Microbial Uptake, Toxicity, and Fate of Biofabricated ZnS:Mn Nanocrystals

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Abstract

Despite their importance in nano-environmental health and safety, interactions between engineered nanomaterials and microbial life remain poorly characterized. Here, we used the model organism E. coli to study the penetration requirements, subcellular localization, induction of stress responses, and long-term fate of luminescent Mn-doped ZnS nanocrystals fabricated under "green" processing conditions with a minimized ZnS-binding protein. We find that such protein-coated quantum dots (QDs) are unable to penetrate the envelope of unmodified E. coli but readily translocate to the cytoplasm of cells that have been made competent by chemical treatment. The process is dose-dependent and reminiscent of bacterial transformation. Cells that have internalized up to 0.5 μg/mL of nanocrystals do not experience a significant activation of the unfolded protein or SOS responses but undergo oxidative stress when exposed to high QD doses (2.5 μg/mL). Finally, although they are stable in quiescent cells over temperatures ranging from 4 to 42°C, internalized QDs are rapidly diluted by cell division in a process that does not involve TolC-dependent efflux. Taken together, our results suggest that biomimetic QDs based on low toxicity inorganic cores capped by a protein shell are unlikely to cause significant damage to the microbial ecosystem.

Introduction

Quantum dots (QDs) are semiconductor nanocrystals that are gaining in popularity over organic fluorophores in applications ranging from bioimaging[1] and analytical assays[2] to electronic displays,[3] solid-state lighting[4] and photovoltaics.[5] QDs commonly consist of a CdSe, CdTe, ZnSe, or PbSe core coated with a ZnS shell to enhance stability and optical properties. In some cases, the shell is further functionalized with thiols or amphiphilic polymers to make the nanocrystals soluble in aqueous solvents and to allow for biomolecule conjugation.[6–8] As QDs become more prevalent in consumer products that will be used, reused, recycled, and landfilled, concerns have been rising about their impact on humans and the environment.[9]

Studies conducted with cultured eukaryotic cells have revealed that QDs can exert cytotoxic effects through a variety of mechanisms. These include leaching of toxic heavy metals from the
inorganic core, (photo)generation of reactive oxygen species that induce oxidative stress, and direct or indirect damage to genomic DNA and biological membranes.[6,10–13] Parameters such as size, shape, composition, and surface coating(s) can all impact cytotoxicity outcomes and do so in a mechanism-specific (and cell-specific) [14] manner. For instance, while ZnS shells or polymer coatings can reduce the cellular toxicity of CdSe nanocrystals,[11] they do little to prevent photo-induced DNA damage.[15] Similar observations have been made in animals, where the situation is further complicated by the route of exposure and where long-term retention in the liver, spleen, kidney, and lymph nodes is of primary concern.[10,16,17]

There is considerably less information on how QDs interact with prokaryotes although these abundant microorganisms will be first to come into contact with engineered nanomaterials that find their way into the environment. Furthermore, most studies conducted to date have focused on cadmium-based QDs (CdS, CdSe and CdTe cores) produced with different synthesis schemes and coatings, and used at different doses with a variety of strains and culture conditions.[18–20]

To answer a growing demand for the production of functional nanomaterials through environmentally friendly processes, we previously described a set of “designer” proteins that support the low-temperature and aqueous fabrication of undoped and transition metal-doped ZnS QDs to which antibodies can be conjugated by simple mixing.[21–23] Because zinc is not as toxic as cadmium,[24] these particles should exhibit low cellular toxicity upon core dissolution, and because they are capped by proteins as part of the manufacturing process, their shell is already biologically-relevant.

Here, we used *E. coli* as a model organism to investigate the penetration requirements, subcellular localization, induction of stress responses, and long-term fate of luminescent ZnS:Mn nanocrystals fabricated with a minimized designer protein.[23] Our results suggest that such protein-coated fluorophores are environmentally benign because their uptake requires membrane destabilization, they only induce oxidative stress at high doses, and they are rapidly diluted by cell division.

