Fabrication of water soluble and biocompatible CdSe nanoparticles in apoferritin with the aid of EDTA

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Apoferritin-coated photoluminescent CdSe nanoparticles generated by an EDTA-mediated *in situ* method are photostable, water soluble and biocompatible.

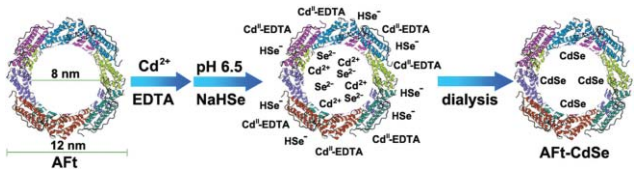
Fluorescent semiconductor nanocrystals (also known as quantum dots, QDs) have shown great potential in cellular imaging, bio-labelling, and deep tissue structure mapping for their high quantum efficiency, long-term photostability, narrow emission, and continuous absorption spectra.[1] However, the biomedical applications of QDs are largely limited by their poor water solubility and inadequate biocompatibility.[2] QDs made directly in water often have narrow size ranges and wide size distribution;[3] while those synthesized in high temperature organic solvents are insoluble in water.[4] On the other hand, QDs are more likely to decompose partially and release ions owing to the enhanced surface-to-volume ratio, and some of the liberated ions such as Cd^{2+} from CdSe are fatal to living cells.[5] Therefore, how to make the hydrophobic and toxic QDs water soluble and biocompatible is the major challenge to the biomedical applications.

Phospholipids,[6] amphiphilic polymers,[7] dendrimers,[8] oligomeric phosphines,[9] and multidentate hydrophilic ligands[10] have been used to modify the surface of QDs in order to improve their water solubility and chemical stability. For example, the hydrophobic QDs synthesized in organic solvents could be rendered hydrophilic by replacing the surface ligands with some water-soluble bifunctional molecules.[11] However, ligand exchange inevitably alters the chemical and physical states of the surface atoms and in most cases dramatically decreases the quantum efficiency of the QDs.[9] In addition, thiol-based ligands may form disulfides over time and come off from the surface and cause the QDs aggregate and precipitate finally out of water.[11a]

CdSe QDs have shown promising properties for biomedical applications;[12] nevertheless, they release Cd^{2+} ions in aqueous solution, which are highly toxic to biological systems. The release of Cd^{2+} is enhanced by oxidation, either through exposure to air or UV irradiation, but is suppressed or even shunned by encapsulating the QDs with appropriate shells.[13] Thus, a direct way to avoid the toxicity of CdSe QDs is to make them well coated to become biologically inert. A variety of strategies have been proposed for CdSe QDs coating. Wrapping QDs with simple molecules such as mercaptopropionic acid cannot prevent the release of Cd^{2+} from the particle surface because the ligand shell is not very stable; covering them with a silica layer is very effective, but the procedures to make a controllable silica layer around hydrophobic QDs are generally complicated.[14] Moreover, silica coating needs to be carried out at dilute conditions, which is not suitable for large quantity production.[15]

Apoferritin (AFt) is the demineralized form of the cellular iron storage protein ferritin. The 24 polypeptide subunits of AFt assemble automatically to form a hollow protein cage with internal and external diameters of 8 and 12 nm, respectively. Eight hydrophilic channels of *ca.* 4 Å permeate the protein cage, which facilitate the influx and efflux of metal ions and small molecules of appropriate size.[16] The rich aspartate and glutamate residues on the inner surface of AFt are believed to promote the formation of nanocrystals in the cage, and the dimensions of AFt confine the upper limit of the crystal growth. As a nanoreactor or biotemplate, AFt has been widely used to prepare different inorganic nanoparticles,[17] including those of CdSe.[18] However, AFt has seldom been used as a coating material to improve the biophysical and biochemical properties of QDs.

In this work, we describe the procedure to encapsulate CdSe QDs in the AFt cage *via* an EDTA-mediated approach, where the protein coat was put on the QDs by forming CdSe particles *in situ* in the AFt cavity (Scheme 1).† The AFt-coated CdSe QDs were characterized by UV-vis spectroscopy, transmission electron microscopy (TEM), and energy dispersive X-ray spectroscopy (EDX). These coated QDs are aqueous soluble and biocompatible.



