Recombinant protein (EGFP-Protein G)-coated PbS quantum dots for in vitro and in vivo dual fluorescence (visible and second-NIR) imaging of breast tumors

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Experimental

Materials

Reduced glutathione (GSH) was purchased from Wako Pure Chemical Industries. Lead acetate (Pb(CH$_3$COO)$_2$·3H$_2$O), and sodium sulfide nonahydrate (Na$_2$S·9H$_2$O) were purchased from Sigma-Aldrich, respectively. All chemical reagents were laboratory grades. Anti-HER2 antibody (Herceptin) was purchased from Chugai Pharmaceutical.

Protein expression

EGFP sequence was amplified by PCR from pEGFP-C1 (Clontech). The PCR fragments were fused with pGEX-6P-1 plasmid (GE Healthcare) by using InFusion HD cloning kit (Clontech). Next, the sequence of Protein G B1 (from pET His6 ProteinG TEV LIC cloning vector (2P-T), Addgene plasmid 29713) was inserted to pGEX-EGFP plasmid. The pGEX-EGFP-Protein G B1 plasmid was transformed into E.coli KRX competent cells (Promega). For large-scale cultures, the transformed cells were grown in
1 L of LB media with ampicillin (100 µg/mL) at 37°C on shaking table, until they approached to 0.5 of O.D. 600 (absorbance). To induce production of the targeted protein, isopropyl β-D-1-thiogalactopyranoside (0.1 mM) and L-Rhamnose (1 %) were added to the LB media, and then incubated with shaking gently for 12 hr at 18°C. The cell pellet of cultured cells was harvested by centrifugation at 4,000 xg for 10 min. The cells were lysed and suspended with 20 mL of binding buffer (50 mM Tris-HCl, pH=8.0). Before cell lysis, AEBSF (1 mM, Wako) as a protease inhibitor were added. The solution was sonicated on ice using 5 min at middle-intensity with a 1 s cooling period between each burst. The lysate was clarified by centrifugation at 10,000 xg for 20 min to eliminate cell debris. The next step was the purification by Glutathione Sepharose 4B (GE Healthcare). Two mL of Sepharose media equilibrated with binding buffer was added to each 20 mL of lysed sample, and incubated with gentle agitation at room temperature for 30 min. After the solution was transferred to an empty column, it was washed with binding buffer five column volumes. Lastly the GST-EGFP-Protein G B1 was drained from the column by the addition of elution buffer (10 mM glutathione in 50 mM Tris-HCL, pH=8.0). The eluted fractions were further purified by gel filtration column (PD-10 columns, GE Healthcare).

**Synthesis of protein-coated PbS QDs**

An aqueous solution (0.25 mL) of lead acetate (10 mM) was added to an aqueous solution (0.3 mL) of GST-EGFP-Protein G B1 protein (1 mg/mL in 250 mM Tris-HCl, pH 8.0). An aqueous solution (0.025 mL) of Na₂S (100 mM) was quickly added. Immediately after the addition of Na₂S, the solution color changed to clear to brown to indicate the formation of protein-coated PbS QDs. The resulting brown solution of the PbS QDs is centrifuged to remove the aggregation of QDs. Unreacted protein and reagents were removed by dialysis (cut off 100 kDa, Spectrum). Nanoparticle-antibody complexes were prepared by mixing of Herceptin (0.1 mg/ml) and QDs (2 µM) at a 1:1 volume ratio.

**Characterization of QDs**

Fluorescence and absorbance spectra of QDs were measured on a SPEX NanoLog (HORIBA) by using 450 nm excitation light and V-670 (JASCO), respectively. Morphology of the QDs was observed using an analytical high-resolution transmission electron microscope H800 (HITACHI) at 200 kV
accelerating voltage. Fluorescence quantum yield of PbS QDs was estimated relatively using standard CdSeTe/CdS quantum dots, which had an emission peak of 840 nm. The quantum yield of this CdSeTe/CdS quantum dots was determined to be 0.5 by an absolute quantum yield measurement systems (C10027; Hamamatsu photonics). By comparing the fluorescence intensities of the standard CdSeTe/CdS quantum dots and PbS QDs with OD = 0.1 at 488 nm, the quantum yields (QYs) of the PbS QDs (1150 nm emission peak) were calculated to be 0.1 in water.

**Fluorescence correlation spectroscopy (FCS)**

FCS analyses of the QDs were carried out on the Compact FCS system (Hamamatsu Photonics KK) using a 473 nm laser as a light source. The measurements were carried out at room temperature in 8-well Lab-Tek chambered coverglass (Thermo Scientific). Sample solution (20 µl) was put on a coverglass as a droplet and measure at 200 µm glass above.

