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# Of Quantum Dots and Microbes

## Smart materials for fluorescence based characterization of environmental microflora

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***Abstract*—Manganese doped zinc sulfide ($ZnS:Mn^{2+}$) quantum dots (QDs) were surface derivatized for use as versatile fluorescent reporters is the study of microorganisms of relevance in environmental bioprocesses. When intracellularly introduced into permeabilized target bacterial cells, mercaptoacetic acid (MAA) or dithiothreitol (DTT) treated $ZnS:Mn^{2+}$ QDs proved to be good visualization enhancers for morphological observations with epifluorescence microscopy. Alternatively, cell surface adhesion of chitosan capped $ZnS:Mn^{2+}$ QDs also led to a similar effect, namely of QD enhanced contrast of target cells from intrinsic background fluorescence. When DTT surface derivatized $ZnS:Mn^{2+}$ QDs were conjugated with oligonucleotide probes and used in fluorescent in situ hybridization (FISH) analysis, specific detection of bacterial strains representative of some proteobacterial classes was achieved.**

***Keywords*—quantum dots, surface functionalization, fluoresence in situ hybridization, fluoresence imaging, microbial characterization**

## I. Introduction

Various sectors of the economy nowadays are being significantly impacted by the development of novel materials in conjunction with nanotechnology. Active research and applications were seen in information technology, biotechnology, medicine, agriculture, energy, and environment, among others. Increasing demand for green technologies and clean energy has all the more stimulated research into applicable engineered nanomaterials for both their benefits and potential impacts to the environment. Potable water has been produced from low-cost nanostructures separation membranes [1]. Cheaper and more efficient solar cells are being developed using nanowires and other nanostructures [2]. Heterogenous nanophotocatalysts have been employed for water splitting to produce hydrogen [3] and gas-phase degradation of odor compounds for indoor air purification [4]. Interestingly, nanotechnology based methodologies that were originally developed for a specific area of application find utility in other areas. An example is the use of medical nanobiotechnology's photoluminescence based imaging technique in the study of microbial communities underlying environmental bioprocesses.

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One of the commonly used technique in molecular and cell biology which has been employed as a standard analytical tool in environmental microbiology, fluorescence in situ hybridization (FISH) employs specific oligonucleotide probes fluorescently labeled with organic fluorophores to visualize and identify microorganisms in biological samples and environmental matrices [5]. The use of organic fluorophores imposes a major limitation in conventional FISH as these are susceptible to photodegradation. Experiments are hence carried out in dark conditions, otherwise expensive additives are used to minimize photobleaching. Alternatively, semiconductor quantum dots (QDs) can be used instead of organic fluorophores. Apart from having a wider excitation spectra, QDs are highly resistant to chemical and metabolic degradation and have a higher photobleaching threshold [6]. In the case of ZnS nanophosphor (Figure 1), UV excitation creates an exciton which proceeds to a shallow trap state and recombines with an electron resulting in violet emission, denoted by (a) and (b). In the presence of the $Mn^{2+}$ dopant, an orange emission either results from recombination of a bound exciton at $Mn^{2+}$ or by trapping a hole by $Mn^{2+}$, denoted by (a') to (c'). The colors hence emitted can serve as specificity reporters in epifluorescence microscopy.

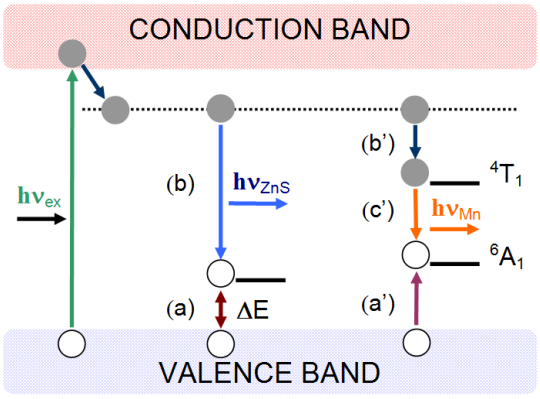


Figure 1. Schema of energy-transfer which leads to photoluminescence emission from ZnS and $Mn^{2+}$; energy levels not drawn to scale [7].