Scheme 1 The route to fabricate hydrophilic and non-toxic AFt-coated CdSe QDs (AFt-CdSe) with the aid of EDTA.

Cd^{2+} reacts quickly with Se^{2-} and induces CdSe aggregation quickly in aqueous solution. To slow down the reaction, ethylenediaminetetraacetic acid disodium salt (EDTA) was selected to stabilize Cd^{2+} by forming Cd^{II}-EDTA species and NaHSe was chosen to supply Se^{2-}. The Cd^{2+} and Se^{2-} ions released from the precursors flow into the AFt cavity through the hydrophilic channels and form CdSe nanoparticles there. The QDs prepared as such wear a protein coat spontaneously. Since the availability of Cd^{2+} in the cavity is mainly determined by the formation constant of Cd^{II}-EDTA ($\log K_f = 16.46$),[19] the supersaturation of Cd^{2+} in solution could thereby be overcome. Considering EDTA could also modulate the reactivity of other metal ions for similar

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purpose, this chelator-mediated slow reaction system may be extended to synthesize other uniform nanoparticles in AFt.

The UV-vis spectrum of the AFt-coated CdSe QDs exhibits a dramatic change as compared to that of AFt (Fig. 1). The typical absorption band of AFt at 280 nm is broadened and a wide absorption band in the range of 400–580 nm appears. The latter is consistent with the absorption characters observed for CdSe nanoparticles.[20] It is estimated from the shape of absorption that CdSe cores in the cavity are polydisperse with diameters about 3–4 nm.[21] As a contrast, the sample prepared without EDTA presents a negligible absorption because of the severe aggregation.

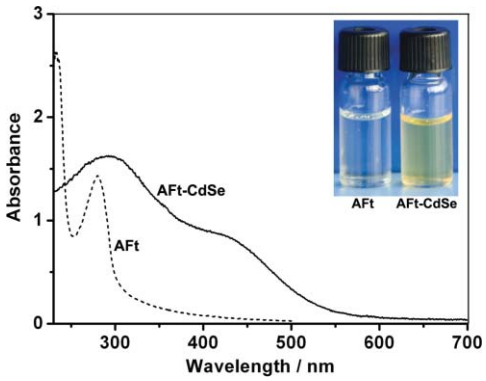

Fig. 1 UV-vis spectra of the solutions of AFt (dashed line) and AFt-coated CdSe QDs (solid line). Inset is the photograph of AFt and AFt-coated CdSe QDs solutions.

AFt-coated CdSe QDs were negatively stained with uranyl acetate (1%) and examined by TEM in order to characterize the CdSe core in AFt. A typical TEM image is shown in Fig. 2A, where discrete electron-dense nanometre-sized CdSe particles are encircled by AFt shells, and the protein shells remain nearly intact (Fig. 2B). It is clear that NaHSe prefers reacting with Cd^{2+} ions inside the AFt cavity because most AFt cages contain QDs, and

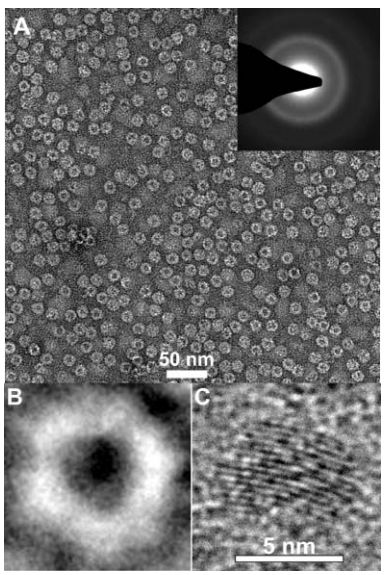

Fig. 2 The TEM image of AFt-coated CdSe QDs stained with uranyl acetate (A), the specific view of a single protein sphere (B) and the high-resolution TEM image of a single CdSe nanoparticle (C). The inset in A is the selected-area electron diffraction pattern.

