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Abstract

The present study examines the interaction of amine- and carboxyl- PEG core/shell quantum dots (QDs) with metal resistant bacterium Cupriavidus metallidurans CH34. The evolution of the number of QDs, their hydrodynamic radius, diffusion coefficients, and single particle fluorescence were characterized before and during the contact with bacterium by fluorescence correlation spectroscopy (FCS). The obtained results showed that at nanomolar concentrations the amine- and carboxyl-PEG-QDs with average hydrodynamic radiiues of 16.4 and 13.5 nm, form stable dispersions in the absence and presence of 15 mgC L(-1) HA. The decrease of the number of fluorescent particles in the bacterial medium, determined by FCS, together with the increase of the fluorescence of bacterial cells over the background, found by flow cytometry (FCM), demonstrated the association of QDs to C. metallidurans. Furthermore, QDs enhanced the level of the reactive oxygen species in the bacterial cells and augmented the percentage of the cells with damaged and leaky membranes as probed by FCM in combination with 5-(and-6)-carboxy-2'7'-dichlorodihydrofluorescein [...]
Amine- and Carboxyl- Quantum Dots Affect Membrane Integrity of Bacterium Cupriavidus metallidurans CH34

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The present study examines the interaction of amine- and carboxyl-PEG core/shell quantum dots (QDs) with metal resistant bacterium Cupriavidus metallidurans CH34. The evolution of the number of QDs, their hydrodynamic radius, diffusion coefficients, and single particle fluorescence were characterized before and during the contact with bacterium by fluorescence correlation spectroscopy (FCS). The obtained results showed that at nanomolar concentrations the amine- and carboxyl-PEG-QDs with average hydrodynamic radiuses of 16.4 and 13.5 nm, form stable dispersions in the absence and presence of 15 mgC L⁻¹ HA. The decrease of the number of fluorescent particles in the bacterial medium, determined by FCS, together with the increase of the fluorescence of bacterial cells over the background, found by flow cytometry (FCM), demonstrated the association of QDs to C. metallidurans. Furthermore, QDs enhanced the level of the reactive oxygen species in the bacterial cells and augmented the percentage of the cells with damaged and leaky membranes as probed by FCM in combination with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate and propidium iodide stains. No difference in the behavior of amine- and carboxyl-PEG-QDs was found, suggesting that different functional groups in the surface coating have no effect on bacterium-QD interactions under the studied conditions. The presence of HA does not affect the hydrodynamic characteristics of the functionalized QDs, but prevented the damage to the bacterial membrane. The slight decrease in the bacterial growth found after exposure of C. metallidurans to these QDs was attributed to the nanoparticles themselves rather the cadmium, zinc, or selenium ions released from the QDs.

Introduction

Engineered nanoparticles (NPs) can be considered as a new class of contaminants with a potential to persist, bioaccumulate, and exercise deleterious action in the environment. The properties of NP that make them useful in manufacturing different materials may also make them potentially biologically disruptive and of risk to the environment if they are in contact with living organisms (1, 2). Nonetheless, current understanding of the stability, fate, and ultimate impact of nanoscale materials in the environment is still very limited as pointed out in a several recent reviews (3–9). Since microorganisms are key players in many biogeochemical cycles and provide different environmental services, the present studies focus on their interaction with NPs and more specifically functionalized quantum dots (QDs).

QDs are semiconductor nanocrystals, identified as a particular class of NPs, and available in different sizes with various capping and surface coverage agents (10), which allow the study of how different characteristics specific to the NP affect their fate and impact on the environment. QDs are increasingly used in a variety of applications ranging from electronic materials and solar cells (11) to different biological applications (12, 13) for in vivo imaging, targeting, and diagnostics (14–16), therefore the potential for their release and impact on the environment is of growing concern. However, there is a paucity of data concerning QDs’ environmental behavior and interactions with environmentally relevant microorganisms. The limited data available are focused mainly on the toxicity of QDs and indicate that their toxicity to bacteria is to a large extent associated with the release of Cd and selenium ions from the weathering of QD cores (17), or intracellular QD concentrations (18, 19).