**Results and Discussion**

**Uptake by *Escherichia coli* requires membrane destabilization**

We recently reported that BB-CT43, a minimized designer protein consisting of a linear ZnS binding peptide (CT43) fused to an antibody-binding domain derived from *S. aureus* Protein A (BB) is suitable for the one-pot synthesis of ZnS:Mn QDs.[23] These luminescent nanocrystals are produced when the designer protein caps the growth of the inorganic core at about 4 nm,[22] a process that is schematically illustrated in Fig 1. With their protein shell, the particles have an overall hydrodynamic diameter of 9.5 ± 2 nm and a zeta potential of -16.5 ± 6 mV. They exhibit a strong emission peak at 590 nm under UV illumination, can be decorated with antibodies by simple mixing and are stable for months without aggregation or degradation of optical properties.[23] Unlike traditional QDs, these fluorophores are manufactured using mild aqueous conditions, do not contain highly toxic heavy metals such as cadmium, and sport a built-in protein shell coat. Thus, they should have a minimal impact on microbial life and the environment.

As a first test of this hypothesis, we studied the uptake of BB-CT43-stabilized QDs by *Escherichia coli*, a well-studied gram-negative organism whose envelope consists of a 5 nm-thick phospholipid bilayer inner membrane, a 12 nm-thick interstitial space known as the periplasm, a 1 to 2 nm-thick peptidoglycan layer, and a 13 nm-thick, negatively charged outer membrane composed of a lipopolysaccharide outer leaflet and a phospholipid inner leaflet.[25]
Like all prokaryotes, *E. coli* lacks the endocytosis pathways responsible for nanoparticle uptake by eukaryotes. In addition, bacterial porins, which allow free diffusion of small molecules across prokaryotic membranes through 1–2 nm pores,[26] should be too small to allow even the smallest QDs to enter the cell. Nevertheless, Hirschey and coworkers reported that CdSe/CdS QDs stabilized by citrate, isocitrate, succinate, or malate readily penetrate *E. coli* when their inorganic core is smaller than 6 nm. By contrast, Wenhua et al. found that the uptake of mercaptoacetic acid-stabilized QDs with 3 to 4 nm CdSe/CdS cores require chemical destabilization of the outer membrane,[27] while Nadeau and coworkers reported that internalization of adenine-coated CdSe QDs strictly depends on photo-induced membrane damage and purine metabolism.[18]

To determine whether BB-CT43-stabilized nanocrystals would be uptaken by unmodified *E. coli*, we incubated 0.5 μg/mL of nanoparticles with mid-exponential phase *E. coli* cells for 2h at room temperature. Cells were washed to remove nonspecifically bound particles, pelleted by centrifugation and exposed to UV light. Under these conditions, there was no detectable nanocrystal uptake (Fig 2A). However, in agreement with the results of Wenhua and coworkers,[27] fluorescent material colocalized with sedimented cells if they were first made chemically competent by incubation with 100 mM CaCl2 at low temperature. This treatment transiently affects the integrity of the outer membrane and is routinely used for introducing naked DNA into cells, although the precise mechanisms at play remain unknown.

Not unexpectedly, uptake was dose-dependent and we observed a linear increase in cell fluorescence at 590 nm (the QD emission maximum) when the particle concentration was increased from 0.5 to 2.5 μg/mL (Fig 2B). We conclude that chemical disruption of the outer membrane is required for the uptake of QDs coated with protein shells and whose inorganic cores are less photo reactive than CdSe/CdS.

**Internalized QDs localize to the cytoplasm**

To confirm that the nanocrystals were not simply adsorbed to the surface of the outer membrane, competent cells incubated with 0.5 μg/mL of BB-CT43-stabilized QDs as above were subjected to spheroplasting (Fig 3). This procedure strips *E. coli* of its outer membrane and peptidoglycan layer, cause release of periplasmic contents in the surrounding medium, and leads to loss of rod shape and the formation of spherical vesicles bounded by the inner membrane.[28] Fig 3B shows that spheroplasts remained fluorescent, indicating that the QDs were either associated with the inner membrane or had translocated to the cytoplasm.

