CdSe/ZnS QUANTUM DOTS CITOTOXICITY AGAINST PHOTOTROPHIC AND HETEROTROPHIC BACTERIA

Ioan I. ARDELEAN [a,c], Iris SARCHIZIAN [c] , Mihaela MANEA [a], V DAMIAN [b], I APOSTOL [b] , Marinela CÎRNU [a], A ARMAŞELU [d], I IORDACHE [b] and D APOSTOL [b]

[a] *Institute of Biology Bucharest, 060031 Bucharest, ioan.ardelean57@yahoo.com;*
[b] *National Institute for Laser, Plasma and Radiation Physics, Magurele; dan.apostol@inflpr.ro;*
[c] *Ovidius University, Constanţa 900470,* [d] *Transilvania University, Brasov, ROMANIA*

Abstract

Cytotoxicity of CdSe/ZnS core-shell quantum dots with long chain amine capping agent (fluorescence at 490nm; 520nm; 560nm and 600nm) was studied against different species of cyanobacteria (*Synechocystis* PCC 6803 and IS-7) and heterotrophic bacteria (*S. aureus* and *E.coli*) as well as natural assemblages of marine microorganisms. QD affects the electron transport related to energetic metabolism both in phototrophic and heterotrophic bacteria. To explain these results one take into account the physico-chemical properties of QD in connection with the ultrastructural differences between Gram-negative and Gram-positive bacteria and with the cellular localization of main energetic processes, respiration and photosynthesis.

Keywords: cyanobacteria, marine microbial populations, heterotrophic bacteria, dehydrogenase activity, resazurine

1. INTRODUCTION

Semiconductor nanocrystals or quantum dots (QD) are known to show desirable characteristics leading to major advances in different domains (medical diagnostics, targeted therapeutics, microbiology, molecular and cell biology etc.,)(1-5) one main tasks in Microbiology being the study of their cytotoxic effects (6-31) The aim of this paper is to investigate the cytotoxicity of CdSe/ZnS core-shell quantum dots with long chain amine capping agent (fluorescence at 490nm; 520nm; 560nm and 600nm) against different species of cyanobacteria (*Synechocystis* PCC 6803 and IS-7), heterotrophic bacteria (*S.aureus* and *E.coli*) as well as natural assemblages of marine microorganisms.

2. MATERIALS AND METHODS

2.1 Dichlorophenol indofenol (DCPIP) microplate assay.

In order o study the possible effect of different QD on natural assemblages of populations, natural sea water was supplemented with yeast extract and peptone 1% to promote cell growth and multiplication. The populations thus obtained after 24 hours of incubation in dark at 18ºC were used as biological material for the following experimental set up: i) 200 µL of natural assemblages of populations have been added in all wells (1-6/A-D) with the exception of blank (column 7- blank) where 200 µL of distilled water was instead; ii) different amount of QD have been added at time zero, as follows: 100 pg, 200 pg, 500 pg and 1000 pg were added in columns 2,3,4 and 5, respectively; iii) after one hour of incubation DCPIP was added in all wheels (1-7/A-D).

2.2 Resazurine microplate assay.

The experiments were done on *Synechocystis* PCC 6803 one of the most studied cyanobacterium (32) and on one of our isolate from mesothermal spring, Obanul Mare (Romania), the unicellular cyanobacterium

called so far IS-7 (Sarchizian and colab., manuscript in preparation). The strains were cultivated as previously reported (33), harvested in late exponential growth, re-suspended in either fresh growth medium (BG_{11}) (plate assay) or in saline solution (quantitative DCPIP measurements) at OD_{750}nm of 1 unit. Preliminary experiments were done on plates to screen for inhibitory effects on resazurine reduction when different QD concentrations were incubated different time with cyanobacterial suspension. The following experimental set up (Figure 2): i) 200 µL of cyanobacterial culture have been added in all wells (1-9/A-D) with the exception of blank (column 10) where 200 µL of distilled water was instead; ii) 200 pg QD (A- 483 nm; B- 522 nm; C-559 nm; D-609 nm) have been added at different time in each wells with the exception of blank(column 10) and control (column 1) in order to incubate the cells in the presence of the same amount of QD (200pg QD/200µl cell suspension) for different periods before the resazurin addition :24 h, 23, 22, 21, 20, 19, 18 and zero hours in columns 2,3,4,5,6,7,8 and 9, respectively; then iii) resazurin was added in all wells (1-7/A-D).