Association of QDs to alga Pseudokirchneriella subcapitata and the food chain transfer to Ceridophenia dubia have been also recently reported (20). Furthermore, toxicity of QDs to alga Chlamydomonas reinhardtii has been assessed at physiological, biochemical, and molecular genetic levels, demonstrating the existence of oxidative stress and up regulation of four stress response genes (21). Clearly, further mechanistic understanding of the interaction of microorganisms is needed as well as new adapted methodologies for their evaluation.

By exploring the capabilities of the single particle/cell methods such as fluorescence correlation spectroscopy and flow cytometry, the present study examines the interaction of functionalized quantum dots with the gram-negative bacterium Cupriavidus metallidurans CH34. The specific emphasis was on the association of QD with bacteria and the effect on the membrane integrity, cellular level of the reactive oxygen species (ROS), and growth. In parallel, QD stability in terms of number, hydrodynamic radius, and fluorescence of the individual particles under conditions of the bacterial exposure experiments was investigated. Gram negative bacterium C. metallidurans CH34 was chosen as a model microorganism because it is known to resist to millimolar concentrations of a variety of heavy metals and metalloids, including Cd (22) and Se (23).

Materials and Methods

Core/shell CdSe/ZnS quantum dots with a polyethylene glycol (PEG) surface coating coupled to carboxyl- and amine-terminal groups (EvITags, Evident technology, NY) were chosen because of their water dispersibility and special tailoring for bioimaging applications. Adirondack green EvITags with an emission at 520 nm, crystal core/shell
diameters of 2.1 nm, and a hydrodynamic radius of around 12.5 at 12 nmol mL\(^{-1}\) in water were purchased and stored at 4 °C in the dark for a maximum of 4 months. Fluorescence correlation spectroscopy (FCS) was used to determine the average diffusion coefficient and hydrodynamic radius, particle number and the fluorescence of individual particles, as well as the effect of humic acid and bacterial cell density on these characteristics. The association of QDs with bacterial cells, as well as their effect on membrane integrity and cellular level of ROS were evaluated by the flow cytometry (FCM). In addition, the total and dissolved (3 kDa - filterable) concentrations of the major components of the QDs, Cd, and Zn in the exposure medium, as well as their content in bacteria were also determined by measurements with inductively coupled plasma mass spectrometry (ICP-MS). The effect of QDs on bacterial cell growth was probed as well. Experimental details can be found in the Supporting Information (SI).

Characterization of the QDs in the Experimental Medium by FCS. FCS measurements were carried out with a Zeiss Confocal Axiovert 135TV (Carl Zeiss) using an argon ion laser for fluorescence excitation at 488 nm. Calibration of the FCS confocal volume was performed with rhodamine 6G (R6G), which has a known diffusion coefficient of \(2.8 \times 10^{-6} \text{cm}^2 \text{s}^{-1}\) (24). In FCS, variations in fluorescence intensity can be attributed to the Brownian diffusion of fluorescent species through a defined confocal volume (\(\sim 1 \mu\text{m}^3\)). Intensity variations were analyzed using the autocorrelation function, \(g(\tau)\) (25):

\[
g(\tau) = N \left( 1 + \frac{\tau}{\tau_c} \right)^{-1} \left( 1 + \frac{\tau}{\mu \tau_c} \right)^{-1/2} + 1
\]

where \(N\) is the average number of fluorescent particles diffusing into the confocal volume, and \(\tau_c\) is the characteristic diffusion time of a particle inside the confocal volume, \(\tau\) is the delay time. The parameter, \(p\), is a structural parameter that is the ratio of the transversal, \(\omega_{xy}\), to longitudinal, \(\omega_z\), radius of the confocal volume, \(p = \omega_{xy}/\omega_z\). Values of \(p\) are obtained from a calibration of the apparatus with rhodamine–6G (R6G). Two fluorescence components fit of the correlation function was used for a system containing QDs and humic acid. Diffusion coefficients are calculated from measured diffusion times, \(\tau\) (eq 2):

\[
D = \frac{\omega^2_{xy}}{4\tau}
\]