Presently, $ZnS:Mn^{2+}$ QDs (see Figure 2) were employed as fluorescent reporters of both unspecific and specific detection of model bacteria representing a range of classes under phylum *Proteobacteria*. Unspecific detection aimed to enhance cell microscopic images by biolabeling target cell cytoplasm or surfaces with MAA, DTT, and chitosan derivatized QDs. Specific detection, on the other hand, was achieved by linking specific oligonucleotide probes directly to $ZnS:Mn^{2+}$ QDs and introducing these into appropriately pretreated target microbial cells. Ensuing detection specificity can be a basis for use in FISH analysis of microflora of significance in environmental biotechnology.



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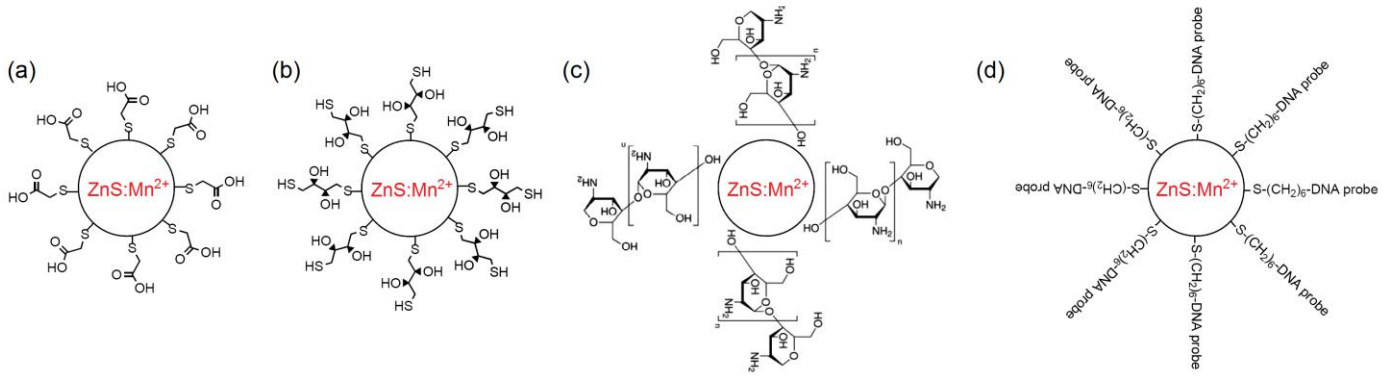


Figure 2. Schema of surface derivatized ZnS:$Mn^{2+}$ quantum dots: (a) MAA functionalized, (b) DTT functionalized, (c) chitosan coated, and (d) oligonucleotide probe ligated via the metal-thiol bond

# II. Materials and Methods

## A. *QD synthesis and characterization*

Details on the synthesis of $Mn^{2+}$ doped ZnS QDs and characterization using such techniques as scanning electron microscopy, high-resolution transmission electron microscopy, X-ray diffraction, and Fourier transform infrared spectroscopy were reported earlier[7,8]. Details on the synthesis and similar physicochemical characterization of chitosan passivated ZnS:$Mn^{2+}$ QDs can be found elsewhere [9].