Because confocal microscopy does not allow one to unambiguously distinguish between these two possibilities, we took advantage of the fact that the emission spectrum of UV-excited ZnS:Mn nanocrystals overlaps the absorption spectrum of the fluorescent protein mCherry.[29] Thus, Forster Resonance Energy Transfer (FRET) should occur between the two fluorophores if they co-localize to the same cellular compartment and are separated by distances smaller than 10 nm.

To test this idea, we first recorded the fluorescence emission spectra of competent cells that had taken up QDs or had been exposed to buffer alone following excitation at 280 nm. Subtraction of the two spectra eliminated the contribution of background fluorescence and revealed a
**Fig 2. Membrane destabilization is required for QD uptake.** (A) AB734 cells made competent (+) or not (-) by treatment with CaCl$_2$ on ice were incubated with 0.5 μg/mL of BB-CT43-stabilized ZnS:Mn nanocrystals for 2h at room temperature. Cells were washed twice, centrifuged, and pellets were photographed under UV illumination. (B) Competent cells were incubated with the indicated concentration of QDs and photographed as above. Fluorescence emission spectra of cell suspensions were recorded following excitation at 280 nm.

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weak but clear peak centered at 590 nm and corresponding to ZnS:Mn emission (Fig 4, orange). Next, we introduced a plasmid expressing mCherry at high level in the cytoplasm of E. coli and confirmed that the emission spectrum of these cells exhibited the characteristic shape and 610 nm emission maximum of mCherry upon excitation at 590nm (Fig 4, inset).[29]

**Fig 3. Spheroplasts remain fluorescent.** Competent AB734 cells were incubated with 0.5 μg/mL of BB-CT43-stabilized QDs, washed and imaged on an optical microscope without further treatment (A) or following spheroplasting (B). Insets show the appearance of pelleted cells or spheroplasts upon UV illumination.

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Finally, we made competent mCherry-producing cells, exposed them to QDs or buffer, and collected fluorescence emission data following excitation at 280 nm. The subtracted spectrum (Fig 4, red) shows that the QD-associated peak at 590 nm peak completely disappeared to the profit of a 610 nm peak corresponding to mCherry emission. We conclude that nonradiative
energy transfer occurs between QDs (donor) and mCherry (acceptor) and therefore that both species are located within a few nanometers of each another in the cytoplasm.

How QDs (or plasmid DNA for the matter) translocate across the peptidoglycan layer, periplasm and inner membrane to reach the cytoplasm remains unclear. One possible explanation is that they rely on the transient and CaCl₂-induced opening of sites where the outer and inner membranes come into intimate contact. Such adhesion zones, known as Bayer’s patches, were identified microscopically over 40 years ago,[30] but their existence has remained controversial in spite of supporting biochemical evidence.[31]

High doses of ZnS:Mn nanocrystals are required to induce an oxidative stress responses

We next investigated how the presence of BB-CT43-stabilized nanocrystals in the cytoplasm would impact cell physiology. Prokaryotes have evolved complex and redundant mechanisms to survive exposure to environmental stresses. Many of these processes rely on increasing the synthesis of protective proteins (e.g., molecular chaperones, proteases, DNA repair enzymes and reductases) through upregulation events that are often controlled at the transcriptional level. Previously, we described E. coli cells harboring single-copy gene fusions between the stress-inducible ibp or sulA promoters and the lacZ gene (which encodes β-galactosidase). [32,33] These strains report on the amount of stress experienced by the cell as a result of cytoplasmic protein misfolding (ibp::lacZ fusion) or DNA damage (sulA::lacZ fusion) by producing the easily assayed enzyme, β-galactosidase. Because QD cytotoxicity has repeatedly been correlated with oxidative damage,[18,34–37] we constructed an additional isogenic strain bearing a single-copy gene fusion between the oxidative stress responsive promoter of the major E. coli catalase (the OxyR-regulated katG gene product)[38] and lacZ.