The data analysis was performed Hamamatsu Photonics Control FCS software. The fluorescence autocorrelation function $G(\tau)$ was calculated by:

$$G(\tau) = \frac{\langle I(t) \cdot I(t + \tau) \rangle}{\langle I(t) \rangle^2}$$

(1)

where $\tau$ denotes the time delay, $I$ is the fluorescence intensity, $G(\tau)$ denotes the autocorrelation function. Acquired autocorrelation curves were fitted by a one-component model as:

$$G(\tau) = 1 + \frac{1 - f + f \exp(-\tau/\tau_t)}{N(1-f)} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{s^2 \tau_D}\right)^{-1/2}$$

(2)

where $N$ is the average number of fluorescent particles in the detection volume defined by radial radius $w$ and axial radius $z$, and $s$ is the structural parameter representing the ratio $s = z/w$. The diffusion time ($\tau_D$) corresponds to the average time for diffusion of fluorescent particles across the detection area, which reflects the size of particles. $f$ is the average fraction of triplet state molecules and $\tau_t$ is triplet relaxation time. Diffusion coefficient was calculated as follows:

$$\tau_D = \frac{w^2}{4D}$$

(3)

The value of $w$ was evaluated by reference measurement by using rhodamine 6G (D = 414 µm²/s).
Hydrodynamic radius of QDs were estimated using:

\[ D = \frac{kT}{6\pi \eta r} \]  

(4)

where \( T \) denotes the absolute temperature, \( k \) is the Boltzman constant, \( \eta \) is the viscosity of the solvent and \( r \) is the hydrodynamic radius of fluorescence particles.

**Cell viability**

HeLa cells were incubated with GST-EGFP-GB1 protein coated QDs (2.5–250 nM, PBS) for 30 min. Then, the cells were washed with PBS and resuspended in a culture media (DMEM). A 10 µL of the cell suspension was mixed with a Trypan blue solution (10 µL), and the cell viability was determined using a cell counter (Countess; Invitrogen).

**Cell line and in vitro imaging**

Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, and 10 µg/mL streptomycin were used for culturing KPL-4 cells. KPL-4 cells were treated by Herceptin (0.1 mg/mL) and QDs (100 nM) sequentially, and the dishes were incubated for 30 min. Unbinding antibodies and probes were washed out with PBS buffer three times. Fluorescence confocal laser-scanning microscopy was carried out by FV1000 (Olympus). The probe was excited by a 473 nm Ar-ion laser and fluorescence was collected through the filter set for FITC and GFP. Cell nucleus was stained by treatment of Hoechst 33342 (1 µM) for 30 min. Hoechst 33342 was excited by 405 nm laser line and the fluorescence was collected through the filter set for DAPI. Image acquisitions of GFP channel and DAPI channel were carried out sequentially.

**Second-NIR imaging**

Fluorescence images of the cells were taken using a home-built second-NIR fluorescence microscope. The imaging system was based on the Macro Zoom System at magnification of 0.63x (MVX; Olympus) equipped with an InGaAs CMOS camera (C10633-34; Hamamatsu photonics KK). A customized filter set consisting of an excitation filter for the 670 nm laser, dichroic mirror to reflect the 670 nm laser, and a band-pass emission filter (1300 ±25 nm) was used for imaging. 670 nm laser diodes
(BWF1 series; B&W TEK) were used as the excitation light for imaging. Maximum excitation powers on the sample stage were 25.5 mW/cm$^2$. Device control and data acquisition were performed by HCImage (Hamamatsu photonics) software and a customized LabVIEW program (National Instruments). Fluorescence signal was collected for 1 min, and background noise was subtracted from images by using ImageJ software (NIH).

**In vivo imaging**

A suspension of KPL-4 cells (0.5x10$^7$ cells per mouse) was transplanted to the dorsal skins of 5-week old female BALB/c nu/nu mice (Charles River Japan). After several weeks, we selected a mouse bearing a tumor less than 10 mm in diameter for imaging. Two hundred µL of the mixture of anti-HER2 antibody and QDs (1 µM) were injected into a xenografted mouse via a tail vein. Second-NIR fluorescence images (ex: 670 nm, em: 1300 nm) of anesthetized mouse were taken 48 hrs after the injection using the second-NIR fluorescence microscope. All experiments were performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Osaka University Animal Care and Use Committee.
**Supporting Figures**

*Figure S1.* Fluorescence spectra of GST-EGFP-GB1-coated PbS QDs (Black line), His-tagged EGFP-coated PbS QDs (Red line) and BSA-coated PbS QDs (Blue line). Fluorescence spectrum differs depending on the template protein.
Figure S2. (a) SDS-PAGE of GST-EGFP-GB1 protein (61.7 kDa). (b) Absorbance spectra of GST-EGFP-GB1 protein (Blue line), protein-QDs without purification (Black line) and protein-QDs after purification by dialysis (Red line). The intensity of absorption peak of EGFP (488 nm) is reduced by the purification. The result indicates that free GST-EGFP-GB1 proteins are removed from the QD solution by dialysis.
Figure S3. (a) Fluorescence laser-scanning confocal microscopy (LSM) of KPL-4 cells treated with anti-HER2 antibody (30 min) followed by GST-EGFP-GB1-coated QDs. (b) DIC image of (a). Fluorescence LSM image (c) and DIC image (d) of KPL-4 cells treated only GST-EGFP-GB1-coated QDs without antibody.
Figure S4. Viability of HeLa cell for GST-EGFP-GB1-coated QDs.
Figure S5. Second-NIR imaging of a breast cancer (KPL-4) tumor without QD injection.

Figure S6. Second-NIR imaging of a breast cancer tumor at 48 hours after the injection of anti-HER2 antibody + GST-EGFP-GB1-coated PbS QDs, where the skin covering the tumor is removed.