## B. *QD surface derivatization*

A volume of as-synthesized ZnS:$Mn^{2+}$ was twice treated with 1.5 volumes of hexane and 4 volumes of ethanol for 2 min followed by centrifugation at 12000 rpm. Ultrasonically resuspended QDs in 1 volume of dimethylformamide were vigorously mixed with 5 volumes of 80% MAA or DTT for 2 min followed by centrifugation at 12000 rpm for 2 min with precipitates discarded. Supernatants (0.5 mL) containing either MAA- or DTT-coated QDs was precipitated with 0.25 mL of 4M NaOH and 0.25 mL of tetrahydrofuran and centrifuged at 12000 rpm for 2 min. Precipitates were redispersed in 100 µL sterile phosphate-buffered saline (0.1M, pH 7.5) and stored at 4°C until further use. Bioconjugation of QDs with DNA probes was done by adding 15 µL thiol-oligonucleotide probes to 35-50 µL to naked ZnS:$Mn^{2+}$ QDs followed by gentle shaking at room temperature overnight. The solution was centrifuged at 4000 rpm for 5 min to remove precipitates and used in FISH experiments.

## C. *Target cell preparation*

Late logarithmic phase cells of *Paracoccus denitrificans* JCM 6892 (α-*Proteobacteria*), *Alcaligenes eutrophus* TISTR 1095 (β-*Proteobacteria*), and *Escherichia coli* TISTR 357 (γ-*Proteobacteria*) were harvested from respective selective liquid media, homogenized by low-ouput ultrasound, and centrifuged at 7000 rpm for 3 min. Cells were twice washed with 0.5 mL sterile MillliQ water followed by centrifugation. To enhance cell wall permeability, harvested cells were treated with 0.1 volume of 1% Nonidet P40 substitute, Tween-80, Tween-20, polyethylene glycol (PEG), cetyl trimethylammonium bromide (CTAB), or sodium dodecyl sulfate (SDS) surfactants followed by centrifugation for 1 min at 6000 rpm. After discarding supernatants, cells were resuspended in 0.5 mL of 0.1% surfactant.

## D. *Cytoplasm or cell surface biolabeling*

For the introduction of QDs into the cytoplasm, 100 µL of pretreated cells were centrifuged and resuspended in 2X SSPE buffer (for 1 L: 17.53 g NaCl, 3.12 g $NaH_2PO_4 \cdot 2H_2O$, 0.74 g EDTA) with 50% formamide and 30 µL of MAA or DTT coated QDs in a total volume of 100 µL, and incubated at 42°C for 30 min. For the fluorescent labeling of cell surfaces, 100 µL of pretreated cells were centrifuged and resuspended in phosphate buffered saline (0.1M, pH7.5) with 30 µL of chitosan derivatized QDs in a total volume of 100 µL, and agitated at ambient temperature for 30 min. In both cases, 3–5 µL of labeled cells were applied on gelatin coated slides and stained with 20–30 µL of 4',6-diamidino-2-phenylindole (DAPI) prior to epifluorescence microscopy

## E. *FISH with QD-labeled probes*

Conventional FISH was carried out according to standard procedures [10,11]. In FISH with QDs, labeled probes, steps were performed according to reference [12] except that specific oligonucleotide probes targeting α-*Proteobacteria* (ALF1b), β-*Proteobacteria* (BET42a), and γ-*Proteobacteria* (GAM42a) were linked to naked ZnS:$Mn^{2+}$ QDs. The universal probe for *Proteobacteria* (EUB338) was labeled with cyanine dye (Cy3). About 100 µL of pretreated cells were centrifuged and incubated with blocking solution 75°C for 3 min and then at 42°C for 20 min. The blocking solution was removed by centrifugation and centrifugate was dispersed again in hybridization solution, incubated at 94°C for 1 min to enhance cell permeability and incubated at 42°C for 30 min. The mixture was centrifuged and washed twice with washing solution 42°C and at room temperature. Labeled cells (3–5 µL) were applied on gelatin coated slides and subjected to epifluorescence microscopy.



