Biosynthesis of Nanoparticles

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Microbial Synthesis of CdS Nanocrystals in Genetically Engineered *E. coli***

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Semiconductor nanocrystals have been shown to possess unique optical, electrical, and optoelectronic properties[1–3] for a wide range of applications. In particular, the exploitation of semiconductor nanocrystals, often referred to as quantum dots, in biological applications has increased dramatically because of their unique spectral properties, which enable simultaneous multiplex labeling and detection.[4,5] More importantly, the spectral properties of these semiconductor nanocrystals can be controlled effectively by tuning the size, composition, surface properties, and crystal structure of the nanocrystals.[6,7] Conventional chemical synthesis based on high-temperature organometallic processes is extremely toxic and expensive, and involves unstable species. For practical purposes, an alternative "green chemistry" scheme that is safe, simple, inexpensive, and suitable for industrial upscaling is extremely attractive.[8,9]

One promising alternative to chemical synthesis is the use of biological templates for the synthesis of nanocrystals. Many different biological templates, including peptides, nucleotides, and fusion proteins, are known to act as capping agents to regulate the synthesis of CdS, CdSe, and CdTe.[10–12] Biological templates not only guide the nucleation of inorganic materials, but also control the crystal structure and size, under aqueous and ambient conditions. Biological approaches to nanocrystal synthesis can also be extended to living biological systems. Peptides capable of nucleating nanocrystal growth were displayed on the surface of M13 bacteriophage; the genetically engineered phage promoted the synthesis of crystalline nanowires, while preserving the exquisite regulation of material composition, size, and shape.[13] Furthermore, the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) has been used to promote the synthesis of CdS nanocrystals.[14] In response to cadmium toxicity, *S. pombe* synthesizes phytochelatins (PCs) with repeating γGlu-Cys units to trap cadmium as nontoxic complexes. This low-molecular-weight complex composed of only cadmium and PCs is then transported actively into the vacuole and converted into high-molecular-weight PC–Cd–S complexes by the incorporation of sulfide. This process results in the formation of nanocrystals.[15,16] During the nucleation process, PCs serve as a binding template/nucleation site for the metal ions and stabilize the nanocrystal core against continued aggregation.

Although *S. pombe* has the intrinsic ability to form semiconductor nanocrystals by storing the peptide–metal complex in the vacuole as a defense mechanism, the multicompartment requirement makes it difficult to control and fine-tune the properties of the nanocrystals produced. Prokaryotes, such as bacteria, are ideal for engineering the synthesis of nanocrystals with precisely tailored size and crystallinity because of their single-compartment property. *Escherichia coli* (*E. coli*) is of particular interest, as the genetic tools and cellular metabolisms associated with this bacterium are well understood. Therefore, the guided assembly of genetic traits necessary for nanocrystal synthesis is possible. A recent study demonstrated the biosynthesis of CdS nanocrystals in *E. coli* without any genetic modification.[17] However, only samples cultured for 24 h were shown to produce CdS nanocrystals, for which a large distribution in size from 2–5 nm was observed. More importantly, the mechanism of nanocrystal synthesis was not elucidated, and only a small subset of strains was able to synthesize nanocrystals. Inspired by the PC-based detoxification mechanism of *S. pombe* and the ability of this microorganism to create CdS nanocrystals, we genetically modified *E. coli* to establish a generalized approach to CdS-nanocrystal synthesis on the basis of the PC-directed method.

To explore the feasibility of using *E. coli* as a biofactory for the controlled synthesis of CdS nanocrystals, the *E. coli* strain JM109 was endowed with the ability to produce PCs by expressing SpPCS, the PC synthase of *S. pombe*. A feedback-desensitized γ-glutamylcysteine synthetase (GSHI*), which catalyzes the synthesis of the PC precursor glutathione (GSH), was cotransformed to enhance the level of PC synthesis 10-fold, as reported elsewhere.[18] Cells designed to synthesize PCs were grown in an LB medium (lysogeny broth) containing the appropriate antibiotics; isopropyl-β-D-1-thiogalactopyranoside (IPTG) and cadmium chloride were added during the early exponential growth to promote PC synthesis and cadmium binding. Sodium sulfide was added 3 h after the addition of cadmium chloride to induce the formation of CdS nanocrystals for an additional 1 h. The formation of PC-templated CdS was first suggested by SDS-PAGE analysis. When the CdS luminescence was visualized

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directly, a strong fluorescent band was detected for cells producing PCs, whereas no such fluorescent band was observed when a control strain was incubated with cadmium chloride and sodium sulfide for the same duration (see Figure S1 in the Supporting Information).