2.3 Gross dehydrogenase activity

Gross dehydrogenase activity following incubation of cells in the presence of QD (200pg QD/ 200µL) was measured (Helios spectrophotometer) in dark and in light following the reduction of DCPIP at 600nm in the presence of lipophilic electron carrier phenazine methosulphate (PMS - 0.1mM final concentration).

2.4 The citotoxicity of QD against heterotrophic bacteria

The citotoxicity of QD against heterotrophic bacteria was done using standard method (34).

2.5 CdSe/ZnS core-shell quantum dots

CdSe/ZnS core-shell quantum dots with long chain amine capping agent (fluorescence at 483nm; 522nm; 559nm; and 609nm) were produced by Evident Technologies.

3. RESULTS AND DISCUSSIONS

The study of marine microorganisms is under huge increase in the last two decades (35) and our ongoing interest is related to their dynamics related to nutrients and pollutants (36-40), including different type of nanoparticles.

As one can see in figure 1 clear differences in the ability of natural assemblages of marine microorganisms to reduce DCPIP appear at 30 minutes after DCPIP addition (and 90 minutes of incubation in the presence of QD) when the decrease in blue color in control (columns 1 and 6) as a result of DCPIP reduction is evident when compared with either blank (column 7) or with the reactions occurring in the presence of QD at given concentrations. The inhibitory effect of 609 QD is visible even at the smallest concentrations in our experiments 0.5pg QD/µL bacterial suspension (2/D).

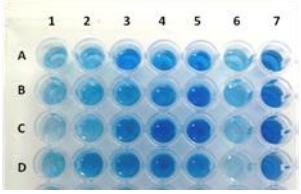
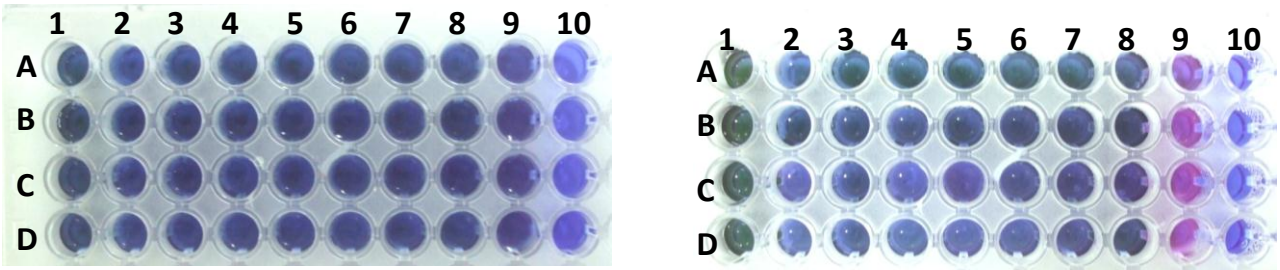


Fig. 1 DCPIP reduction by natural assemblages of marine microorganisms after 30 minutes of its addition to microorganisms previously incubated with different concentration of QD (see materials and methods for details).

The inhibitory action of these QD against natural assemblages of marine microorganisms, composed of many different cells belonging to different microbial species concerns the problem of the fate of these

nanoparticles in marine ecosystems, including the effect on different biological components performing specific task in that environment. The quantity of QD used in these preliminary experiments is not very low (pg/μL) but the inhibitory effect become evident in relatively short time , one hour after the addition of QD to the samples. In this respect, special attention is focused in this paper on the interaction between QD and cyanobacteria for longer periods of time, as these oxygenic phototrophic prokayotes are major contributors in aquatic environments to organic matter synthesis, carbon dioxide consumption and molecular oxygen production.

