Published in final edited form as:

Biochem Eng J. 2014 August 15; 89: 28–32. doi:10.1016/j.bej.2013.09.009.

Biofabrication of ZnS:Mn luminescent nanocrystals using histidine, hexahistidine, and His-tagged proteins: a comparison study

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Abstract

The ubiquitous hexahistidine purification tag has been used to conjugate proteins to the shell of CdSe:ZnS quantum dots (QDs) due to its affinity for surface-exposed Zn²⁺ ions but little attention has been paid to the potential of His-tagged proteins for mineralizing luminescent ZnS nanocrystals. Here, we compare the ability of free histidine, a His tag peptide, His-tagged thioredoxin (TrxA, a monomeric protein), and N- and C-terminally His-tagged versions of Hsp31 (a homodimeric protein) to support the synthesis of Mn-doped ZnS nanocrystals from aqueous precursors under mild conditions of pH (8.2) and temperature (37°C). We find that: (1) it is possible to produce poor quality QDs when histidine is used at high (8 mM) concentration; (2) an increase in local histidine concentration through repetition of the amino acid as a His tag decreases the amount of needed reagent ≈10-fold and improves optical properties; (3) fusion of the same His tag to TrxA allows for ZnS:Mn QDs mineralization at micromolar concentrations; and (4) doubling the local hexahistidine concentration by exploiting Hsp31 dimerization further improves nanocrystal luminescence with the brightest particles obtained when His tags are spatially co-localized at the Hsp31 N-termini. Although hexahistidine tracts are not as efficient as combinatorially selected ZnS binding peptides at QD synthesis, it should be possible to use the large number of available His-tagged proteins and the synthesis approach described herein to produce luminescent nanoparticles whose protein shell carries a broad range of functions.

1. Introduction

Semiconductor nanocrystals (*a.k.a.* quantum dots; QDs) have attracted widespread interest as optical labels for biological applications due to their small size (1–12 nm), narrow emission but wide excitation spectra, high quantum yield and superior photostability [1–3]. QDs are typically synthesized by colloidal chemistry using toxic precursors, organic solvents and high temperatures [4]. To interface with biology, their surface must be made hydrophilic and further derivatized with biomolecules such as proteins or oligonucleotides.

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While a number of elegant synthetic chemistries have been developed for this purpose [2], an alternate route is to use peptides and proteins having high affinity for transition metals to template and control nanocrystal nucleation and growth [5–12].

We recently described an aqueous, low temperature and environmentally friendly route for the integrated synthesis, solubilization and biological functionalization of zinc sulfide (ZnS) QDs [13, 14]. The biofabrication process relies on the use of BB-TrxA::CT43, a “designer” protein consisting of a ZnS-binding dodecapeptide (CT43) inserted within the active site loop of *E. coli* thioredoxin A (TrxA) which is itself fused to an antibody binding module (BB) derived from *Staphylococcus aureus* protein A. Addition of sodium ions to a solution of BB-TrxA::CT43 dispersed in a zinc electrolyte leads to the formation of fairly monodisperse luminescent particles consisting of an ≈ 4 nm ZnS crystalline core capped by a protein shell that is fully functional for antibody binding [14]. Of interest for multiplex applications, the maximum emission wavelength of these QDs can be tuned from blue to the green and orange regions of the visible spectrum by doping the ZnS core with Cu^{2+} and Mn^{2+} ions, respectively [13].

Previous work focused on the disulfide-constrained CT43 peptide (amino acid sequence *CGPAGDSSGVDSRSVGPC* where italics identify invariant residues) because it yields highly luminescent nanocrystals when used within the context of the BB-TrxA framework [14]. However, our original cell surface display screen yielded a number of additional ZnS binders due to the fact many different peptides can bind to the same solid [15, 16]. For instance, peptide CT81 (*CGPKGRPGRAEEEEQGQGPC*) also supported ZnS nanocrystal formation when used as a BB-TrxA::CT81 fusion, but the maximum photoluminescence of the nanocrystals was 20–50% lower than that obtained with BB-TrxA::CT43 [14]. Remarkably, neither of these peptides, nor most of the ZnS binders that we previously described [14], contain histidine (H), an amino acid that is known to form strong coordination bonds with transition metal ions such as Zn^{2+} , Cu^{2+} , Ni^{2+} , and Co^{2+} through the electron donor groups of its imidazole ring [17]. In fact, polyhistidine extensions fused to the N- or C-termini of target proteins not only allow their rapid purification by immobilized metal affinity chromatography (e.g., with nickel-nitrilotriacetate columns), but have also been used to decorate water soluble CdSe-ZnS core-shell QDs with proteins and peptides by coordinating Zn^{2+} ions on the nanocrystal surface (e.g., references [18] and references within). Although Aryal and Benson previously described the use of His-tagged proteins to produce CdSe nanocrystals with weak optical emission properties [5], a detailed analysis of the potential of His tags for ZnS nanocrystal fabrication has not so far been reported.