To further validate the formation of CdS nanocrystals, cells were disrupted to release the CdS nanocrystals from the water-soluble fraction by repeated freezing and thawing. The released nanoparticles were separated from other cellular components by anion-exchange chromatography. Successive fractions eluted from the column were collected and analyzed. All cellular proteins were eluted in the early fractions, whereas cadmium was associated exclusively with much later fractions (Figure 1a). The observation that only the Cd-rich fractions contained sulfide suggested the presence of CdS complexes (Figure 1a). The fractions at the various positions were pooled and concentrated for PC analysis.

The PC levels also agreed with the Cd and S profiles, thus implying that the CdS complexes formed are coated with PCs (Figure 1b). To confirm the formation of CdS nanocrystals, the Cd- and sulfide-rich fractions were subjected to further analysis. High-resolution transmission electron microscopy (HRTEM) was used to probe the CdS complexes; a unique crystal-lattice distance of 3.3 Å was detected for the CdS nanocrystals (Figure 1c). Elemental analysis also supported the presence of Cd and sulfide in the nanocrystals (see Figure S2 in the Supporting Information). The water-soluble CdS nanocrystals were fairly polydisperse with a size distribution of 2–6 nm, a result attributed to the heterogeneous population of PCs (PC2, PC3, and PC4 in a roughly 1:2:3 ratio) used as the capping agents. The nanocrystals exhibited an absorption peak at $\lambda = 318$ nm and a fluorescence peak at $\lambda = 384$ nm (excitation at $\lambda = 320$ nm; Figure 3a). The strong blue shift in both the absorption edge and the fluorescence peak again supports the presence of nanocrystals (band gap of the bulk material: 2.41 eV; absorption edge at $\lambda \approx 515$ nm) and the good surface passivation by PCs. These results are