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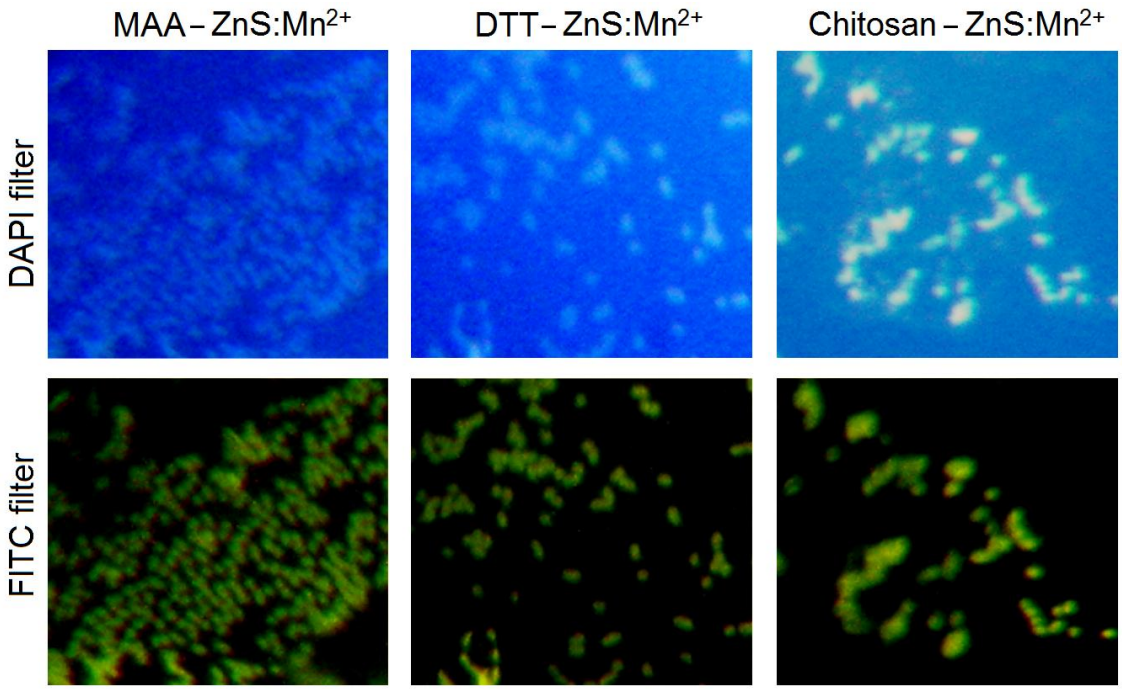


Figure 3. Representative images of intracellularly (MAA or DTT derivatized QDs) and extracellularly (chitosan coated QDs) biolabeled *E. coli* cells. Images obtained with the FITC filter (QD signals) show better contrast than with the DAPI filter (conventional DAPI stainig).

## III. Results and Discussion

Quantum dots are nanometer-sized crystalline semiconductors having unique fluorescent properties that gain popularity for potential application in advanced biosensors, bioanalytical assays, cell imaging, and in vivo animal targeting. A novel biological labeling material, QDs are considered complementary or even an alternative to the routinely used organic fluorophores [12]. The narrow emission spectra of QDs allows for multi-color excitation which implies the possibility for simultaneous use of differently functionalized QDs for a number of biological targets at the same time [13]. An example is the use of QDs in multiplex immunohistochemistry assay [14]. Unlike organic fluorophores, QDs are also photostable and resistant to biodegradation [6]. Although some are not known for cytotoxicity, techniques have been explored to avoid potential cell toxicity [15]. On the other hand, to fully exploit the potential of QDs for various diagnostic ends, issues of monodispersivity and specificity have to be addressed. Presently, monodisperse ZnS:$Mn^{2+}$ QDs were obtained by surface functionalization with MAA, DTT, chitosan, and oligonucleotide probes. Unspecific intracellular biolabeling of target cells has been achieved by translocating MAA- or DTT-derivatized QDs into the cytoplasm. Unspecific extracellular biolabeling of target cells has also been achieved via adhesion of chitosan coated QDs. Both techniques has improved the contrast of target cells in epifluorescence microscopy which can potentially be used in routine characterization of constituents of environmental microflora. Specific detection of target bacterial cells by using group-specific oligonucleotide probes labeled with ZnS:$Mn^{2+}$ QDs was also achieved, very useful in the study of microorganisms underlying fundamental biological processes in both the natural and engineered environments.