For particles that are rigid spheres, diffusion coefficients, \(D\), can be related to an equivalent hydrodynamic radius, \(R_{H}\), using the Stokes–Einstein relationship:

\[
R_{H} = \frac{RT}{6\pi\eta D N_a}
\]

where \(R\) is the gas constant, \(N_a\) is Avogadro’s number, \(T\) is the absolute temperature, and \(\eta\) is the viscosity of the solvent. The fluorescence corresponding to the individual particles (CPP) was calculated as a ratio between measured fluorescence and the number of particles in the confocal volume. Three series of nine measurements each were performed with 20 nM amine- and carboxyl-PEG–CdSe/ZnS and average diffusion time, hydrodynamic radius, number of particles, and count per particle were determined in the model experimental medium in the absence and presence of 15 mg C L\(^{-1}\) humic acid, different amounts of bacteria and bacterial supernatant. A standard humic acid isolated from the Suwannee River (SRHA) Georgia, obtained from the International Humic Substances Society (St. Paul MN), was used as a model of dissolved organic matter.

Study of Quantum Dot–Bacterium Interactions. Cupriavidus metallidurans CH34 (also Ralstonia metallidurans, Alcaligenes eutrophus) was cultured aerobically in a mineral salts liquid medium 284 with 10^{-2} M MOPS (3-(N-morpholino)-propanesulfonic acid) at pH 7.0 with 0.2% Na-glucanate as a carbon source at 30 °C in incubator INFOR with rotary shaking at 160 rpm. At the late log phases (OD\(_{600}\) = 1.8 ± 0.1) bacteria were isolated by gentle centrifugation at 3000 rpm for 10 min and washed two times with 10^{-2} M MOPS buffer solution at pH 7.0, then resuspended in the experimental medium containing 20 or 200 nM amine- or carboxyl-PEG-QDs with and without 15 mg C L\(^{-1}\) humic acid. Bacterial suspensions were passed through the flow cytometer (CyAn ADP from Beckman Coulter, formerly Dako) and signals were collected. Individual cells were identified using side scatter (SSC) and the pulse width of SSC. The flow cytometer was set to threshold on the side scatter signal, and this signal was also used to identify the main population of bacteria and thus excludes debris and the bulk of laser noise. Fluorescence of QDs associated with bacteria was measured with green laser excitation and a 530/40BP detector. Bacteria alone were used as negative controls. Bacterial membrane integrity was assessed by measurement of propidium iodide (PI) staining of cells pre-exposed to QDs in the absence and presence of HA (blue laser excitation and a 610/20BP detector). Measurements of the membrane damage in the presence of \(10^{-4}\) M of NaN\(_3\) were used as positive control. Cellular level of ROS in the absence and presence of QDs was probed by measurement of the fluorescence (blue laser excitation and a 525/50A detector) of cells stained with 1 µM 5-(and-6)-carboxy-2′-7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA). Cells were preloaded with carboxy-H2DCFDA for 15 min in the dark, exposed for 60 min to 200 nM QDs, and washed with and resuspended in 10^{-2} M MOPS. Measurements in the presence of \(10^{-3}\) M H\(_2\)O\(_2\) were used as positive control. In parallel, the association of QDs with bacteria was followed by measurement with violet laser excitation and a 660/10A detector. QDs with similar characteristics, but emitting at 655 nm were used in ROS—measurement experiments to avoid the overlap between QDs and carboxy-H2DCFDA emission spectra. All experiments were conducted in triplicate.

Statistical Treatment. Statistical differences in hydrodynamic radius, number of particles and fluorescence of QDs as well as membrane integrity were evaluated by one-way ANOVA followed by Student-Neuman-Keuls test at \(p < 0.05\) using Sigm Ans Plot 11.0.