The functionality of the reporter panel was first confirmed using hydrogen peroxide, nalidixic acid and ethanol at concentrations known to cause extensive oxidative stress, DNA damage, or protein misfolding, respectively.[32,33] These chemicals caused an about 3-fold induction of the corresponding promoters (Fig 5, positive controls). Next, the three strains were made chemically competent, exposed to QDs, and cultures were assayed for β-galactosidase activity after 3h. While there was no detectable induction of any of the stress promoters when QDs were supplied at the concentration used in all above experiments (0.5 μg/mL), addition of 2.5 μg/mL nanocrystals was as effective as the use of 10 μM H₂O₂ in inducing the katG promoter. Of note, however, there was no statistically significant activation of either the ibp or sulA promoter under the same conditions (Fig 5). While the dependency of toxicity on QD dose is not particularly surprising,[11] our results indicate that it takes highly concentrated solutions of nanocrystals to fully induce the bacterial oxidative stress response and that BB-CT43-stabilized QDs do not cause appreciable protein misfolding or DNA damage under the same conditions.

QD fluorescence is rapidly lost in growing cells

The persistence of toxicants in the environment can lead to their long-range transport and bioaccumulation at toxic doses in animal and human tissues. To gain information on the in vivo stability of BB-CT43-stabilized QDs, we first incubated cells that had internalized nanocrystals in phosphate buffered saline (PBS) for 24h at temperatures ranging from 4 to 42°C. There was no significant change in the fluorescence of cell pellets indicating that protein-capped nanocrystals are stable for extended periods of time in quiescent cells exposed to a physiologically relevant range of temperatures (S1 Fig).
To determine if growth or metabolic activity would influence this outcome, QD-loaded cells were taken in LB medium or PBS and incubated at 37°C, the optimum growth temperature for *E. coli*. While non-growing cells held in PBS did not lose their initial fluorescence (S2 Fig), we observed a linear decrease in fluorescence over time and nearly complete disappearance of the signal after 3h of cultivation in LB medium (Fig 6A, closed symbols). Because cells experienced a 1h lag phase and exponential growth only started about 2h after transfer to LB (Fig 6A, open symbols), the nearly 50% loss of fluorescence that occurs over the first 1.5h of cultivation cannot be attributed to QD dilution by cell division. Indeed, when the experiment was repeated in the presence of the translational inhibitor kanamycin, we observed a similar fluorescence loss over the first 1.5h but, remarkably, no further decrease thereafter (Fig 6B). Thus, although *de novo* protein synthesis and/or cell growth are not implicated in initial signal loss, they are necessary for complete elimination of QD fluorescence.

There are several possible explanations for the initial fluorescence loss: dissolution or extrusion of about 50% of the internalized nanocrystals or substitution of the BB-CT43 shell by host species that change the QD optical properties. We do not believe that chemical dissolution of...
the nanocrystals is a likely mechanism since it would be unlikely to abruptly stop in kanamycin-treated (Fig 6B) or quiescent cultures (S1 and S2 Figs).

To directly test the possibility that active extrusion was involved, we repeated the experiment of Fig 6A in isogenic cells containing or lacking TolC, a trans-periplasmic protein that functions as an exit duct for the expulsion of a wide variety of small drugs and proteins from the cytoplasm to the growth medium.[39] The lack of significant difference in the kinetics and extent of fluorescence loss in \textit{tolC} and \textit{tolC} ruled out the involvement of TolC-dependent QD export (S3 Fig).