Here, we demonstrate that “generic” Zn^{2+} binders such as histidine, hexahistidine-containing peptides, and His-tagged proteins also support ZnS:Mn QD fabrication with increased efficiency as a protein framework is used and the local histidine concentration is increased.

2. Materials and Methods

2. 1. Plasmids and DNA manipulations

Plasmid pH-TrxA which encodes a N-terminally His-tagged version of TrxA was obtained by subcloning the *NdeI-HindIII* fragment from pT-Trx [19] into the same sites of pET28a (+) (Novagen). Plasmids pNT-hchA and pCT-hchA which encode variants of the molecular chaperone Hsp31 with N- and C-terminal hexahistidine tags, respectively, have been described previously [20, 21].

2. 2. Protein purification

Wild type TrxA and BB-TrxA::CT43 were purified by thermal shock and ion exchange as described [14]. Wild type Hsp31, its hexahistidine-tagged variants and the MGSSHHHHHHSSGLVPA peptide were purified as before [20]. For His-TrxA purification, BL21(DE3) cells harboring pH-TrxA were grown at 37°C to mid-exponential phase in LB medium supplemented with 30 µg/ml kanamycin, transferred to 30°C for 10 min, and induced with 0.4 mM IPTG. After 4h of growth at the same temperature, cells were collected by centrifugation at 3,500 g for 15 min, and resuspended into 20 mM Na₂HPO₄-NaH₂PO₄, pH 7.5 and 1 mM PMSF to an A₆₀₀ ≈ 50. Cells were disrupted by three cycles of homogenization on a French pressure cell operated at 10,000 psi and the lysate was clarified by centrifugation at 14,000 g for 10 min. The clarified lysate was incubated at 80°C for 10 min and aggregated proteins removed by centrifugation as above. The solution was diluted 4-fold with buffer A (20 mM Na₂HPO₄-NaH₂PO₄, pH 7.5, 300 mM NaCl, 20 mM Imidazole) and loaded onto a Ni-NTA agarose (Qiagen) column (7.0 cm × 1.0 cm I.D.) equilibrated in buffer A and developed at 1 mL/min. Contaminants were removed by washing the column with 20 mL of buffer A, followed by 20 mL of buffer A supplemented with 50 mM imidazole. The protein was eluted in 20 mL of buffer A supplemented with 125 mM imidazole. Fractions were pooled, desalted using a 3 kDa-MWCO SnakeSkin dialysis tube (ThermoSci) against 20 mM Na₂HPO₄-NaH₂PO₄, pH 7.5. Protein concentrations were determined using the BCA kit (Sigma). Purity was greater 95% as determined by videodensitometric analysis of silver-stained gels.

2.3. QD synthesis

Mn-doped ZnS nanocrystals were synthesized essentially as described [13]. Briefly, 180 µL of 5 mM Zn(CH₃COO)₂ and 20 µL of 5 mM Mn(CH₃COO)₂ were mixed in an 18 mm diameter round-bottom test tube. Next, 200 µL of 40 mM NH₄(CH₃COO) and the test biological reagent were added at concentrations varying between 2 µM and 40 mM (for the amino acid histidine or purified MGSSHHHHHHSSGLVPA peptide) and between 1 and 10 µM (for the various proteins studied here). The pH was adjusted to 8.2 by addition of 250 µL of 10 mM NH₄OH and the volume brought to 2.3 mL with ddH₂O. After 1h incubation at room temperature, 200 µL of 5 mM Na₂S was added dropwise with vortexing and the solution was transferred to 37°C incubator for a 5-day aging period. Long-term nanocrystal storage was at 4°C.

