Violet ZnSe/ZnS as an Alternative to Green CdSe/ZnS in Nanocrystal−Fluorescent Protein FRET Systems

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Fluorescent proteins from the green fluorescent protein family strongly interact with CdSe/ZnS and ZnSe/ZnS nanocrystals at neutral pH. Green emitting CdSe/ZnS nanocrystals and red emitting fluorescent protein dTomato constitute a 72% efficiency FRET system with the largest alteration of the overall photoluminescence profile, following complex formation, observed so far. The substitution of ZnSe/ZnS for CdSe/ZnS nanocrystals as energy donors enabled the use of a green fluorescent protein, GFP5, as energy acceptor. Violet emitting ZnSe/ZnS nanocrystals and green GFP5 constitute a system with 43% FRET efficiency and an unusually strong sensitized emission. ZnSe/ZnS-GFP5 provides a cadmium-free, high-contrast FRET system that covers only the high-energy part of the visible spectrum, leaving room for simultaneous use of the yellow and red color channels. Anisotropic fluorescence measurements confirmed the depolarization of GFP5 sensitized emission.

INTRODUCTION

Semiconductor nanocrystals and fluorescent proteins can be assembled in fluorescence (or Förster) energy transfer (FRET) systems. Both nanocrystals and fluorescent proteins present outstanding properties as biological probes (*1*), and their coupled use provides a research tool of promising potential.

Semiconductor nanocrystals have large absorption cross sections, high photoluminescence (PL) quantum yields (QY), size-tunable PL spectra, exceptional photochemical stability (*2, 3*), and good dispersibility in biological fluids (*3*−*7*). The green fluorescent protein (GFP) family is composed of a group of homologous, colored, compact proteins that spontaneously generate their own fluorophore (*8*−*10*). They have been extensively used as gene-based probes in molecular and cell biology (*11*). The diversity of emission and absorption profiles of these elements provide a variety of possible donor−acceptor FRET pairs.

The nanocrystal−fluorescent protein FRET pairs commonly used to date are composed of green emitting CdSe/ZnS nanocrystals and yellow or red emitting fluorescent proteins (*12*−*15*). Such systems have been used to create three chromophore FRET systems (*13*) and to research cell penetration and nanoparticle mediated drug delivery (*14*).

In the present work, we extend the available nanocrystal−fluorescent protein FRET pairs by substituting the previously used red emitting protein HcRed1 (quantum yield 5% (*12*)) with the high quantum yield dTomato (quantum yield 69% (*16*)) and by introducing ZnSe/ZnS as a cadmium-free, blue-shifted nanocrystal donor substitute for CdSe/ZnS. The CdSe/ZnS−dTomato system exhibits highly efficient FRET with larger overall PL alteration under complex formation than nanocrystal−fluorescent protein pairs used previously (*12*−*15*). ZnSe/ZnS nanocrystals are advantageous not only because they are free of toxic cadmium (*17*), but also because their emission peak is blue-shifted to 400 nm, leaving all color channels from blue to red available for other probes. The ZnSe/ZnS−GFP5 system presents particularly attractive characteristics such as unusually intense sensitized emission and excellent contrast due to the large separation, 100 nm, between donor and acceptor PL peaks.

EXPERIMENTAL PROCEDURES

Nanocrystals. Mercaptoundecanoic acid (MUA) coated CdSe/ZnS nanocrystals were purchased from NN Laboratories. The emission spectrum of these CdSe/ZnS nanocrystals is provided in Figure 1. Mercaptopropionic acid (MPA) coated ZnSe/ZnS nanocrystals were synthesized in accordance with a protocol derived from ref 18. MPA was added to 60 mL of Milli-Q water containing 1 mM zinc perchlorate, to a final concentration of 2.4 mM. The pH of the solution was raised to 10.5 by the addition of sodium hydroxide. The solution was then stirred and put under a nitrogen flux for 10 min. In parallel, 9.6 mg of selenium powder and 10 mg of sodium borohydride were mixed in a 15 mL Falcon flask. To this mixture 2 mL of Milli-Q water was added, and the solution was sonicated for 5 min, keeping the Falcon's cap loose to let the evolving gas escape. 0.5 mL of this solution was injected in the zinc perchlorate solution and stirred. Thirty milliliters of the resulting solution were collected and poured inside a 50 mL total volume Teflon-lined autoclave. The Teflon set was then placed inside an oven at 70 °C for 24 h. 0.85 mL of the autoclaved solution was collected and placed inside a 1 mL quartz cuvette. 4.4 μL of MPA were diluted in 1 mL of Milli-Q water containing 0.1 mM sodium hydroxide, and 0.15 mL of this solution was also