While we cannot rule out extrusion through other systems, we favor a mechanism in which endogenous species replace at least some of the bound BB-CT43 at the ZnS:Mn surface and cause a decrease in emission intensity through fluorescence quenching. Such quenching phenomena have previously been described and exploited for ZnS:Mn QDs.[40] This explanation is consistent with our previous finding that ZnS:Mn nanocrystals fabricated with BB-TrxA::CT43 have about 30% lower emission intensity than those synthesized with BB-CT43 owing to fluorescence quenching by the TrxA domain.[23] It is also in agreement with the fact that the fluorescence of kanamycin-treated cultures reaches a plateau after 1.5h (Fig 6B), a time that is presumably needed to modify the surface of all internalized nanocrystals. Why samples taken in PBS do not experience a similar initial decrease in fluorescence (S2 Fig) remains unclear but the process appears to require metabolic activity. Irrespective of the precise mechanism of initial fluorescence loss, the data of Fig 6A shows that the QD signal is rapidly lost in actively growing cultures due to dilution by cell division.

**Conclusions**

We have shown here that protein-coated ZnS:Mn nanocrystals can translocate in a dose-dependent manner to the cytoplasm of \textit{E. coli}. The process requires transient destabilization of
the cell outer membrane and is reminiscent of bacterial transformation. Once in the cytoplasm, biofabricated QDs do not cause a significant induction of the unfolded protein or SOS responses. However, they lead to oxidative stress when supplied at very high concentrations (2.5 μg/mL). Although internalized QDs are stable over a broad range of temperature in quiescent cells, they are rapidly diluted in dividing cells. Taken together, our results suggest that biomimetic fluorophores designed with low toxicity cores and biologically-relevant shells are unlikely to cause significant damage to the microbial ecosystem. These design principles may prove useful for the production of other environmentally benign nanomaterials.

Materials and Methods

QD uptake by competent cells

AB734, an *E. coli* K-12 strain containing a mutation in the *lacZ* gene but otherwise wild type was obtained from the *E. coli* Genetic Stock Center. To prepare competent cells, 500 mL cultures were grown in LB medium at 37°C to *A*600 = 0.4, and cells were sedimented by centrifugation at 8,000g for 8 min and resuspended in 100 mL of 100 mM CaCl₂ or phosphate buffered saline (PBS; 150 mM NaCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄). After 30 min incubation on ice and centrifugation at 8,000g for 8 min, cells were taken into 12.5 mL of 100 mM CaCl₂ (or PBS for a non-competent control) and held on ice overnight. Glycerol was added to a 10% (v/v) final concentration and aliquots (200 μL) were stored at -80°C for future use. For uptake experiments, competent or control cells were thawed at room temperature, washed twice with PBS with intervening cycle of centrifugation at 4,000 rpm for 5 min in a microfuge, and resuspended in 900 μL of the same buffer. QDs (approximately 100 μL for a dose of 0.5 μg/mL) were added and the mixture was incubated at room temperature for 2h without shaking. Cells were washed twice with PBS to remove unincorporated QDs.

QD subcellular localization

Cells that had uptaken QDs were stripped of their outer membrane and peptidoglycan layer by spheroplasting.[28] Briefly, samples prepared as above were resuspended in 200 μL of buffer A (100 mM Tris-HCl, pH 8.0, 0.5 M sucrose, 0.5 mM EDTA), and 10 μL of a 2 mg/mL solution of lysozyme was added, followed by 400 μL of buffer A, and 400 μL of ddH₂O. After 20 min at room temperature, spheroplasts were recovered by centrifugation at 12,800g for 30 s and resuspended in 100 mM Tris-HCl, pH 8.0, 0.3 M sucrose, 10 mM MgCl₂. Samples were visualized on an optical microscope at 50x magnification.

