Fluorescence Quenching of CdSe/ZnS Quantum Dots by Using Black Hole Quencher Molecules Intermediated With Peptide for Biosensing Application

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Quantum dots (QDs) have recently been investigated as fluorescent probes for detecting a very small number of biomolecules and live cells; however, the establishment of molecular imaging technology with on–off control of QD fluorescence remains to be established. Here we have achieved the fluorescence off state of QDs with the conjugation of black hole quencher (BHQ) molecules intermediated with peptide by using streptavidin-QDs585 and biotin-pep-BHQ-1. The fluorescence of streptavidin-QDs585 was decreased by the addition of biotin-pep-BHQ-1 in a dose-dependent manner. It has been suggested that the decrease in QDs585 fluorescence occurred through a Förster resonance energy transfer (FRET) mechanism from the analysis of fluorescence intensity and lifetime of streptavidin-QDs585 and QDs585-pep-BHQ-1. QDs585 fluorescence could be quenched by more than 60% efficiency in this system. The sequence of intermediate peptide (pep) was GPLGVRGK, which can be cleaved by matrix metalloproteinases (MMPs) produced by cancer cells. QDs585-pep-BHQ-1 is thus expected to detect the MMP production by the recovery of QDs585 fluorescence as a new bioanalytical agent for molecular imaging.

Key words: Quantum dots (QDs); Black hole quencher (BHQ); Fluorescence resonance energy transfer; Matrix metalloproteinase

INTRODUCTION

Quantum dots (QDs) are inorganic probes that generally consist of cadmium selenide/zinc sulfide (CdSe/ZnS) core/shell semiconductor nanocrystals. QDs have several fluorescence advantages such as a high luminance, resistance to photobleaching (long time labeling), a range of excitation wavelengths, and narrower emission bandwidths, compared with conventional inorganic and organic agents (1–3,7,8,10). Owing to these characteristics, QDs recently have been applied as the material of 4K television display, which can achieve high definition, ultrahigh sensitivity, ultralong lifetime, energy saving, and ultralow cost (10). In addition, QDs have recently been developed as fluorescent probes for detecting a very small number of biomolecules and live cells in analytical applications for diagnostics (5,19).

Bakalova et al. showed that QD-based Western blotting protocols were useful for the ultrasensitive detection of “tracer” proteins in cell lysates (5), and QD-conjugated

hybridization probes were effective for a rapid procedure for the selection of highly effective small-interfering RNA (siRNA) sequences for RNA interference in mammalian cells (4). Zhelev et al. reported that QD–lectin conjugates could identify leukemia cells from normal lymphocytes using fluorescent confocal microscopy and flow cytometry (20). Biju et al. indicated that QDs could be transfected into the cytoplasm and even the nucleus of the cells using insect neuropeptide, namely, allatostatin I from *Drosophila melanogaster* (9).

On the other hand, stem cells labeling technology using QDs has recently received attention due to the extremely significant development of regenerative medicine based on stem cell therapy (11,15–18). Various approaches to label stem cells with QDs have been applied, such as micro-injection, electroporation, liposome-based transduction, and special peptide delivery. We have already reported that stem cells including induced pluripotent stem cells

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(iPSCs) and adipose tissue-derived stem cells (ASCs) could be labeled safely and efficiently with QDs by using cationic liposome or cell-penetrating peptides (15,17). Especially, octa-arginine peptides were effective for the introduction of QDs into various cells including stem cells and the labeling for a long time (12).

Moreover, QD labeling has proven to be effective for in vivo fluorescence imaging of the transplanted stem cells in comparison to other traditional fluorescence agents because of the fluorescence properties of QDs. We have succeeded in observing the behavior and organ-specific accumulation of transplanted ASCs labeled with QDs in acute liver failure mice by using an in vivo fluorescence imaging system, and we could analyze the relationship between the therapeutic effects and the organ-specific accumulation rate of transplanted ASCs (18). However, the diagnostic approach for detecting the changes of the condition of transplanted stem cells, such as cancer development or abnormal differentiation, remains to be fully established. Therefore, the establishment of molecular imaging technology with on-off control of QD fluorescence is expected to be useful for detailed diagnosis of the stem cell state.