Results and Discussion

Characterization of the Behavior of Amine- and Carboxyl-PEG-QDs. To correctly evaluate the potential impact of the QDs on bacteria, the behavior of QDs in the experimental solutions was assessed in terms of hydrodynamic radius, number of particles, and fluorescence per particle. The average diffusion coefficients of freely diffusing particles in dispersions containing 20 nM of amine- or carboxyl-PEG-QDs were evaluated to be equal to \((13 ± 2.9) \times 10^{-12} \text{m}^2 \text{s}^{-1}\) and \((16 ± 2.5) \times 10^{-12} \text{m}^2 \text{s}^{-1}\), respectively. The corresponding average hydrodynamic radii were 16.4 ± 2.5 and 13.5 ± 3.9 nm for amine- or carboxyl-PEG-QDs, respectively (Figure 1). The obtained \(R_H\) values are comparable with those found by asymmetrical flow field flow fractionation coupled with UV detection, giving weight average hydrodynamic radii of 17.0 and 13.4 nm for amine- or carboxyl-PEG-QDs (SI Figure S1). Addition of 15 mg C L\(^{-1}\) of humic acid or increasing amount of bacteria had no detectable effect on these measures. Furthermore no difference in the number of particles and the fluorescence of individual particle was found in the absence or presence of HA. The addition of bacteria to 20 nM QD dispersions resulted in a decrease of the number.
of fluorescent particles in the experimental medium. The number of dispersed QDs in contact with 10^4 or 10^5 cell mL^-1 bacteria decreased by about 5 and 18%, respectively, during 60 min incubation. No significant change in the fluorescence of individual particles was seen during this 60 min time period at cell density of 10^4 cell mL^-1 (Figure 1). When bacterial cell density was increased 10 times, a 30% decrease in the fluorescence of the individual QDs was found. Given the lack of decrease in fluorescence of the individual particles in the exposure medium and bacterial supernatant (Figure 1), the reduction in fluorescent intensity in the presence of higher bacterial biomass was attributed to the interaction of QDs with bacterial cells. The effect was more pronounced at higher bacterial biomass since the collision efficiency between QDs and cells increases. The above observations were further confirmed by following the changes in the number of QDs and single particle fluorescence intensity as a function of the contact time with bacteria (SI Figure S3). The obtained results are highly suggestive for the association of QDs with bacteria and/or loss of the fluorescence due to the changes in the QDs electronic environment, or surface degradation upon the interaction with bacteria.

Interestingly, similar tendencies were obtained for 20 nM carboxyl-PEG-QDs (SI Figures S2 and S3), suggesting that different functional groups of the surface coating (amine- or carboxyl-) have no significant effect on bacterium-QD interactions under the studied conditions (pH 7.0 and 10^-2 M MOPS).

**Association of Functionalized QDs to Bacteria.** FCM measurements showed that QD treated cells produced an increase over background fluorescence thus indicating that QD associated to the cells of *C. metallidurans* (Figure 2, Quadrants P2 + P3 and SI Figure S4). The addition of 15 mg CL^-1 HA resulted in no changes in the percentage of the cells emitting at QDs wavelength, suggesting that under the studied conditions HA do not significantly affect the association of QDs with bacteria. Furthermore, no statistically significant difference (p > 0.05) was found between bacterial cells exposed to amine- or carboxyl-PEG-QDs in FCM experiments (SI Figure S4), confirming further that the different functional groups in the surface coating do not influence bacterium-QD interactions under the studied conditions. These observations further imply that electrostatic interactions are of secondary importance in the possible adhesion of QDs to *C. metallidurans*. Indeed, the negative surface charge of the PEGylated QDs with carboxyl terminal groups is higher than that of QDs with amine groups (26, 27). Consequently, higher electrostatic repulsion could be expected with a negatively charged bacterial surface (28) and less carboxyl-PEG-QDs would associate with bacteria when compared with amine-PEG-QDs. Instead, no difference between the carboxyl- and amine-PEG-QDs treated cells was found implying that other mechanisms regulate the interaction with bacteria. These findings are consistent with other published data showing that negatively charged Ag NP accumulated on the negatively charged bacterial surface (29), but contrast to those found for the interaction of CeO_2 NP with *E. coli*, where adhesive interactions were considered to be important (30).