FRET experiments

AB734 (pmCherry-mut2) cultures were grown to *A*600 = 0.4 at 37°C in LB medium supplemented with 50 μg/mL kanamycin. Production of mCherry was induced by addition of 0.2% L-arabinose and cultures were collected after 3 h of growth at 37°C. Cells were made competent by CaCl₂ and ice treatment as above and stored in 200 μL aliquots. After two wash cycles and resuspension in 900 μL PBS, one sample was incubated for 2 h with 100 μL of BB-CT43-stabilized QDs or the same volume of PBS to serve a control. After 2 wash cycles with PBS, samples were diluted 20-fold in PBS and fluorescence emission spectra were recorded with excitation at 280 nm or 590 nm. Control samples of AB734 cells lacking the pmCherry-mut2 plasmid and incubated or not with QDs were prepared as above and fluorescence spectra were recorded following excitation at 280 nm. The spectra of Fig 4 show QD-free AB734 emission subtracted from QD-treated AB734 emission with excitation at 280 nm (orange), and QD-free mCherry-
producing AB734 subtracted from QD-treated mCherry-producing AB734 with excitation at 280 nm (red).

QD fate
Aliquots (200 μL) of AB734 cells that had uptaken QDs as above were used to inoculate 2 mL of LB media in multiple 15 mL culture tubes supplemented or not with 50 μg/mL of the translational inhibitor kanamycin. Cultures were transferred to 37°C water bath. At the indicated time points, culture absorbance was recorded at 600 nm and samples (2 mL) were subjected to centrifugation at 5,000 rpm for 5 min in a microfuge. Cells were resuspended in 50 μL of PBS, deposited on quartz microscope slide and photographed on a UV table with excitation at 303 nm. Median fluorescence in square areas encompassing about 60% of the droplets and excluding their edges was quantified in the red channel using the histogram function of Adobe Photoshop. Fluorescence loss was quantified by subtracting the fluorescence of control samples from that of QD-loaded cells at the indicated time points.

Stress responses
Strains ADA110 (AB734 λφibp::lacZ)) and ADA510 (AB734 λφsulA::lacZ)) have been described previously.[32,33] ADA710 (AB734 λφkatG::lacZ)) was constructed by lysogenizing AB734 with a bacteriophage λ derivative bearing the oxidative stress-responsive katG::lacZ translational fusion and isolated from BGF931 (a kind gift from Dr. Gonzalez-Flecha) through standard protocols.[41] The three strains were made chemically competent by CaCl2 treatment and incubated or not with QDs as above. After resuspension in buffer, 200 μL of culture was used to inoculate 5 mL of LB medium. Samples were either exposed to buffer (negative control), known stress response inducers (4% ethanol, 15 μg/mL nalidixic acid, or 10 μM H2O2) or 0.5 μg/mL or 2.5 μg/mL of BB-CT43-stabilized QDs. After 3h incubation at 37°C, cells were lysed and β-galactosidase activities determined as described. [42]

Analytical techniques
UV-visible absorption spectra were recorded on a Beckman DU640 spectrophotometer. Fluorescence and phosphorescence emission spectra were recorded using 1 mL of sample on a Hitachi F4500 fluorescence spectrophotometer with excitation at 280 nm and excitation and emission slit widths set at 2.5 nm (fluorescence) or excitation at 316 nm and excitation and emission slit width at 2.5 nm and 10 nm, respectively (phosphorescence). The wavelength region corresponding to the second order diffraction peak of the excitation light was omitted.

Supporting Information
S1 Fig. Quiescent cells that have internalized QDs remain fluorescent over a broad range of temperatures. Competent AB734 cells incubated with 0.5 μg/mL of BB-CT43-stabilized QDs remain fluorescent after 24h of incubation in PBS buffer at temperatures varying from 4 to 42°C. (PDF)

S2 Fig. Quiescent cells that have internalized QDs remain fluorescent over time. Competent AB734 cells incubated with 0.5 μg/mL of BB-CT43-stabilized QDs remain fluorescent after 3h of incubation in PBS buffer at 37°C while they completely lose fluorescence under the same conditions in LB medium. (PDF)
S3 Fig. Impact of a tolC null mutant on fluorescence loss. Inactivation of tolC has no obvious impact on the loss of fluorescence in AB734 cells experiencing balanced growth in LB medium at 37°C. (PDF)

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Author Contributions
Conceived and designed the experiments: FB BJFS. Performed the experiments: BJFS. Analyzed the data: FB BJFS. Wrote the paper: FB BJFS.

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