In this study, we tried to achieve the fluorescence off state of QDs conjugated with black hole quencher (BHQ) molecules intermediated with peptide, which can be cut by matrix metalloproteinases (MMPs) produced by cancer cells for cancer detection. We investigated their fluorescence intensity and lifetime conjugated with BHQ intermediated with peptide (QDs-pep-BHQ), and analyzed the quenching efficiency of QDs-pep-BHQ.

MATERIALS AND METHODS

QDs and Peptide Samples

Streptavidin-functionalized CdSe/ZnS QDs585 (streptavidin-QDs585) was purchased from Life Technologies (Tokyo, Japan). The streptavidin-QDs585 solution was diluted to 3 nM with purified water (Millipore Milli-Q Lab Purification System; Tokyo; Japan). A custom-made peptide biotin-glycine-proline-leucine-glycine-

valine-arginine-(lysine BHQ-1)-amide {GPLGVRG[K(BHQ-1)]-CONH₂; Biotin-Pep-BHQ-1} was purchased from Custom Peptide and Antibody Service, Eurofins Genomics K.K. (Tokyo, Japan). The biotin-Pep-BHQ-1 was diluted to 1,494 nM with purified water (Millipore Milli-Q Lab Purification System).

Conjugation of QDs With BHQ

QDs585 and BHQ-1 conjugates (QDs585-pep-BHQ-1) were prepared by reacting streptavidin-QDs585 with biotin-pep-BHQ-1. Figure 1 gives the graphical representation of the synthesis procedures. The biotin-pep-BHQ-1 solution (2 μ l, 1,494 nM) was added into the streptavidin-QDs585 solution (330 μ l, 3 nM). After this adding process, the concentration of the peptide in biotin-pep-BHQ-1 was 9 nM. It was increased up to 117 nM by the continuous addition of biotin-pep-BHQ-1.

Fluorescence Emission Spectrum Measurement

Spectral fluorescence analysis of QDs585-pep-BHQ-1 was performed by using a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan). Between the 1-min time gap of successive excitation, the fluorescence intensity was measured. Time-resolved fluorescence spectra were recorded using an assembly of polychromator (250IS; Chromex, Tokyo, Japan) and a photon counting streak-camera (C4334; Hamamatsu, Hamamatsu City, Japan). The QDs585 was selectively excited using 400-nm pulses (150 fs) generated from the second harmonic generation (SHG) crystal of an optical parametric amplifier (OPA) (OPA 9400; Coherent, Tokyo, Japan). The OPA was pumped by 800-nm pulses (200 kHz) from a regenerative amplifier (RegA 9000; Coherent) that was seeded by a mode-locked Ti:sapphire laser (Mira 900F; Coherent). Fluorescence signal from the QDs585 was collected through a suitable band-pass filter and focused at the entrance slit of the polychromator.

Photographic Image Capture

The fluorescent pictures of streptavidin-QDs585 (3 nM) and QDs585-pep-BHQ-1 solution (streptavidin-QDs585:

Figure 1. A schematic diagram of the quenching of quantum dot (QD) fluorescence by using black hole quencher (BHQ) molecules. This diagram shows that streptavidin-QDs are conjugated with biotin-glycine-proline-leucine-glycine-valine-arginine-(lysine BHQ-1)-amide {biotin-GPLGVRG [K(BHQ-1)]-CONH₂; biotin-pep-BHQ-1}, and QD fluorescence was quenched by the BHQ molecules.

biotin-pep-BHQ-1 = 1:10, 1:20, 1:30, and 1:40) upon UV excitation in the reaction cuvette were taken by an ordinary digital camera.

Data Analysis

Stern–Volmer plot was performed by using the fluorescence intensity data. Relative changes in the fluorescence intensity rate (I_0/I) of streptavidin-QDs585 as a function of the concentration of biotin-Pep-BHQ-1 were plotted and analyzed by sigmoidal fit. The slope of the graph was calculated from a linear fit of the data points. The plotting and analysis was performed using Microcal Origin 6.0 software (Microcal, Northampton, MA, USA). I_0 and I are the emission intensity of the donor CdSe/ZnS in the absence and presence of Biotin-Pep-BHQ-1 acceptor, respectively.

Evaluation of FRET Efficiency

The efficiency of energy transfer in a FRET system is given by $E = 1/[1 + (r/R_0)^6]$, where r is the distance between the donor and the acceptor, and R_0 is the Förster distance of the donor acceptor pair, that is, the distance at which efficiency of the energy transfer is 50%. The value of R_0 is given by $R_0 = [(9,000\Phi_0(\ln 10)\kappa^2 J)/(128\pi^5 n^4 N_A)]^{1/6}$, where Φ_0 is the fluorescence quantum efficiency of the donor, κ^2 is the donor-to-acceptor dipole orientation factor, J is the spectral overlap integral, n is the refractive index of the medium, and N_A is the Avogadro's number. The spectral overlap integral is given by $J = \int f_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda$; where f_D is the normalized emission spectrum of the donor, λ is the wavelength, and ϵ_A is the molar extinction coefficient of the acceptor (12–14). The experimental estimation of all the above parameter is tedious, so FRET efficiency is evaluated more directly using $E = 1 - (\Phi_{D-A}/\Phi_D)$ or $E = 1 - (\tau_{D-A}/\tau_D)$, where Φ_{D-A} and τ_{D-A} are, respectively, the fluorescence quantum efficiency and lifetime of the donor in the FRET pair system, and Φ_D and τ_D are, respectively, the fluorescence quantum efficiency and lifetime of pristine donor (6,12). We calculated the FRET efficiency using the average fluorescence lifetime of QDs. The average lifetime values are estimated as $\tau_{av} = (\tau_1\alpha_1 + \tau_2\alpha_2 + \tau_3\alpha_3)/(\alpha_1 + \alpha_2 + \alpha_3)$, where τ_1 , τ_2 , and τ_3 are individual lifetime values, and α_1 , α_2 , and α_3 are weighted amplitudes.

RESULTS AND DISCUSSION

Quenching of QD Fluorescence by BHQ Molecules

To confirm the changes in the fluorescence of QDs585 by the addition of BHQ-1 molecules, biotin-GPLGVRG [K(BHQ-1)]-CONH₂ (biotin-pep-BHQ-1) was added to streptavidin-QDs585 in microtubes, and then the fluorescence images were checked by using a digital camera. The fluorescence of streptavidin-QDs585 could not be observed under day light; however, it could be detected as

orange fluorescence under UV light (Fig. 2A-a,b). The fluorescence of streptavidin-QDs585 was attenuated by the addition of biotin-pep-BHQ-1 in a dose-dependent manner (Fig. 2A-c).

Fluorescence Spectral Changes of QDs by the Addition of BHQ Molecules

To investigate the changes in the fluorescence spectra of QDs585 by the addition of BHQ molecules, biotin-pep-BHQ-1 was added to streptavidin-QDs585 in the cuvette, and then the fluorescence spectra of QDs585-pep-BHQ-1 were checked by using the fluorescence spectrophotometer. There were little changes in the peak wavelength of fluorescence spectra of streptavidin-QDs585; however, the fluorescence intensity of streptavidin-QDs585 was decreased by the addition of biotin-pep-BHQ-1 in a dose-dependent manner (Fig. 2B). These data suggested that the fluorescence of streptavidin-QDs585 was quenched by biotin-pep-BHQ-1.

Fluorescence Intensity Rate of QDs585-pep-BHQ-1 to Streptavidin-QDs585

The fluorescence intensity rate of the QDs585-pep-BHQ-1 to streptavidin-QDs585 was calculated from the intensity of peak wavelength in Figure 2B. The fluorescence intensity rate was reduced to about 50% and 34% at a concentration of 63 nM and 117 nM of biotin-pep-BHQ-1, respectively (Fig. 3).

Relative Change in the Fluorescence Intensity rate (I_0/I) of Streptavidin-QDs585 (I_0) and QDs585-pep-BHQ-1 (I) to the Concentration of Biotin-pep-BHQ-1

The relative change in the fluorescence intensity rate (I_0/I) of streptavidin-QDs585 (I_0) and QDs585-pep-BHQ-1 (I) to the concentration of biotin-pep-BHQ-1 was shown as a Stern–Volmer plot. The nature of binding between streptavidin-QDs585 and biotin-pep-BHQ-1 was analyzed by the Stern–Volmer plot. The Stern–Volmer constant (K_{sv}) was determined by the following equation.