The association of the both amine- and carboxyl-PEG-QDs with *C. metallidurans* was further explored by measurement of the concentration of Cd, Zn and Se in the bacterial cells by ICP-MS (SI Figure S5). 4.9 (1.4% and 4.7 (1.1% of the total Se and Cd present in the bacterial medium containing 20 nM of amine-PEG-QDs were accumulated by bacterium following 60 min of contact time, whereas for Zn this percentage was about 51%. Furthermore, dissolved Zn concentrations determined by ICP-MS in the filtrates of the microcon ultrafiltration units...
FIGURE 2. Cytorams characterizing cells preloaded for 15 min with 1 µM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) and incubated for 1 h at different experimental conditions: (A) bacterial cell not exposed to QDs (negative control); (B) cells exposed to the carboxyl-PEG-QDs in the absence (C-PEG-QD); and (C) presence of 15 mg Cl L⁻¹ HA (C-PEG-QD+HA). Individual bacterial cells are represented by single points. Quadrant P2 includes red-fluorescent cells and indicates association of the QDs with bacteria. Quadrant P3 contains green plus red-fluorescent cells identified as cells containing QDs and increased ROS level. Quadrant P4 includes green-fluorescent cells, which are identified as cells with measurable ROS levels. (D) Fluorescence as a percentage of the maximum of C. metallidurans preloaded with carboxy-H2DCFDA and incubated for 1 h in the absence (NC), in the presence of 200 nM of carboxyl- (C-PEG-QDs); 200 nM of carboxyl- and 15 mg Cl L⁻¹ HA (C-PEG-QDs+HA); Zn and Cd denote bacteria in the presence of 200 nM of Zn²⁺ as Zn(NO₃)₂ and Cd²⁺ as Cd(NO₃)₂. PC denotes a positive control, which was 10⁻³ M H₂O₂. Note that in this experiment QDs with emission at 655 nm were used to avoid overlap with the emission of the carboxy-H2DCFDA dye used as ROS probe.

Effect on Cellular ROS Level. The influence of the 200 nM QDs on production of the ROS by C. metallidurans cells was probed by FCM using carboxy-H2DCFDA stain. This is nonfluorescent cell-permeant indicator, which is transformed to fluorescent deacetylated product (DCF, with 488 nm excitation/525 nm emission) by intracellular esterases and oxidation within the cell. Exposure to QDs induced an increase of the ROS level in the bacterial cells, as revealed by the appearance of cell population exhibiting both enhanced fluorescence signal at QDs and ROS-dye wavelengths (Figure 2, Quadrant P3). The addition of HA to the bacterial exposure medium containing QDs has no effect on ROS level (Figure 2C, D). Exposure of the bacterial cells to 200 nM of Zn or Cd nitrates, and 15 mg Cl L⁻¹ HA, resulted in a percentage of carboxy-H2DCFDA stained cells (ca. 4%) comparable with that for unexposed cells (Figure 2D). ROS were shown to be important players in mediating QD-induced cellular damage by "naked" QDs, which induce damage to the plasma membrane, mitochondrion, and nucleus, leading to cell death (31, 32). In the case of biotin, functionalized CdSe/ZnS, the induction of the DNA nicking has been proposed via ROS generation mechanism, involving the oxidation of the ZnS cap producing soluble SO₂ forming SO₄²⁻ free radical, which further oxidize to O₂⁻ and *OH (33).

Effect on Bacterial Membrane Integrity and Growth. The effect of 200 nM amine- and carboxyl-PEG-QDs on cell membrane integrity was probed by FCM using PI, a fluorescent nucleic acid stain that does not penetrate cells with intact membranes, while staining cells with damaged or leaky membranes (34). FCM analysis of PI stained cells demonstrated that both amine- and carboxyl-PEG-QDs induce an increase in the number of damaged/leaky membranes of C. metallidurans cells (Figure 3). The addition of HA to the bacterial exposure medium containing QDs resulted in a significant decrease (p < 0.05) in the percentage of membrane damaged cells. No increase in PI labeled cells with respect to control was found in the presence of Zn or Cd nitrates.
Membrane damage has been already observed for different metal containing NPs and bacteria as recently reviewed in ref 7. Examples include 14 nm-size ZnO and gram-negative bacterium E. coli (35); 4 nm halogenated MgO and three bacterial species: gram positive Bacillus megaterium, endospore forming bacterium Bacillus subtilis, and gram negative E. coli (36) as well as 12 nm Ag and E. coli (29) or Ag of different shape and E. coli (37). Fullerene water suspensions nC60 altered bacterial membrane lipid composition, phase transition temperature, and membrane fluidity of gram negative (P. putida) and gram positive (B. subtilis) bacteria (38).