CdSe was formed only in the cavity. This explains the scarcity of bulk precipitation in the reaction mixture and the stability of the QDs in aqueous solution. The selected-area electron diffraction pattern (Fig. 2A, inset) indicates that CdSe adopts a cubic zinc blende structure (JCPDF no. 19-0191). The high-resolution TEM of individual cores (Fig. 2C) exhibits continuous lattice spacings of 3.52 Å, which are analogous to the (111) facet of CdSe single crystals. The dimensions of the CdSe cores and the protein spheres are determined statistically according to the sizes of 100 particles in the TEM image. The mean diameter of the former is 4.2 nm ($\sigma = 0.7$) (Fig. 3A) and that of the latter is 11.8 nm ($\sigma = 1.2$). The result is basically consistent with the size obtained from UV-vis analysis. The dimensions of the recombinant protein cages were also measured by dynamic light scattering, which gives a mean diameter of 12.5 nm ($\sigma = 2.2$) (Fig. 3B). The results indicate that the protein cages hardly changed after the encapsulation. EDX spectroscopy further confirms the presence of Cd and Se (Fig. 4). Based on the relative areas under the peaks for Cd and Se, the atomic ratio of Cd to Se is evaluated to be approx. 1 : 1.

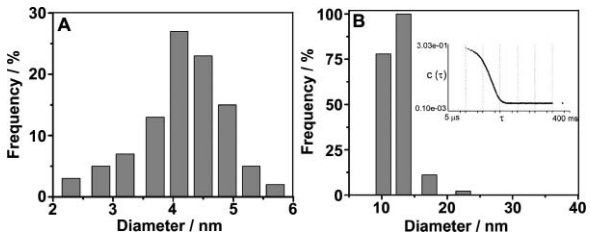

Fig. 3 Dimensions of the CdSe cores determined by statistic method (A) and the AFt-CdSe spheres analyzed by dynamic light scattering (B).

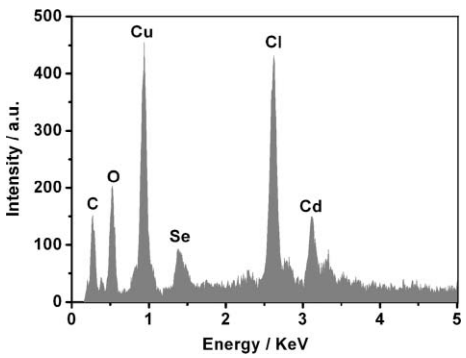

Fig. 4 EDX spectrum of AFt-coated CdSe QDs showing Cd and Se peaks. Peaks for Cu and C arise from the supporting grid of Cu and covering film of C; peaks for O and Cl come from the protein shells and NaCl introduced during dialysis.

The photoluminescence spectrum of AFt-coated CdSe QDs was measured with the excitation wavelength of 442 nm (Fig. 5). An emission peak centered at 582 nm is observed, with the Raman scattering of water appearing around 442 nm. Similar phenomena were observed in the CdS nanoparticles synthesized in the rLiDps cavity.[22] As judged by the emission position, the luminescence should be resulted from the core within the AFt cavity. Thus, covering CdSe QDs with AFt does not exert a radical influence on their photoluminescent property.

The cytotoxicity of the AFt-coated CdSe QDs was tested on the human cervical cancer cell line Hela and the human

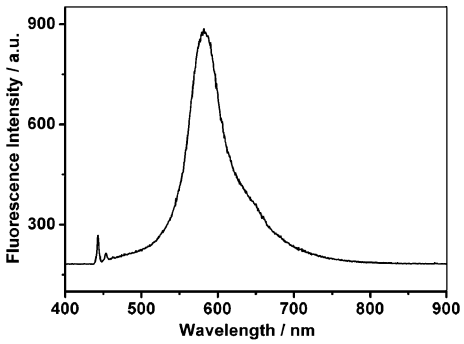

Fig. 5 Photoluminescence spectrum of the AFt-coated CdSe QDs (λ_{ex} = 442 nm).

hepatocarcinoma cell line HepG2 by MTT assay.‡ AFt-coated CdSe QDs demonstrate a comparable cytotoxic profile with that of AFt (Fig. 6), suggesting the potential toxic effect of CdSe QDs can almost completely be shielded by the protein coat and hence their biocompatibility is greatly enhanced.