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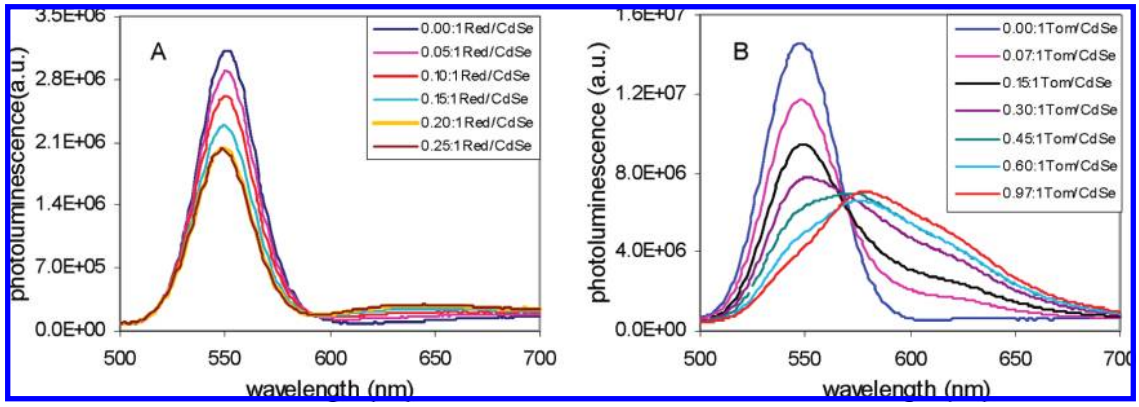

Figure 1. (A) Evolution of the photoluminescence spectra of CdSe/ZnS nanocrystals in CdSe/ZnS-HcRed1 ("Red") assemblies versus increasing HcRed1 to CdSe/ZnS molar ratio. (B) Evolution of the photoluminescence spectra of CdSe/ZnS nanocrystals in CdSe/ZnS−dTomato assemblies versus increasing dTomato to CdSe/ZnS molar ratio.

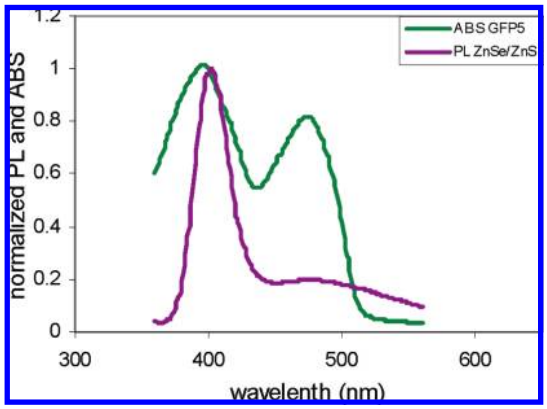

Figure 2. Normalized photoluminescence (PL) spectrum of ZnSe/ZnS nanocrystals and absorption (ABS) spectrum of GFP5 solutions.

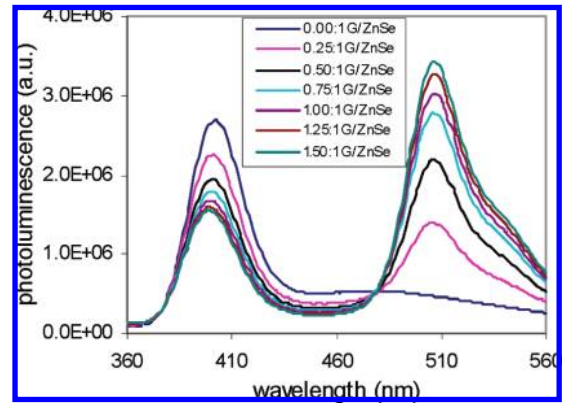

Figure 3. Evolution of the photoluminescence spectra of ZnSe/ZnS-GFP5 assemblies versus increasing GFP5 (G) to ZnSe/ZnS molar ratio. Excitation at 310 nm, pH 7.4.

added to the quartz cuvette. The cuvette was then capped and placed 20 cm from a commercial 27 W UV light (emission peak at 370 nm) inside an oven held at 60 °C, where it was kept for 4 h. Absorption and emission spectra of the ZnSe/ZnS nanocrystals are shown in Figures 2 and 5. The approximate concentration, 2.7 nM, and diameter, 3 nm, of the nanocrystals were inferred in accordance to *refs* 19 *and* 20. The quantum yield of the colloidal solution, 50%, was estimated by comparison with quinine in aqueous 0.5 M sulfuric acid (quantum yield: 54.5% (*21*)).

Fluorescent Proteins. HcRed1 on plasmid pHcRed1 was purchased from Clontech. Plasmid pHcRed1 was used without modifications. HcRed1 does not encode a hexa-histidine sequence. dTomato was generously provided by R. Y. Tsien (University of California at San Diego) on plasmid pRSET-B dTomato. pRSET-B (Invitrogen) encodes a hexa-histidine sequence at the N-terminus of the dTomato coding sequence. pRSET-B dTomato plasmid was used without modification. GFP5 was generously provided by J. Haseloff (University of Cambridge) on plasmid pBIN m-GFP5-ER. The gene encoding GFP5 was excised by digestion with *Eco*RI and *Sac*I and ligated in the same sites at the multiple cloning site of the pET-28a (+) (Novagen). pET-28a (+) also encodes a hexa-histidine sequence at the N-terminus of the GFP5 coding sequence. Each plasmid was respectively introduced into BL21(DE3) *E. coli* cells (Invitrogen) and expression induced with 0.16 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) for 20 h at 30 °C, before harvesting the cells by centrifugation. His-tagged proteins were purified from soluble cell lysates using Ni-NTA Resin (Qiagen) eluted with 500 mM imidazole and dialyzed. The absorption spectrum of GFP5 is shown in Figure 2. Figure 3 shows the emission spectrum of the ZnSe/ZnS-GFP5 system as a function of the GFP5 (G) to ZnSe/ZnS molar ratio.

Nanocrystal−Fluorescent Protein Conjugates. To assemble CdSe/ZnS-HcRed1 and CdSe-dTomato, the proteins at the appropriate molar ratios were added to 33 nM of CdSe/ZnS (capped with MUA) in 5 mM phosphate, 5 mM NaCl, pH 6 at room temperature. To assemble ZnSe/ZnS-GFP5, GFP5 at the appropriate molar ratios was added to 120 nM of ZnSe/ZnS (capped with MPA) in phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4, PBS) at room temperature. Conjugates were characterized through the quenching of the nanocrystals' fluorescence and enhancement of the proteins' fluorescence, which was monitored by measuring the steady-state fluorescence using a SPEX Fluorolog 1691 (Spex industries) spectrophotometer equipped with a 450 W Xenon lamp. The SPEX software MCORRECT.SPT was used to correct the responsivity of the spectrophotometer. Polarizers were introduced into the SPEX to measure the polarization anisotropy (r). Anisotropy was calculated using the equation:

$$r = \frac{VV - gVH}{VV + 2gVH}$$

Vertically polarized excitation of the donor was used in combination with capture of both horizontal (VH) and vertical (VV) polarizations. The factor g corrects for polarization bias in the instrument (*22, 23*). g was calculated using the equation

$$g = \frac{HV}{HH}$$

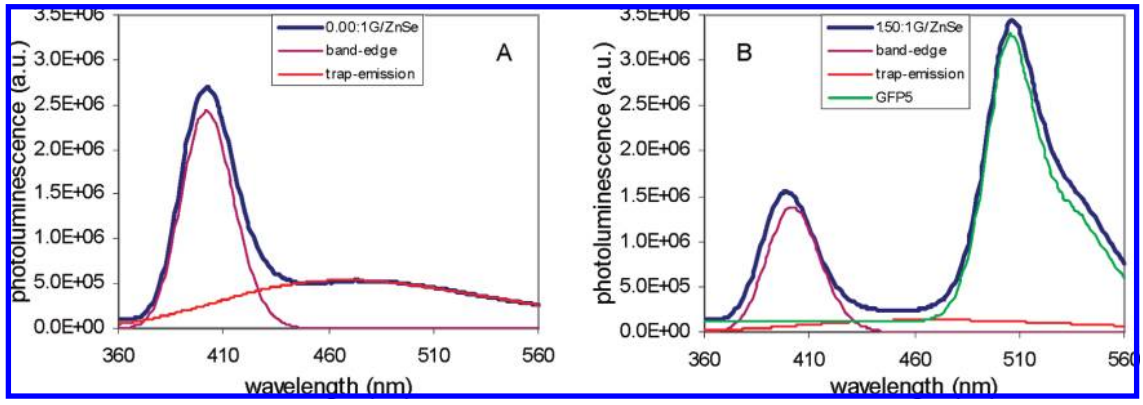

Figure 4. (A) Deconvolution of the ZnSe/ZnS nanocrystals photoluminescence spectrum with two Gaussians corresponding to band-edge and trap emission. (B) Deconvolution of the ZnSe/ZnS−GFP assembly photoluminescence spectrum at GFP5 (G) saturation.

In this case, horizontally polarized excitation of the donor was used in combination with capture of both vertical (*HV*) and horizontal (*HH*) polarizations.

RESULTS AND DISCUSSION

We have previously reported the occurrence of FRET from green emitting CdSe/ZnS nanocrystals to the red emitting protein HcRed1 (*12*), as shown in Figure 1A. At that time it was pointed out that the low quantum yield of HcRed1, 5%, was the reason for the weak intensity of the sensitized emission. We have now replaced HcRed1 with the red emitting protein dTomato (*16*), which has a quantum yield of 69%, and a substantial increase of the sensitized emission intensity was observed, as shown in Figure 1B.

In both cases, the titration was conducted at pH 6. The FRET efficiency for the pair CdSe/ZnS−dTomato, calculated by using the relative fluorescence intensity of the donor, in the absence and presence of acceptor is 72% (*22*). The strong overall fluorescence transition undergone by the CdSe/ZnS−dTomato system during complex formation makes it an excellent tool for FRET-based characterization.

Bulk CdSe exhibits band gap emission centered at 713 nm. Due to quantum confinement, it is possible to tune the emission of CdSe nanocrystals over the visible spectrum by controlling their diameter. However, blue emitting CdSe nanocrystals require very small diameters, which renders them unstable and restricts their commercial availability (*20*). This limitation is the primary reason there are no reports of CdSe nanocrystals as FRET donors in the blue spectral region. To overcome this limitation, we have substituted CdSe nanocrystals. Bulk ZnSe emits at 459 nm, and there are a large number of references reporting aqueous synthesis of efficient ZnSe/ZnS nanocrystals (*18, 20, 24*). We decided to synthesize ZnSe/ZnS nanocrystals at our lab, which demanded a considerable effort in refining the available protocols and adapting them to the lab conditions. At pH 10.5, immediately after synthesis, our nanocrystals presented a quantum yield of 50%, with emission centered at 400 nm. The quantum yield slowly decreases with time and was 25% one month after synthesis. Such a decrease in quantum yield is possibly related to the long-term dynamics undergone by the MPA ligand at the nanocrystal surface (*25*). ZnSe/ZnS nanocrystals are promising candidates for FRET donors at the shorter wavelength end of the visible spectrum. Moreover, the violet emission of these nanocrystals overlaps well with the GFP5 absorption spectrum, as can be seen in Figure 2. GFP5 is a mutant form of GFP especially designed to be expressed in *E. coli* (*26*).

Unlike the MUA coated CdSe/ZnS nanocrystals, the lab synthesized ZnSe/ZnS nanocrystals were coated with MPA. MPA, however, confers poor protection for pH changes. At a pH below 7, the ZnSe/ZnS nanocrystals barely fluoresce. Consequently, assays with ZnSe/ZnS were made in PBS buffer at pH 7.4, whereas assays made with CdSe/ZnS nanocrystals had been conducted at pH 6. At pH 7.4, the ZnSe/ZnS nanocrystals present a quantum yield of 15%, which is substantially smaller than the quantum yield of 50% at pH 10.5 but is still satisfactory for FRET characterization, as can be seen in Figure 3. Actually, there is a tradeoff between lowering the pH to increase the electrostatic attraction among nanocrystals and fluorescent proteins (*12*) and augmenting the pH to increase the nanocrystal fluorescence quantum yield. As a compromise, we decided to conduct the assays at an intermediate pH value, 7.4.