The observed impairment of membrane integrity in the presence of QDs may disturb and alter several functions associated with the plasma membrane, respiratory activity, permeability barrier, etc.) and could be life threatening for bacterial cells (39). Indeed, a small decrease was observed in the bacterial biomass (measured by optical density, OD) for bacteria exposed for 60 min to the 200 nM of amine- or carboxyl-PEG-QDs (SI Figure S6). No effect of 200 nM Cd2+ and Zn2+ on membrane integrity and bacterial growth was found.

This is not unexpected, because C. metallidurans is known to resist to millimolar concentrations of a variety of heavy metals and metalloids, including Cd (22) and Se (23). The decrease in OD observed for bacteria exposed to QDs was thus attributed to the QD interaction with bacteria rather than to the Cd or Se ions release in the experimental medium. Similarly, the cytotoxicity to E. coli and B. subtilis of adenine- and AMP-conjugated QDs (diameters smaller than 5 nm) was correlated to intracellular QD concentrations (19). In contrast, coated QD or weathered QD aggregates were not found to be taken up by bacterial cells of B. subtilis, E. coli, and Pseudomonas aeruginosa, although the growth of these bacteria was still inhibited, in particular the respiration activity, respect QDs (17). In this case the bactericidal toxicity was related to the rapid release of Cd and selenium ions following QD destabilization under acidic or alkaline conditions.

The exact mechanism of impairment of membrane integrity by the QDs under the studied conditions is still unclear. Contact mediated lipid peroxidation via production of the reactive oxygen species can be assumed as a possible mechanism of membrane damage. This assumption is consistent with both the existing literature (7, 9, 40) and experimental observations of ROS generation in the present work. The experimental relationship between ROS generated by addition of increasing concentrations of H2O2 to C. metallidurans cultures, to simulate oxidative stress conditions, and the significant shift of PI fluorescence intensities, used as a measure for membrane damage (41) also support this assumption.

Overall, nanomolar concentrations of the amine- or carboxyl-PEG-QDs with hydrodynamic radius of 16.4 ± 2.5 and 13.5 ± 3.9 nm form stable dispersions in 10-7 M MOPS at pH 7.0. The particle number, fluorescence of individual particles and their average hydrodynamic radius were not significantly modified by the addition of 15 mg C L-1 HA. The decrease in the number of QDs in the exposure medium together with the increase of the percentage of the cell fluorescing at QDs emission wavelength demonstrated the association of QD with C. metallidurans. The interaction of QDs with C. metallidurans resulted in increased cellular ROS level and about 2.5-fold increase of the cells with damaged and leaky membranes as probed by FCM in combination with H2CFDA and PI stains. The presence of HA does not affect the hydrodynamic characteristics of QDs, but reduced significantly the damage to the membrane of bacterium C. metallidurans.

Although specific to the particular bacterium and amine- and carboxyl-PEG-QDs employed, the broad findings of the present study can have important implications for a more general understanding of the interaction of the nanoparticles with microorganisms. Functionalized NPs are not expected to aggregate in an aquatic environment and have a potential to interact with microorganisms and cause cell damage. Humic substances, representing the predominant portion of dissolved organic matter, do not have significant effect on their size, but seem to prevent membrane damage of bacterium. Since microorganisms are the foundation of all known ecosystems and provide key environmental services ranging from nutrient cycling and waste decomposition, disruption of the microorganism community may result in a degradation of environment quality. Consequently, an understanding of the interaction between metal containing NPs, representing about one-third of all NPs (3), and microorganisms, as well as the role of dissolved organic matter in these interactions is of utmost importance to evaluate their potential environmental impacts. Identifying the environmental impacts of nanotechnology in the early stages of its development may result in safer, greener products, and less long-term liability for industry.

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Supporting Information Available

FC measurements of the fluorescence per particle, average diffusion coefficient, hydrodynamic radius, and number of carboxyl-PEG-QDs, FCM results about the association of QDs to bacterium, effect of QDs on the bacterial growth as well as ICP-MS measurement of the metal content in bacteria and dissolved metal ion concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited