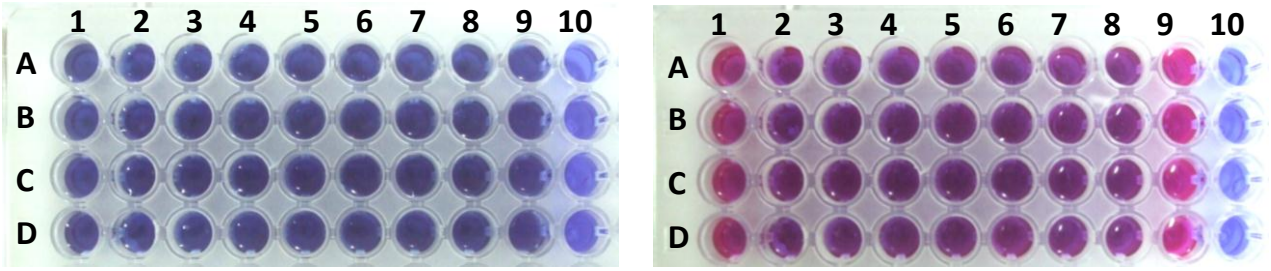
In light (Figure 2) and in darkness (Figure 3), in *Synechocystis* PCC 6803 the first differences were obtained at 3 hours after the addition of resazurin when column 9 (QD addition just before resazurine) shown a slight change in color as a result of resazurin reduction for all type of QD (483nm; 522nm; 559nm; and 609nm), these differences being more evident after 7 hours of reaction (results not shown). More differences between the effects of QD incubated together with cyanobacteria either in light or in dark appeared after 24 hours. In light, at 24 hours of incubation in the presence of resazurine in column 1 and 9 the reduction is more advanced then at 7 hours, whereas very low changes in color are visible in other wells, arguing that in light with all types of QD the suppression of metabolic activity by these nanoparticles is very severe.



Time zero: light

24 hours : light

Fig. 2 Resazurin reduction (pink color) by *Synechocystis* PCC 6803 incubated in light with 200 pg QD/ 200μL cell suspension (A- QD:483nm; B- QD:522nm; C-QD:559nm; D-QDE:609nm), for different periods of time (see Materials and methods for details).



Time zero: darkness

24 hours : darkness

Fig. 3 Resazurine reduction by *Synechocystis* PCC 6803 incubated in darkness with 200 pg QD/200μL cell suspension (A- QD:483nm; B- QD:522nm; C-QD:559nm; D-QDE:609nm), for different periods of time (see Materilas and methods for details).

In darkness after 24 hours in column 1 and 9 the reduction is more advanced then at 7 hours; moreover different degrees of reduction are visible in other wells arguing that even in longer incubation times in darkness with all types of QD the ability to reduce resazurin to its pink, semi reduced form (resorufin) is present in all experimental conditions. This is an important difference as compared with light incubation, suggesting that the cytotoxicity of these QD against *Synechocystis* PCC 6803 is stronger in light than in darkness. Up to our best knowledge this is the first report concerning the higher citotoxicity of QD against

cyanobacteria in light than in darkness, results which suggest that the interactions between photosynthetic cells and QD is stronger in light than in darkness.

However, taking into account the reduction of resazurin by *Synechocystis* PCC 6803 in the absence of QD (column 1 A-D in all figures) one can see that, after 24 hours, the reduction in light is practically complete (there is any pink color visible, suggesting that resazurin has been reduced to its incolor form) as compared with dark conditions where the control (column 1 A-D in figure 3) is pink, resazurin being (half) reduced to resorufin. In light (figure 2 column 1 A-D) it is visible the color of cyanobacterial suspension (blue-green) whereas in darkness the color of cyanobacterial suspension is masked by the pink resorufin, suggesting that this cell permeable redox substance can be reduce also inside the cell by photosynthetic electron transport.

Taking into account the well known higher chemical reactivity in light of these semiconductor nanoparticles (14, 15) one can think that this higher reactivity could be involved in the higher cytotoxicity of these QD in light. Whether or not there is an interaction of QD in light with the photosynthetic metabolism of the intact cyanobacterium is another interesting question. One can think that QD located at cell wall or cell membrane should interact with the thylakoids, membranes located inside the cytoplasm, *via* an unknown mechanism or/and because of very small diameters 4-6 nm of these QD their entry inside the cytoplasm could be taken into account, as well.

In connection with these assumptions, there are several reports concerning interaction between QD and photosynthesis. *In vitro*, it was experimentally demonstrated excitation energy transfer from the CdSe/ZnS quantum dots to the phycobiliprotein complexes isolated from the cyanobacterium *Acaryochloris marina* (24) and *in vivo* it was shown that the adsorption of CdSe/ZnS quantum dots on eukaryotic photosynthetic microorganism, the green alga *Chlamydomonas* sp. inhibits algal photosynthesis (41). Further research is needed to deeper understand the interactions between photosynthesis occurring in prokaryote or eukaryote and QD or other nanoparticles.

3.1 Gross dehydrogenase activity

Preliminary experiments shown that after 21 hours of incubation in darkness or in light with all the 4 types of QD used in these experiments at a concentration of 1 pg QD/ 1 µL the ability of either *Synechocystis* PCC 6803 or IS-7 to reduce DCPIP alone or in the presence of PMS is completely abolished, showing the cytotoxic effect of these QD in theses experimental conditions.

Following these results, new experiments have been designed to measure the cytotoxic effect -if any- at shorter incubation time, namely one or two hours. The incubation of cell suspension with QD were performed in light as well as in darkness in order to further test the interaction in light as compared with the dark incubation, as suggested by microplate assays done with resazurin. Incubation of *Synechocystis* PCC 6803 cultures in darkness together with QD for one or two hours induces interesting effects on the ability of these cells to reduce DCPIP in the presence of PMS (tables 1 and 2). As one can see in table 1 a stimulation of DCPIP reduction occurs in *Synechocystis* PCC 6803 incubated in darkness with QD as compared with the control (no QD).

Table 1 The effect of QD on DCPIP (via PMS) reduction by *Synechocystis* PCC 6803 incubated in darkness

darkness	Control	QD 483nm	QD 522nm	QD 559nm	QD 609nm
One hour	100%	380%	260%	1300%	1500%
Two hours	100%	1000%	460%	800%	1180%

In table 2 there are presented the quantitative results concerning DCPIP reduction in the presence of PMS by the unicellular cyanobacterium IS-7 cell, incubated in darkness for 1 or 2 hours with the four types of QD, The signification of these results remains to be elucidated.

Table 2 The effect of QD on DCPIP (via PMS) reduction by IS-7 incubated in darkness

darkness	Control	QD 483nm	QD 522nm	QD 559nm	QD 609nm
One hour	100%	68%	73%	92%	91%
Two hours	100%	60%	62%	71%	65%

Interestingly, Incubation in light of *Synechocystis* PCC 6803 or IS-7 cultures together with QD for one or two hours totally abolish the ability of these cells to reduce DCPIP in the presence of PMS (results not shown), arguing once again the higher cytotoxicity of QD in light than in darkness in these cyanobacteria.