2.4. Analytical techniques

All measurements were made using 1 mL of samples. UV-visible absorption spectra were obtained on a Beckman DU640 spectrophotometer. Photoluminescence emission spectra were acquired from 300 to 800 nm on a Hitachi F4500 fluorescence spectrophotometer with excitation at 280 nm and slit widths at 2.5 nm. The wavelength region corresponding to the second order diffraction peak of the excitation light was omitted. Hydrodynamic diameters were measured on a Malvern Zetasizer Nano-ZS dynamic light scattering instrument equipped with a 633 nm laser filter using 1 mL samples. For transmission electron microscopy (TEM) analysis, samples (5 μ L) were deposited on plasma-cleaned carbon-coated copper TEM grids and air-dried. TEM images were collected on a FEI Tecnai G2 F20 S/TEM operated at an accelerating voltage of 200 kV.

3. Results and discussion

3.1. Biofabrication of ZnS:Mn QDs using histidine and a polyhistidine-containing peptide

With an equilibrium dissociation constant (K_d) of 8.7×10^{-13} [22], zinc-histidine complexes can act as nucleation centers for sulfide incorporation and the production of luminescent ZnS and CdS nanocrystals [23, 24]. Typical synthesis schemes require high concentration of histidine (in the 2–30 mM range), alkaline conditions (pH 9.5) and elevated temperatures ($T \approx 60^\circ\text{C}$), which can all affect the structural and functional integrity of proteins. To determine if histidine might be useful to fabricate ZnS:Mn nanocrystals under conditions that we routinely employ for protein-aided biofabrication, we first dissolved the amino acid at a concentration of 2 μ M in an electrolyte containing Zn^{2+} and Mn^{2+} ions. Addition of sodium sulfide led to the formation of orange precipitates that sedimented at the bottom of the tube and could be removed by filtration through a 0.2 μ m membrane (Fig. 1A). This outcome reflects uncontrolled growth and precipitation of bulk ZnS:Mn due to lack of particle capping. No luminescence was detected at histidine concentrations up to 800 μ M. However, solutions that retained weak fluorescence after filtration were obtained with 8 mM of the amino acid with deterioration of the optical signal at higher concentrations (Fig. 1A). DLS measurements confirmed that nanoparticles of hydrodynamic diameter (D_h) 7 ± 2.5 nm were produced in the presence of 8 mM histidine. However, their maximum emission intensity at 590 nm was less than 10% that of nanocrystals obtained with 2 μ M BB-TrxA::CT43 (Fig. 1C, compare purple and orange spectra).

To determine if a high local concentration of histidine residues would yield higher quality nanocrystals, we conducted similar experiments with MGSSHHHHHHSSGLVPA, a hexahistidine-containing peptide that is liberated upon thrombin cleavage of N-terminally His-tagged proteins cloned into plasmid pET28(+) [20]. Figure 1B shows that results were similar to those observed with the free amino acid except that luminescent material was produced at lower concentrations (100 μ M to 10 mM). The brightest nanoparticles ($D_h = 8.5 \pm 3$ nm) were obtained with 1 mM peptide but their maximum emission intensity at 590 nm was only about a third that obtained with 2 μ M BB-TrxA::CT43 (Fig. 1C, compare blue and orange spectra).

The above results indicate that it is possible to synthesize luminescent ZnS:Mn nanocrystals using histidine and hexahistidine-containing peptides and the $\text{Zn}(\text{NH}_4^+)_4 - \text{S}^{2-}$ system under mild conditions of pH (8.2) and temperature (37°C). They also demonstrate that an increase in local chelating and/or capping capability through histidine repetition reduces the amount of reagent needed and improves the material's optical properties. However, both reagents must be used at relatively high concentrations that are comparable to those of the Zn^{2+} and S^{2-} precursor ions (0.4 mM). This suggests that free histidine and the hexahistidine-containing peptide function as traditional ligands rather than as selective surface active agents which affect inorganic growth and morphology at much lower concentrations, like designer proteins do [25]

3.2. His₆-TrxA supports ZnS:Mn QD synthesis

If a peptide encompassing hexahistidine is suitable for ZnS:Mn QD fabrication, we next asked whether His-tagged proteins could be used for the same purpose and what would be the impact of a larger framework on the concentrations required and properties of the nanomaterial produced. To answer this question, we constructed His₆-TrxA, a derivative of TrxA containing a ≈ 2 -kDa MGSSHHHHHSSGLVPAGSH N-terminal extension, and compared the ability of untagged and tagged proteins to mediate nanocrystal formation under our experimental conditions. Fig. 2 shows that luminescent material could be obtained with both proteins at much lower (μM) concentrations than those needed with histidine or the hexahistidine-containing peptide. There were however important differences. Wild type TrxA yielded material with major emission peaks centered at 350 nm and 460 nm (which correspond to a combination of ZnS band edge emission and protein tryptophan fluorescence and to a ZnS surface trap state, respectively) [14], but there was little Mn^{2+} -associated emission at 590 nm. Furthermore, solutions were turbid and contained large particles, even after filtration through a 0.2 μm cartridge (the D_h were approximately 250 nm and 40 nm at TrxA concentrations of 2 and 5 μM , respectively). By contrast, His₆-TrxA yielded slightly turbid (2 μM) to completely transparent (5 μM) solutions that exhibited the 590 nm peak characteristic of Mn-doped QDs (Fig. 2B). At the higher protein concentration, and as expected from previous studies [13], the band edge and trap state peaks disappeared at the profit of the 590 nm peak. Nevertheless, with a measured D_h of 17.5 ± 1.5 nm, the particles produced were larger than expected for quantum-confined nanocrystals.

How wild type TrxA supports the formation of luminescent nanoparticles remains unclear but it is possible that the highly reactive Cys-32 residue in the protein's Cys-Gly-Pro-Cys active site mediates binding to ZnS particles and quenches their growth [14, 26]. The process is however inefficient since the colloidal solutions produced contain large structures that scatter light, and because the particles do not efficiently incorporate Mn^{2+} ions and have poor optical properties. Appending a N-terminal His tag to TrxA and using the resulting protein at 5 μM concentration allows for production of ZnS:Mn QDs that exhibit the expected photophysical characteristics. While the nanoparticles thus fabricated have low brightness and a larger D_h than the expected value (≈ 10 nm), these results nevertheless illustrate how fusion of a solid binding peptide to a protein scaffold can lower the effective concentration needed for mineralization by 200-fold compared to free peptide. Clearly, the protein backbone plays an important role in the synthesis process, most likely by

contributing non-specific interactions with ZnS inorganic nuclei through surface-exposed amino acids (Fig. 2A), but also perhaps by enhancing the solubility or optimizing the presentation of the fused His tag.

3.3. Influence of the number and localization of hexahistidine tags

Previously, we constructed N- and C-terminally His-tagged variants of Hsp31 a homodimeric molecular chaperone composed of 31-kDa subunits [27, 28]. Fig. 3A shows that while the N-termini of the two subunits are in close proximity (34Å) and at the end of unstructured segments, Hsp31 C-termini are 83Å apart and lie at the end of α -helices located on opposite sides of the dimer. We used this distinct placement of His tags to investigate how an increase in the number of hexahistidine extensions and their spatial co-localization would influence the biomineralization process. At a concentration of 2 μ M, only His₆-Hsp31 was capable of supporting the formation of optically active nanoparticles ($D_h = 20 \pm 5$ nm) exhibiting the expected Mn-associated peak but the maximum emission intensity at 590 nm was low (about 20% that observed at the same concentration of BB-TrxA::CT43; Fig. 1C and 3B). When used at 5 μ M, both proteins yielded ZnS:Mn QDs (Fig. 3C–D). However, His₆-Hsp31 mediated the formation of nanoparticles that were 1.7-fold brighter and 40% smaller than those obtained with Hsp31-His₆ (D_h of 19.5 ± 4.5 nm vs. 32 ± 5 nm, respectively).

To rule out the possibility that the difference in hydrodynamic diameters was related to an increase in the size of the inorganic core, preparations were imaged by TEM. Fig. 3E–F shows that the two proteins yielded spherical particles of comparable diameters (4.3 ± 1 nm for His₆-Hsp31 vs. 4.8 ± 0.6 nm for His₆-Hsp31). These values are similar to those we previously reported for undoped (4.3 ± 1 nm) [14] and Mn-doped (4.1 ± 0.7 nm) [13] ZnS nanocrystals mineralized in the presence of BB-TrxA::CT43. Thus, the larger hydrodynamic diameter of QDs produced with His₆-TrxA relative to BB-TrxA::CT43 is most likely related to the fact that the two proteins have very different sizes (62 vs. 14 kDa), while the larger D_h of QDs mineralized with Hsp31-His₆ relative to His₆-Hsp31 can be accounted for by the differential orientation of Hsp31 at the surface of nanocrystals, as schematically illustrated in Fig. 4. The proximity of the two hexahistidine extensions in the His₆-Hsp31 construct also explains why this protein is more efficient at supporting ZnS nanocrystal synthesis at low concentrations (Fig. 3B), and consistent with our observation that repetition of histidines in a hexahistidine motif lowers the effective concentration needed for QD mineralization (Fig. 1).

4. Conclusions

We have shown here that His-tagged proteins are suitable for the direct mineralization of luminescent ZnS:Mn nanoparticles in aqueous solvents and under mild conditions of temperature and pH. Considering how prevalent the polyhistidine purification technology has become [17, 29, 30], it should be possible to use the large number of available His-tagged proteins and the synthesis approach described herein to produce water-soluble QDs that combine luminescence properties with ligand binding, enzymatic activity or other useful function conferred by the capping protein shell.

This study also highlights differences and commonalities in how free amino acids, peptides and proteins influence functional inorganic mineralization. In particular, we have found that increasing the local concentration of histidine by repetition in a polyhistidine tag or spatial co-localization through N-terminal fusion to Hsp31, can significantly improve the quality of ZnS:Mn nanocrystals produced. We have also demonstrated that fusing His tags to a protein scaffold leads to a two orders of magnitude reduction in the concentration of biological reagent required for QD mineralization. This simple step converts a solid binding peptide exhibiting synthesizing capability from a ligand that is effective at millimolar concentration into a surface-specific agent capable of controlling inorganic materialization at micromolar concentrations [31]. These lessons should prove valuable in informing the design of artificial proteins that will provide unparalleled control on the morphology and function of next generation hybrid materials.

Acknowledgments

This work was supported by NIH-NIEHS award 1U19ES019545. TEM characterization was performed at the University of Washington NanoTech User Facility, a member of the NSF National Nanotechnology Infrastructure Network (NNIN).

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- We show that His-tagged proteins can mineralize ZnS:Mn QDs
- Increasing histidine local concentration improves QD optical quality
- Increasing histidine spatial proximity improves QD optical quality

Figure 1.

Histidine repetition lowers the concentrations require for QD production and improves optical properties. Appearance of colloidal suspensions obtained at the indicated concentrations of free histidine (A) or hexahistidine-containing peptide (B) after filtration through 0.2 μm membranes and under UV illumination. (C) Emission spectra obtained with 8 mM of free hisidine (purple), 1 mM of peptide (blue) or 2 μM of BB-TrxA::CT43. The left inset shows the appearance of BB-TrxA::CT43-mineralized QDs under UV illumination. The right inset shows the corresponding absorption spectra.

Figure 2.

His-TrxA supports the mineralization of ZnS:Mn QDs at micromolar concentrations. TrxA (A) or its N-terminally His-tagged variant (B) were used for colloid production at the indicated concentrations. Samples were filtered through 0.2 μm membranes, emission and absorption spectra were collected, and samples were photographed under UV illumination.

Figure 3.

His₆-Hsp31 yields higher quality ZnS:Mn QDs relative to Hsp31-His₆. (A) Ribbon structure of the Hsp31 dimer. Subunits are identified with different colors and the location of the N- and C-termini are shown with purple and green circles, respectively. (B) Emission spectra obtained post-filtration with 2 μM of the indicated proteins. The inset shows the appearance of the samples under UV illumination. (C) As in panel B but with 5 μM of proteins. (D) Absorption spectra obtained post-filtration with 5 μM of the indicated proteins. TEM images of QDs obtained with 5 μM of His₆-Hsp31 (E) or 5 μM of Hsp31-His₆ (F).

Figure 4. Possible scheme for the capping of ZnS:Mn nanoparticles by His₆-Hsp31 (A) and Hsp31-His₆. Hydrodynamic diameters are represented by dashed circles.