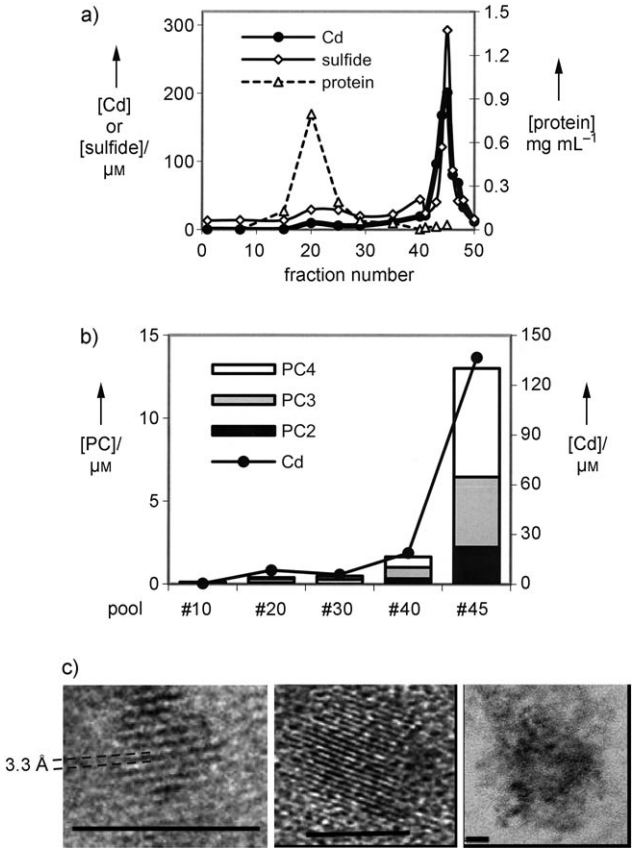

Figure 1. a) Cd, sulfide, and protein profile of fractions obtained from ion-exchange chromatography of engineered JM109 after disruption of the cells. b) Analysis for Cd and PCs of pooled fractions eluted during ion-exchange chromatography of engineered JM109. Two or three fractions were pooled in each case and concentrated for analysis. The PC concentration is given in terms of thiol-group equivalents. c) HRTEM micrograph of CdS nanocrystals obtained from a Cd- and sulfide-rich fraction pool (pool #45) from engineered JM109. Scale bars: 5 nm.

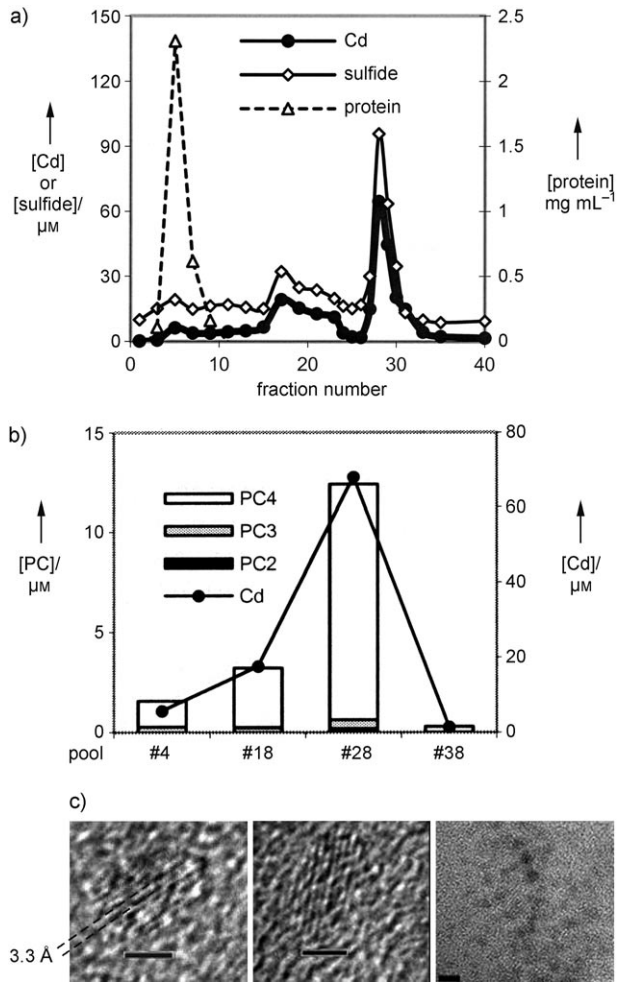

Figure 2. a) Cd, sulfide, and protein profile of fractions obtained from ion-exchange chromatography of engineered R189 after disruption of the cells. b) Analysis for Cd and PCs of pooled fractions eluted during ion-exchange chromatography of engineered R189. Two or three fractions were pooled in each case and concentrated for analysis. The PC concentration is given in terms of thiol-group equivalents. c) HRTEM micrograph of CdS nanocrystals obtained from a Cd- and sulfide-rich fraction pool (pool #28) from engineered R189. Scale bars: 2 nm.

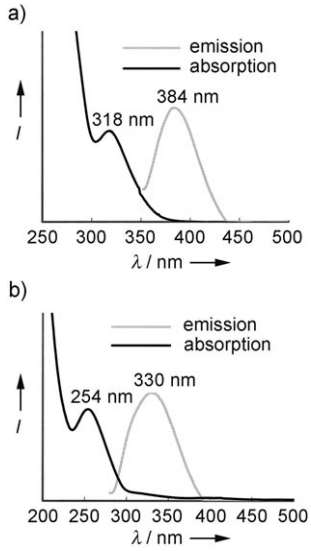

Figure 3. UV/Vis (solid line) and fluorescence spectra (dotted line) of PC-capped CdS nanoparticles in an aqueous solution: a) from engineered JM109 ($\lambda_{exc} = 320$ nm); b) from engineered R189 ($\lambda_{exc} = 260$ nm).