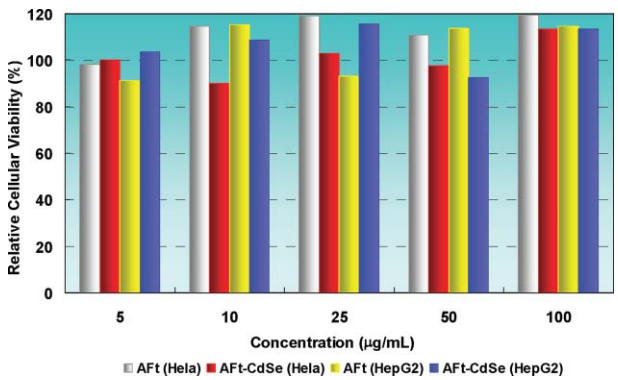

Fig. 6 Cytotoxicity of AFt-coated CdSe QDs (AFt-CdSe) against human cervical cancer cell line Hela and human hepatocarcinoma cell line HepG2 at concentrations of 5–100 μg mL^{-1} after 24 h, with AFt as a negative reference.

In summary, CdSe QDs were successfully encapsulated into the AFt cage by an *in situ* generation method with the aid of EDTA. AFt acts as a nanoreactor for CdSe mineralization firstly and serves as a coating material for the formed QDs subsequently. The water solubility of CdSe QDs is largely improved and the potential cellular toxicity is efficiently prevented by this approach. Moreover, the active sites throughout the protein surface provide further possibilities for interactions or couplings with antibodies, peptides and proteins, which will broaden the applicability of QDs in biomedical fields. This strategy could be extended to the solubilization of other QDs in aqueous solution.

Acknowledgements

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Notes and references

† AFt was obtained from the horse spleen ferritin (85 mg mL^{-1}, 0.15 mol L^{-1} NaCl–H_2O, Sigma) by demineralization according to the established procedures.[23] The protein concentration was determined by BCA method using bovine serum albumin as a standard. NaHSe (50 mM) was prepared following the literature method.[24] The mineralization of CdSe QDs in AFt was completed as follows. Deaerated AFt (*ca.* 1 mg mL^{-1}) prepared with NaCl solution (150 mM) was mixed with air free $CdCl_2$ (100 mM, 10 μL) ([Cd^{II}]/[AFt] ≈ 440). After continuous stirring for 1 h, EDTA (100 mM, 10 μL, pH 6.5) and freshly prepared NaHSe (50 mM, 20 μL) were added to the mixture successively, which turned the colourless solution yellow immediately. The reaction was kept going under stirring for 2 h and the resulting solution was centrifuged at 10 000 rpm for 10 min and then was dialyzed against NaCl (150 mM) thoroughly. Every step in the process was taken in an argon atmosphere. Two control experiments on the formation of CdSe in the presence of AFt but absence of EDTA, or in reverse, were carried out in the same conditions as those for the primary experiment. Dark red precipitates were observed instantly or after 5 min.

‡ MTT assay was carried out referencing to the literature method.[25] Briefly, Hela or HepG2 cells (*ca.* 5000) were seeded in each well of 96-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 12 h. The cells were then treated in triplicate with AFt-coated CdSe QDs or AFt (100, 50, 25, 10 and 5 μg mL^{-1}) at 37 °C for 24 h. Aliquot of MTT (10 μL, 5 mg mL^{-1}) was added to each of the wells. The supernatant was taken off after 4 h of incubation at 37 °C and DMSO (150 μL) was added. The culture was subjected to spectrophotometric determination for the amount of MTT formazan product at 490 nm in DMSO. Optical density was used to calculate the percentage of cell growth inhibition as with respect to the control. The cytotoxicity result is the mean value of three replicate tests.

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