### *A. QD translocation in cell cytoplasm*

MAA and DTT, directly adsorbed onto the QDs surface, can help stabilize QD nanocrystals against flocculation which rendered the QDs soluble in polar solvents such as ethanol and dimethylformamide. The presence of partially or highly polar functional groups such as –OH and –SH makes the nanocrystals soluble in water. MAA- or DTT-surface derivatized ZnS:$Mn^{2+}$ can therefore easily translocate into the cytoplasm of surfactant pretreated target cells and interact with a range of biomolecules therein. As Figure 3 shows, fluorescence outlined by cell shape suggest throrought penetration and perhaps dispersion of QDs in the cytoplasm. On the other hand, of all the surfactants tested, the use of 1% Nonidet P40 substitute gave the best fluorescence signal (data not shown).

### *B. QD adhesion on cell surfaces*

Chitosan, alternatively, can stabilize QD nanocrystals as well against flocculation and could render them soluble in intermediate polar solvents such as ethanol and



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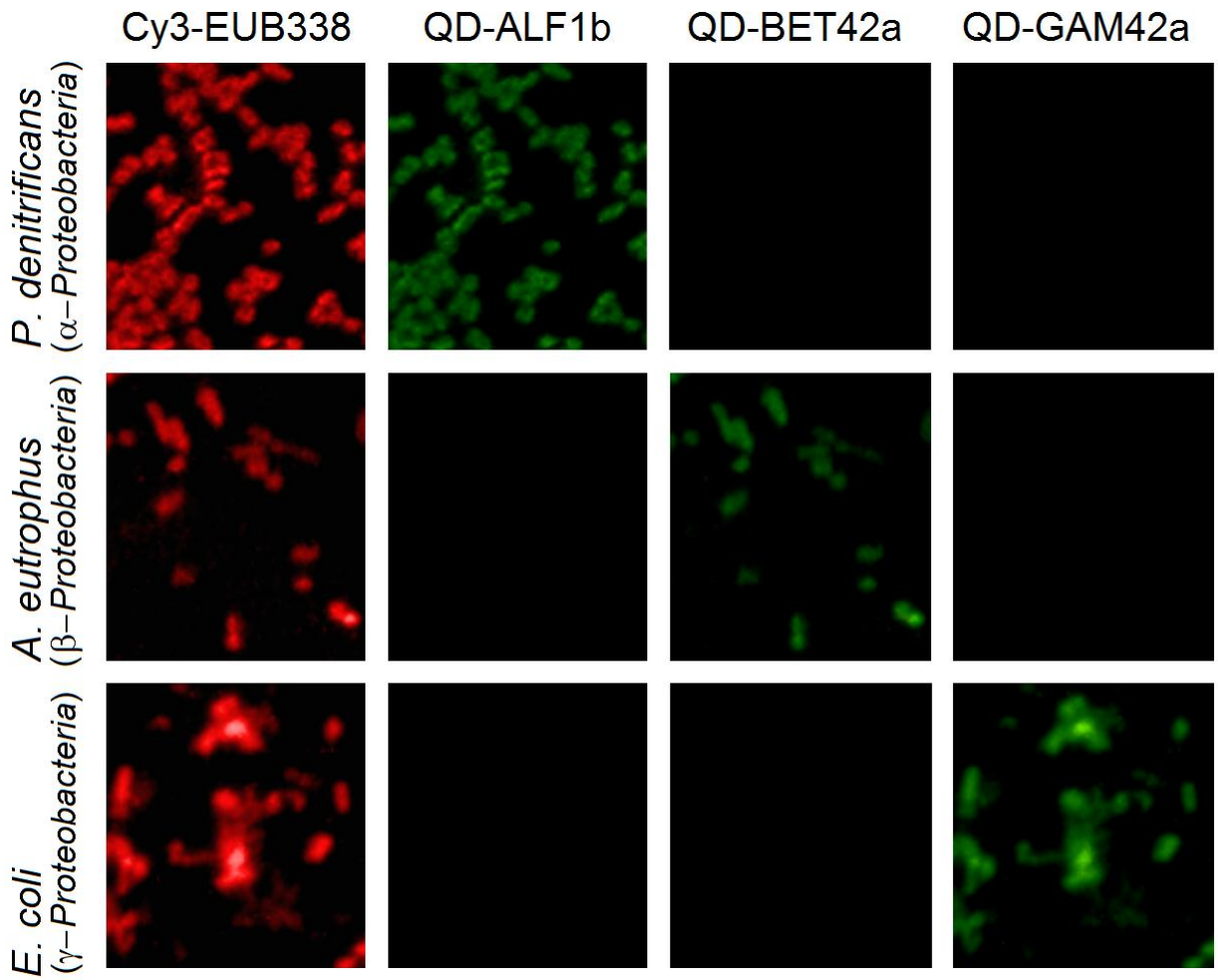