similar to those observed for CdS nanocrystals that emit yellow–orange radiation by deep-trap photoluminescence.[19]

To verify that our strategy for the synthesis of CdS nanocrystals can be generalized to any *E. coli* strain that produces PCs, the *E. coli* strain R189, which was reported not to produce CdS nanocrystals upon the addition of Cd and sulfide sources,[17] was chosen for investigation. We used the same procedure to produce the CdS nanocrystals, except that $CdCl_2$ was used at a concentration of 100 μM rather than 20 μM to promote the synthesis of a more uniform population of PCs, as reported previously.[20] The distribution of cellular proteins, cadmium, and sulfide was similar to that observed with the strain JM109 (Figure 2a); however, the distribution of the PCs differed. Primarily PC4 was synthesized at this higher cadmium concentration, with PC2 and PC3 accounting for less than 10% of the PCs present (Figure 2b). A TEM image again confirmed the presence of CdS nanocrystals in the engineered R189 strain (Figure 2c). In contrast to the size distribution in the range of 2–6 nm observed for the engineered JM109 strain (Figure 1c), the nanocrystals synthesized by strain R189 were uniform in size in the range of 3–4 nm (Figure 2c). This difference in size distribution is the result of using a homogeneous PC4 population (see Table 1 in the Supporting Information) as the primary capping agent[21,22] and demonstrates the ready tunability of our approach for controlling the size of the nanocrystals synthesized. Both the absorption and fluorescence peaks were observed at a shorter wavelength, a result that is consistent with the smaller size of the nanocrystals (Figure 3b). As expected for aqueous-phase synthesis,[17] the observed quantum yield is relatively low at 0.007%.

In conclusion, we have described the PC-mediated intracellular synthesis of CdS nanocrystals in engineered *E. coli*. By controlling the population of the capping PCs, *E. coli* cells were engineered as an ecofriendly biofactory to produce uniformly sized PC-coated CdS nanocrystals. To our knowledge, this is the first systematic approach toward the tunable synthesis of semiconductor nanocrystals by genetically engineering bacteria. We envision that similar nanocrystals, such as PbS or ZnS, can be produced by using engineered *E. coli*.

Experimental Section

Nanocrystal formation: The bacterial strains used for nanocrystal synthesis were the *E. coli* strains JM109 (Stratagene, La Jolla, CA) and R189 (gift from Dr. Georgiou). The plasmid pQE60-SpPCS containing the ampicillin marker was used to express SpPCS. The plasmid pMMB277-Ptac-isoILR1-gshI*[23] containing the chloramphenicol-resistance marker was used for the expression of γ-glutamylcysteine synthetase (GSHI*). These two plasmids were transformed into JM109 or R189. Cells were inoculated into 25 mL of LB medium containing the appropriate antibiotics in a 125 mL flask at an optical density $OD_{600} = 0.1$ from a seed culture grown overnight. When the OD_{600} value reached 0.5, the expression of SpPCS and γ-ECS was induced with IPTG (0.5 mM; Sigma). Cadmium chloride was added to a final concentration of 20 μM. After incubation of the samples for 3 h, freshly prepared sodium sulfide (20 μM) was added slowly. The samples were incubated at room temperature with end-over-end rotation for 1 h.

Isolation of nanocrystals: Harvested cells were washed and resuspended in 10 mM Tris buffer (pH 7.6; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol). The resuspended cells were frozen at −70 °C for 30 min and then thawed at room temperature. This process was repeated at least five times, then a lysozyme (1 mg mL^{-1}) and deoxyribonuclease (0.2 mg mL^{-1}) were added. After end-over-end rotation overnight, the cell lysate was spun down. The supernatant was filtered with a 0.2 μM syringe filter that was cooled to 4 °C prior to use. An anion-exchange resin, DEAE–sephadex G-50 (DEAE = diethylaminoethyl), was equilibrated with Tris buffer (pH 7.6) containing KCl (125 mM). The filtered supernatant was loaded onto the column at a flow rate of 2 mL min^{-1} and eluted with a buffered KCl salt gradient to fractionate the cell materials and purify the released CdS semiconductor nanocrystals.