3.2 The citotoxicity of QD against heterotrophic bacteria.

In order to study the interaction between QD and intact bacterial cell one question concerns the detection of any bactericide effect of the selected QD. In the next tables there are presented the inhibition of bacterial growth both dakness and in light for two species, *Staphylococcus aureus* (table 3) and *Escherichia coli* (table 4)

Table 3 The effect of CdSe/ZnS QD on the growth of *Staphylococcus aureus* in darkness and in light (the diameter of inhibitory growth zone is expressed in mm)

	1µg QD		1,5 µg QD		2µg QD	
	light	dark	light	Dark	light	Dark
483	0	2 mm	0	2 mm	2	3 mm
522	0	0	0	0	0	0
559	2	1 mm	2	1 mm	2	2 mm
609	2	2 mm	2	2 mm	3	3 mm

As one can see, the degree of inhibition is higher in darkness than in light in the Gram- pozitive *Staphylococcus aureus* as well as in Gram- negative *Escherichia coli* .It is difficult to explain why in darkness QD cause in these heteotrophic bacteria a stronger inhibition of growth than in light whereas in darkness QD cause in cyanobacteria ,*Synechocystis* PCC 6803 and IS-7 a lower inhibition of DCPIP reduction, at short time level (1-2 hours of incubation); however, at similar times of incubation (24 hours), the DCPIP reduction is compety abolished.

The inhinbition of growth in darkness is higher in *Escherichia coli* than in *Staphylococcus aureus.* However, to understand these differences and to search for the mechanism(s) of interactions there is the need to use more appropriate experimental designs and analytic methods.

Table 4 The effect of CdSe/ZnS QD on the growth of *Escherichia coli* in darkness and in light (the diameter of inhibitory growth zone is expressed in mm)

	1µg QD		1,5 µg QD		2µg QD	
	light	dark	light	Dark	light	Dark
483	1	2.5 mm	1	3 mm	2.5	7 mm
522	0	2 mm	1	3 mm	1	3 mm
559	1	3 mm	2	3 mm	3	3.5 mm
609	1	3 mm	1	3.5 mm	3.5	3.5 mm

Control experiments were carried out with toluene only or with inactivated QD (suspended in water for 2 days, when no fluorescence was visible at the microscope) showed no growth inhibition thus further arguing that, in these experimental conditions, QD themselves are responsible, directly or indirectly, for the reported results.

When it comes to the mechanism(s) responsible for the inhibitory effects of QD no original experiments have been done but, in agreement with the literature, one could think that the interactions between cells and QD causes the production of reactive oxygen species (ROS) (6, 11, 12) but other mechanism(s) could also be

involved. One important task in all these experiments concerns the physical relationship between QD and different microbial populations, with special emphasis on the position of QD towards cell wall and cell membrane. It seems logically to assume that the first site of interaction between these nanoparticles and cells is at the level of cell wall; however cell wall has rather different structure in Gram- negative bacteria (including cyanobacteria) and Gram-pozitive bacteria. The physical access of QD at the external face of the cell membrane (toward cell wall) is still an open question as well as the ability-if any- of these CdSe/ZnS core-shell quantum dots (with long chain amine capping agent) with dimensions in the range of few nanometers to pass through the intact (or previously damaged!) cell membrane to enter the cytoplasm.

5. CONCLUSIONS

The main conclusions obtained all four type of QD used in these experiments (1pgQD/µL culture) are:

1. In light, QD totally inhibit the gross dehydrogenase activity both in *Synechocystis* PCC 6803 and IS-7 even after one hour of incubation. Up to our best knowledge this is the first report on higher toxicity of QD against cyanobacteria incubated in light as compared with dark incubation.
2. In darkness, 1-2 hours of incubation with QD induce in *Synechocystis* PCC 6803 a strong increase in grosss dehydrogenase activity (from 260% to 1000%!) whereas in IS-7 occurs a decrease to 60-90 %.
3. In darkness, the inhibition of growth measured in the presence of CdSe/ZnS (1µg - 2µg in difusimetric method) is higher than in light, both in *Escherichia coli (*Gram- negative) and in *Staphylococcus aureus* (Gram - positive).

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