$$I_0/I = 1 + k_q \tau_0 [Q] = 1 + K_{sv} [Q] = \tau_0/\tau$$

where $[Q]$ is the concentration of acceptor (biotin-pep-BHQ-1), k_q is the bimolecular quenching constant, τ_0 and τ are the fluorescence lifetime in the absence (donor only) and presence of acceptor, respectively. The plot of the relative change showed a linear relationship between fluorescence intensity rate (I_0/I) and the concentration of acceptor (biotin-pep-BHQ-1). The Stern–Volmer constant (K_{sv}) was estimated to be $1.7 \times 10^7 \text{ M}^{-1}$ from the slope of the plot (Fig. 4A). Then, the bimolecular quenching constant (k_q) could be estimated to be $1.69 \times 10^{15} \text{ M}^{-1}\text{s}^{-1}$ from the value of K_{sv} and the lifetime of the donor (τ_0). Large value of k_q showed that the interaction between streptavidin-QDs585 and

Figure 2. The fluorescence images and fluorescence spectral changes of streptavidin-QDs585 and streptavidin-QDs585 conjugated with biotin-GPLGVRG[K(BHQ-1)]-CONH₂ (biotin-pep-BHQ-1). (A) The fluorescence images of streptavidin-QDs585 and QDs585-pep-BHQ-1. The fluorescence images of streptavidin-QDs585 (3 nM) under day light (a) or UV light (b), and QDs585-pep-BHQ-1 under UV light (c) are shown. (B) The fluorescence spectral changes of QDs585-BHQ-1. Biotin-pep-BHQ-1 was added to streptavidin-QDs585 at various concentrations (0–117 nM). The fluorescence intensity of QDs585 was decreased by the addition of biotin-pep-BHQ-1 in a dose-dependent manner (excitation wavelength: 400 nm).

biotin-pep-BHQ-1 was predominantly static, so these data suggested that streptavidin-QDs585 and biotin-pep-BHQ-1 were conjugated.

Fluorescence Lifetime of Streptavidin-QDs585 and QDs585-pep-BHQ-1

The fluorescence lifetime of streptavidin-QDs585 (τ_D) only was 10.03 ns, and then the fluorescence lifetime of QDs585-pep-BHQ-1 (τ_{D-A}) was decreased to 6.18 ns by the addition of 90 nM of biotin-pep-BHQ-1 (Fig. 4Ba, b). The efficiency of energy transfer [$E = 1 - (\tau_{D-A}/\tau_D)$] could be calculated by using the fluorescence lifetime and was found to be about 38% at 90 nM of biotin-pep-BHQ-1. The efficiency of energy transfer obtained from the fluorescence lifetime as well as steady-state fluorescence intensity was in good agreement with each other. These data suggested that FRET occurred with high efficiency between streptavidin-QDs585 and biotin-pep-BHQ-1.

CONCLUSIONS

We have achieved the fluorescence off state of QDs by the conjugation of black hole quencher (BHQ) molecules intermediated with peptide actually using streptavidin-QDs585 and biotin-pep-BHQ-1. These molecules could be conjugated in a simple process, and then the QD

fluorescence could be reduced with high quenching efficiency about 60% at 90 nM of biotin-pep-BHQ-1. It has been suggested that the quenching of QDs585 occurred as a result of FRET between streptavidin-QDs585 (donor) and biotin-pep-BHQ-1 (acceptor) by the analysis

Figure 3. The fluorescence quenching efficiency of QDs585-pep-BHQ-1 to streptavidin-QDs585. The graph shows the fluorescence quenching efficiency (%) of QDs585-pep-BHQ-1/streptavidin-QDs585 (3 nM) at various additional concentrations (0–117 nM) of biotin-pep-BHQ-1.

A

B

Figure 4. The relative change in the fluorescence intensity rate and fluorescence lifetime decay of QDs585-pep-BHQ-1. (A) The relative changes in the fluorescence intensity rate (I_0/I) of streptavidin-QDs585 (I_0 (3 nM) and biotin-pep-BHQ-1 (I) (0–117 nM) are shown as a Stern–Volmer plot. (B) The fluorescence lifetime decay of streptavidin-QDs585 (3 nM) in the absence (black trace) and presence of biotin-pep-BHQ-1 (90 nM) (red trace) are shown using femtosecond laser at 200 nanosecond (ns) time scale (a) and 50 ns time scale (b) (excitation wavelength: 400 nm).

of the fluorescence intensity and lifetime of streptavidin-QDs585 and QDs585-pep-BHQ-1. The sequence of intermediate peptide was GPLGVRGK, which can be cut by matrix metalloproteinases (MMPs) produced by cancer cells. Thus, QDs585-pep-BHQ-1 may be able to detect the MMP production from the recovery of QDs585 fluorescence with more improvement.

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