SDS-PAGE: Cells grown as described above were harvested, washed with 5 mM HEPES buffer containing 0.8% NaCl, and concentrated to a cadmium concentration of 1 mM. Resuspended cells were mixed with SDS gel-loading buffer containing 5% 2-mercaptoethanol, lysed by boiling at 95 °C for 10 min, and spun down. The supernatant was separated on a 7.5% triglycine gel at 100 V for 1.5 h. The gel image was captured by a UV transilluminator (Bio-Rad Laboratories).

Cadmium and protein assay: The cadmium content was measured by atomic adsorption spectroscopy (analyst 800, Perkin–Elmer, MA), and the protein concentration was determined by the Bradford method (Biorad).

PC analysis: The derivatization procedure with monobromobimane (mBBr) and fluorescence detection was adapted from the procedure described by Sneller et al.[24] Fractions were pooled and freeze dried for analysis. The lyophilized samples were resuspended in 6.3 mM diethylenetriamine pentaacetic acid (DTPA; 1 mL) with 0.1% trifluoroacetic acid (TFA) for the extraction of thiol-containing peptides. A quantity of 250 μL of the resuspended samples was mixed with 200 mM 4-(2-hydroxy-ethyl) -piperazine-1-propane-sulfonic acid buffer (450 μL; pH 8.2) containing DTPA (6.3 mM) and mBBr (10 μL, 25 mM). After derivatization of the samples at 45 °C in the dark for 30 min, 1M methane sulfonic acid (300 μL) was added to stop the reaction. The peptides were separated on a reversed-phase gemini C18 column (pore size: 110 Å; particle size: 5 μm; dimensions: 4.6 × 150 mm; Phenomenex, Torrance, CA) with a binary-linear-gradient elution program. The two eluents used were methanol containing 0.1% (v/v) TFA and water containing 0.1% (v/v) TFA. The column

was equilibrated with 12% methanol containing 0.1% TFA, and the peptides were eluted with methanol/water, whereby the proportion of methanol was increased from 12–100% over 60 min, at a flow rate of 0.5 mL min^{-1}. Fluorescence was monitored by an Agilent 1200 series HPLC fluorescence detector. An excitation wavelength of 380 nm and an emission wavelength of 470 nm were used. PC2 was synthesized chemically by GenScript Corporation (Piscataway, NJ), and PC3 and PC4 were purchased from AnaSpec (San Jose, CA).

Sulfide analysis: The quantity of acid-labile sulfide (inorganic sulfide) was determined by the methylene blue assay described by King and Morris.[25] A quantity of 400 μL of a sample fraction was mixed with 2.6% (w/v) zinc acetate (250 μL) at room temperature for 1 min, and then 0.1% (w/v) *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride (125 μL) in 5 M HCl was added. The resulting mixture was shaken until clear. $FeCl_3$ (0.0115 M) in 6 N HCl (50 μL) was then added, and the mixture was mixed for 1 min and then incubated at room temperature for 30 min. Finally, distilled water (425 μL) was added, and the suspension was mixed and centrifuged for 1 min. Samples were read for their methylene blue content at 670 nm in a spectrophotometer against a calibration series containing 0–20 nmol of sodium sulfide per sample.

TEM: The Cd-rich fractions obtained by anion-exchange chromatography were dialyzed for 3–7 days against water to remove potassium chloride before the images were recorded. One drop of the fractionated sample was placed on a carbon-coated 400-mesh copper grid and dried under an incandescent lamp for about 15 min. Transmission electron microscopy (TEM) was performed with a FEI CM300 transmission electron microscope operated at an accelerating voltage of 300 kV.

Quantum-yield calculation: The fluorescence quantum yields were calculated by using anthracene and tryptophan as references for nanoparticles obtained from the engineered *E. coli* strains JM109 and R189, respectively.[26]

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