The calculated FRET efficiency using the relative fluorescence intensity of the donor, in the absence and presence of acceptor, is 43% (*22*). Despite the lower FRET efficiency when compared to the CdSe/ZnS−dTomato pair, the high intensity of the sensitized emission is an outstanding characteristic of the new system. GFP5 has an elevated quantum yield, 77% (*26, 27*), and the nanocrystals, at pH 7.4, have a quantum yield of only 15%. Probably, some of the nanocrystal energy is being rapidly transferred to the GFP5 before it is emitted or nonradiatively recombined, causing the substantial enhancement of the GFP5 emission (*28*). Bidentate anchors like dihydrolipoic acid (DHLA) confer superior stability for the nanocrystals than single sulfur molecules like MPA, improving their quantum yield at neutral pH (*13, 14*). Tests are being conducted to verify the effect of DHLA on the energy transfer among ZnSe/ZnS and GFP5. Preliminary results show that the addition of 5 mM DHLA, after the UV processing, immediately increases the nanocrystal emission intensity as well as has a positive effect on retaining the nanocrystal quantum yield at neutral pH. Studies concerning the long-term stability and dilution effects over the nanocrystal quantum yield at neutral pH are being conducted.

The ZnSe/ZnS nanocrystal emission spectrum has two components, one related to the band-edge emission, centered at 402 nm, and the other related to the trap emission, centered at 468 nm (*18*), which can be represented by two Gaussians, as shown in Figure 4A. In Figure 4B, the two Gaussians, together with a curve corresponding to pure GFP5, are used to deconvolute the emission spectrum of a ZnSe/ZnS−GFP5 assembly at a saturating concentration of GFP5. Analyzing Figure 4B, two considerations were made. First, the nanocrystals suffer a blue shift of 3 nm when put into close proximity to GFP5, which is characteristic of an environment change at the nanocrystal surface (*15*). Second, comparing the deconvolution curves for the emission spectra of ZnSe/

ZnS alone and for the assembly ZnSe/ZnS−GFP5 it comes out that the deconvolution factor for the trap emission was more strongly reduced, 75%, than the deconvolution factor for the band-edge emission, which was reduced by only 43%, possibly because the ratio of the Förster exchange rate from the traps to the GFP5 is proportionally larger than the Förster exchange rate from the band-edge states to the GFP5. In spite of the possible high trap emission Förster exchange rate, we intend to improve the postprocessing protocol for the ZnSe/ZnS nanocrystals to further reduce the trap emission. As can be seen in Figure 4B, the trap emission causes a signal contamination in the GFP5 emission spectrum, and its elimination would override any spectral overlapping, further improving the resolution of this FRET system.

The ZnSe/ZnS−GFP5 constitutes a sensitive and well-resolved, cadmium-free nanocrystal−fluorescent protein FRET system. Moreover, due to its low wavelength position in the visible spectrum, this system is particularly suitable for extension to a multifluorophore FRET system. For example, GFP5 emits at 506 nm, and a ternary FRET system having yellow fluorescent protein as the second energy acceptor can be envisioned. Such a system would include FRET among fluorescent proteins along with nanocrystal donors. Multifluorophore FRET systems are useful for the elucidation of multicomponent molecular assemblies, characteristic of highly coordinated biological supramolecular interactions (*13*).

The enhanced fluorescence emission of GFP5 was also characterized by polarization fluorimetry to confirm the FRET. The direct emission of fluorescent proteins is highly polarized, while the depolarization of its fluorescence is observed only in the presence of energy transfer (*22, 23*). This characteristic provides the means to detect the FRET with great accuracy in high-throughput screening assays (*23*). In Figure 5, it is possible to verify that the anisotropy of GFP5 complexed with the ZnSe/ZnS tends to zero when the absorption of the nanocrystals becomes strong, i.e., when the emission of GFP5 is mainly due to energy transfer. The anisotropy of GFP5 alone, on the other hand, is essentially wavelength independent.

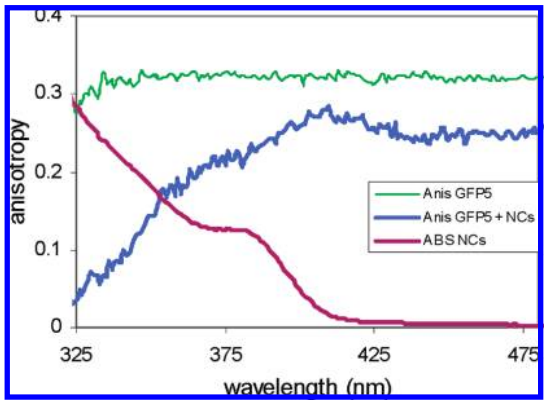

Figure 5. Steady-state anisotropy was calculated from fluorescence excitation spectra with emission set at 506 nm for solutions containing GFP5 alone and GFP5 complexed with ZnSe/ZnS nanocrystals. The absorbance spectrum of the nanocrystals is also depicted.

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