Figure 4. Representative FISH images show the hybridization of the *Proteobatceria* universal probe EUB338 to bacterial cells belonging to class α-, β-, and γ-*Proteobacteria*. Specific probes only hybridized with cells according to proteobacterial class.

dimethylformamide. The presence of polar functional groups such as $–NH_2$ and protonated counterparts makes the nanocrystals soluble in water at slightly acidic pH. Also, depending on the degree of decarboxylation in chitosan some –COOH may be present. The polycationic behavior of chitosan induce steric hindrances which results in the dispersivity of coated QDs. It also readily and unspecifically interacts with the negatively charged biomolecules on the cell surface. Since the target bacterial cells were pretreated with surfactants and formamide, the cracks induced by these agents on cell surface also allows the penetration of chitosan coated QDs into the cytoplasm with it unspecifically binds with any sub-cellular components therein. As Figure 3 shows, treated cells appear bulky due to both extracellular adhesion and intracellular uptake of QDs.

### C. *FISH specificity of QD-labeled probes*

Checking the specificity of oligonucleotide conjugated probes requires that these be used in FISH assays on both target and non-target bacterial cells. An alternative method would be to mix a known ratio of representative microorganisms and use a particular probe in FISH assay to check if signals reflect the ratio of the actual cell targets. As shown in Figure 4, all the targeted bacteria representative of different proteobacterial sub-classes hybridized with the universal probe EUB338 as expected among the *Proteobacteria*. This indicates that conventional FISH-wise, the assay procedure works well. Occasionally some cross-hybridizations were observed but specific QD-labeled oligonucleotide probes tend to be specific to the intended target bacterial cells. We found that the fluorescence intensity of QDs is higher than those determined by conventional dyes (results not shown).

## IV. Concluding Remarks

Whether used intracellularly (i.e. MAA-/DTT-surface passivated, oligonucleotide probe conjugated) or extracellularly (i.e. chitosan capped), $ZnS:Mn^{2+}$ QD biolabeling have consistently resulted in enhanced target cell



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epifluorescence which can be useful in routine observational study of environmental microbial communities. In permeabilizing the target cells for the internalization of the QDs, the use of Nonidet P40 substitute has resulted in better cell epifluorescence as compared to the use of other surfactants (e.g. Tween 20, Tween 80, PEG, CTAB, and SDS). Preliminary results suggest there is scope for cost-effective FISH based detection of multiple microbial groups in environmental samples with the use of specific oligonucleotide probe conjugated QDs that emit multi-color fluorescence. Anticipatedly, the latter can be achieved by incorporating various dopant ions in ZnS QDs for the multi-colored luminescence (i.e. different emission wavelengths within the doped metal sulfide bandgap) or size selective synthesis (i.e. quantum confinement) for use in the simultaneous detection of several target organisms in environmental bioprocess.

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