, which are two-dimensional grids of pixel intensity values. These images have the width and height defined by the number of pixels in *x* (rows) and *y* (columns) directions. Thus, the pixels are the smallest single components of images, holding numeric values (i.e., pixel intensities) that range between black and white. The obtained microphotographs were red, green, blue channels images, RGB/HSB stacks, and composite. In Fig. 4 the results concerning image color analysis of filamentous cyanobacterium at the start of the experiment,T0, with no QD addition, and at different times T1, T2, and T3 are presented.

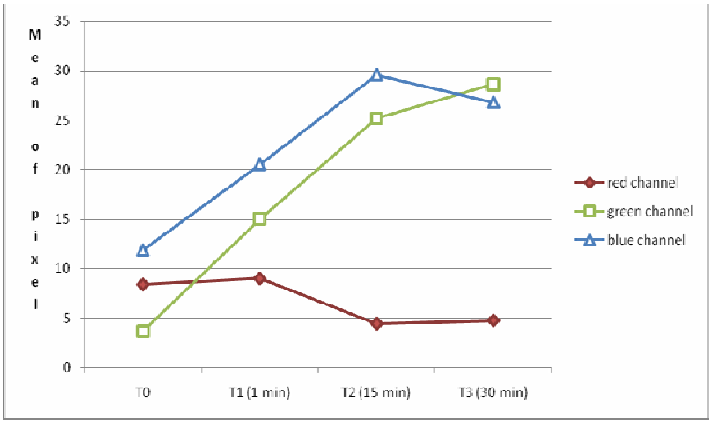


Fig. 4. The increase of mean value of pixels in green and blue channels, after QD 0560 addition.

Table 2. The mean value of pixels in RGB channels.

QD addition time	Red channel	Green channel	Blue channel
T0	8.41	3.65	11.87
T1 (first quantity of QD)	9.01	15.04	20.56
T2 (second quantity at 15 minutes).	4.42	25.21	29.60
T3 (third quantity at 30 minutes)	4.75	28.61	26.83

The above results show that the red channel pixel values remain small (T0-T3) after adding QD 0560, while in the case of green and blue channel the values increase after 30 minutes (Table 2).

Digital image analysis allowed us to distinguish from each analyzed images that green color appears immediately in filamentous cyanobacteria after adding QD 0560 and this value increases after supplementary quantities of QDs (30 minutes). Furthermore, ImageJ software allowed us to display simultaneously several selections or regions of interest (ROI). In order to increase the specificity of image, the analysis was further done only on ROI, according to Image J user guide.The processing of the original pictures was performed by subtracting the smooth background from the image. This command uses a "sliding paraboloid" or a legacy "rolling ball" algorithm that can be used to correct for uneven illuminated background, like in our pictures. This obtained light background allowed us the processing of images with bright background and dark objects and to visualize the color changes of the cyanobacterial filaments. The final results are shown in the Table 3.

Table 3. The mean value of pixels for filamentous cyanobacteria fluorescence in RGB channel with subtracted background.

QD addition time	Red channel	Green channel	Blue channel
T0	236.43	5.29	75.33
T1 (first quantity of QD)	223.20	27.96	168.09
T2 (second quantity)	128.93	92.32	225.94
T3 (third quantity)	117.97	96.55	228.46

The value of intensity in red channel decreased very softly after first QD treatment, but after second treatment this value drops to half and then becomes stationary. This behavior can be attributed to variation in red fluorescence of filamentous cyanobacteria. Green channel can be considered as following variation of intensity of fluorescence green QDs. Note that intensity in green channel increases about 5 times after adding the first quantity of QDs. After adding the second QD amount, the intensity of green channel grows more, while after

implementing the other quantities, the green intensity remains almost constant. The fluorescence intensity in the blue channel is greater than in the case of red and green channel even in the natural state (the blue channel was observed in images from the beginning of digital automated analysis of filamentous cyanobacteria).

3.3 Results analysis

We are supposing that the observed attachment of the QDs to the surface of the filamentous cyanobacteria is of electrostatic nature. In fact, QDs used (i.e., QD 0560) possess positive charges on their lateral amino groups. In the presence of the bacterial cell with the negatively charged carboxyl groups, the QDs are attracted on the external envelope of the cells forming the filaments. Due to this fact we have used in title the term "passive labeling" which indicates that between the cell envelope and QDs a passive attraction, basically of electrostatic nature is manifested. The main advantage of passive labeling is its simplicity: it does not require a further functionalization of the QD surface with a targeting ligand for labeling.

For the unicellular bacteria, present also in the natural samples, we have not observed the QD attachment. In this case, the use of coupling reagents or crosslinkers are recommended, in order to realize a coupling between QDs and bacterial cells. For example, it is reported the presence of the fluorescence signals of *Escherichia coli* cells labeled with CdSe/ZnS/SiO2 QDs and with the addition of the glutaraldehyde as crosslinker. In contrast, the same technique of labeling, but without the addition of the glutaraldehyde, showed that the fluorescent signals were not present [31]. This is a proof that the amino groups of CdSe/ZnS/SiO2 QDs and the surface of bacteria could be effectively coupled by covalent bounded glutaraldehyde.

By analyzing the decomposed images obtained by us, it is obvious that a mixing of colors (of the fluorescent emissions of the system components) does not occur. Indeed, by addition of the two basic colors (red-chlorophyll and green - quantum dots) will give yellow.

In order to understand the increase in the blue signal and the decrease in the red signal of cyanobacteria treated with QD 0560 suspended in toluene, we further treated the cell smears with toluene only. As one can see in the next image analysis (Table 4) there is an increase in the blue signal following the addition of pure toluene on smears, in the same portions, as in the experiment with QDs. The increase in the blue signal could be attributed to the effect of toluene on the intact cyanobacteria.

The mechanism of this effect is unknown, but we put forward that the increase in blue signal could be due to the interaction of toluene (and QDs) with an organic molecules having a blue fluorescence. This new radiation is superimposed over the remaining red from the chlorophyll fluorescence and green from the non-degraded QD particles (Fig. 3 C, D).

As for the red signal, as one can see in the Table 4, there is no decrease in it, but actually a small increase from 62 to 68 units, suggesting that the previous observed decrease in the red signal, when the cell was treated with QD 0560 suspended in toluene, could not be explained by pure toluene addition.

Table 4. The mean values of pixels for filamentous cyanobacteri fluorescence in RGB channels with subtracted background following toluene addition.

Channel/sample	Control sample	2 µL toluene	4 µL toluene	6 µL toluene
Red	62.00	64.25	66.22	68.13
Green	4.70	18.89	15.48	24.27
Blue	19.38	45.05	37.80	51.55

4. Conclusions

We have analyzed the nonspecific labeling of cyanobacteria both in natural samples and enriched cultures with CdSe/ZnS core-shell QDs with dimensions of about 4 nm and the effect of CdSe/ZnS QDs on the overall color of the epifluorescence microscopy images. The unicellular and the filamentous bacteria were considered in the samples.

We have observed that QDs are migrating along the direction of filamentous cyanobacteria and are becoming fixed on their sheaths. The epifluorescence microscopy images were analyzed by a digital color analysis method. Digital image analysis allowed us to distinguish the changes in color of cyanobacteria filament labeled with QDs and to analyze the mean value of pixels in red/green/blue channels of the specific region of interest. Up to our knowledge this is the first report showing the change in the color of epifluorescence images of filamentous cyanobacteria, suggesting the possible toxic effect of QDs on the cyanobacteria as shown by the decrease in red color following QD addition.

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