Fluorescent, Recombinant-Protein-Conjugated, Near-Infrared-Emitting Quantum Dots for in Vitro and in Vivo Dual-Color Molecular Imaging

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Near-infrared (NIR)-emitting fluorescent probes are widely used for molecular imaging at the whole-body level. However, NIR-emitting fluorescent probes emitting over $\lambda = 700$ nm are not suitable for molecular imaging at the cellular level, because most of the conventional fluorescence microscopes have very low optical sensitivity in the NIR region. Thus, to achieve fluorescence imaging at the cellular and whole-body levels by using single probes, visible and NIR-emitting dual-color fluorescent probes are desirable. For dual-color fluorescence molecular imaging, we synthesized fluorescent, recombinant-protein-conjugated, NIR-emitting quantum dots (QDs), in which the recombinant protein consists of enhanced green fluorescent protein (EGFP) and the immunoglobulin binding domain (B1) of protein G. This dual-color fluorescent QD probe binds the Fc region of immunoglobulin G (IgG) through its B1 domain at the QD surface and acts as a molecular-imaging probe at both the cellular and whole-body levels. In this paper, we present the synthesis of fluorescent, recombinant protein (HisEGFP-GB1)-conjugated, NIR-emitting QDs and their application to the dual-color molecular imaging of breast cancer cells in vitro and in vivo.

Introduction

Fluorescence molecular imaging is an indispensable tool in the fields of biology and life sciences.[1] In conventional fluorescence microscopes, the sensitivity of optical detection is limited to the visible region ($\lambda = 400–700$ nm).[2] Thus, visible-emitting, fluorescent probes are usually used for fluorescence molecular imaging in vitro (at the cellular level).[18] However, visible-emitting probes are not suitable for whole-body fluorescence imaging because of the strong absorption and scattering of visible light by blood and tissues.[19] Compared to visible light, near-infrared (NIR) light over $\lambda = 700$ nm is highly permeable in living tissues.[20] Thus, NIR-emitting fluorescent probes are used for deep-tissue imaging at the whole-body level.[20] To achieve in vitro and in vivo molecular imaging by using single fluorescent probes, probes that have dual-color emissions from the visible region to the NIR region are desirable.

Recently, several reports have appeared on visible- and NIR-emitting dual-color probes[5] for fluorescence imaging. Kwon et al. developed dual-color-emitting ($\lambda = 450–850$ nm) upconversion nanocapsules for different cancer bioimaging in vitro[5d] and in vivo.[5e] They modified nanocapsule probes with the anti-MUC1 antibody or TCP1 peptide for molecular imaging of cancer cells. Zhang et al. developed InP/ZnSe/ZnS quantum dot (QD)-based probes with strong visible and NIR emission ($\lambda = 550–1000$ nm) for in vitro and in vivo tumor imaging, for which the QD probe was functionalized with the cRGD peptide.[5f] More recently, Ma et al. reported a fluorescent unmolecular micelle probe for in vitro and in vivo cancer imaging: the micelle probe included different types of organic fluorescent dyes having dual emission in the visible and NIR regions ($\lambda = 500–750$ nm).[5j] In previous work, we developed fluorescent recombinant protein capped PbS QDs that showed dual emission at $\lambda = 515$ and 1100 nm.[5k] We used a recombinant protein (GST-EGFP-GB1; GST = glutathione s-transferase, EGFP = enhanced green fluorescent protein, GB1 = immunoglobulin binding domain of protein G) as a protecting reagent to prepare PbS QDs with dual-color emission resulting from EGFP and QDs. Although GST-EGFP-GB1-capped PbS QDs are useful for dual-color molecular imaging, this probe cannot be applied in the conventional NIR-wavelength region ($\lambda = 700–900$ nm).

The objective of this work was to develop a dual-color (visible- and NIR)-emitting QD probe that could be used for conventional fluorescence microscopes and in vivo imaging instruments (spectral response: $\lambda = 400–900$ nm). Fluorescence molecular imaging of specific cancer cells is crucial for tumor detection and diagnosis. For cancer-cell-targeted imaging, antibodies, peptides, carbohydrates, and ligands towards surface receptors have been used for the functionalization of QDs.[6] Among such QD probes, monoclonal-antibody-functionalized probes are highly specific towards membrane receptors such as epidermal growth factor receptors (EGFRs) on cancer cells.[7]
To date, for the preparation of antibody-functionalized QDs, cross-coupling reactions have typically been used to conjugate the antibody to the QDs, and the antibody molecules bind to the functional groups, such as the carboxyl and amino groups, at the surface of the QDs. However, this conjugation method results in nonspecific binding of the antibody to the QDs, which leads to aggregation of the QDs and lowers the activity of the antibody.

To overcome this disadvantage, adaptor proteins such as streptavidin and protein A/G have been used for the functionalization of QDs with antibodies. Recently, we reported that a small adaptor protein, immunoglobulin binding domain (B1), could be used to mediate antibody conjugation for the preparation of antibody-functionalized QDs. We found that recombinant protein (HisGB1)- conjugated QDs could bind monoclonal anti-HER2 (human epidermal growth factor 2) S2 antibody through the B1 domain present at the QD surface. In this work, we applied this conjugation strategy for the preparation of a visible and NIR dual-color-emitting fluorescence probe with antibody-binding ability.

In this study, we used the antibody-binding ability of the HisEGFP-GB1 protein (Scheme 1). As the recombinant protein (HisEGFP-GB1) has a His tag at its N terminus, this protein directly binds to the surface of a CdS layer of CdSeTe/CdS (core–shell) QDs. Given that histidine has high binding affinity for Zn$^{2+}$ ions as well as Cd$^{2+}$ ions, histidine-tagged proteins also can bind to a ZnS layer of CdSeTe/CdS (core–shell) QDs. HisEGFP-GB1-conjugated QDs were easily prepared only by mixing GSH-QDs and HisEGFP-GB1 protein in an aqueous solution. GB1-EGFP-QDs can bind the IgG antibody through the B1 domain of the recombinant protein present at the QD surface. In this study, we used the anti-HER2 antibody for molecular imaging of HER2 overexpressing breast tumor cells in vitro and in vivo.

**Results and Discussion**

**Design and synthesis**

To achieve in vitro and in vivo fluorescence molecular imaging by using single probes, visible- and NIR-emitting dual-color probes are desirable. We synthesized fluorescent recombinant protein (HisEGFP-GB1)-conjugated, NIR-emitting quantum dots (QDs) that have the ability to bind immunoglobulin G (IgG) on the QD surface. We show that a histidine-tagged protein (HisEGFP-GB1) easily binds to the surface of the NIR-emitting CdSeTe/CdS QDs to form HisEGFP-GB1-conjugated QDs. These recombinant-protein-conjugated QDs allow visible and NIR dual-color molecular imaging to be achieved at the same time. We demonstrate the capability of the dual-color-emitting QD probe for fluorescence molecular imaging of breast tumor cells in vitro and in vivo.

**Figure 1.** Agarose gel electrophoresis of 1) GSH-QDs, 2) GSH-QDs + HisEGFP-GB1, and 3) a mixture of GSH-QDs and EGFP-GB1, for which c(GSH-QDs)/c(GB1-EGFP-QDs) = 1:30. The left image shows the NIR fluorescence of the QDs at $\lambda = 830$ nm. The right image shows EGFP fluorescence at $\lambda = 515$ nm. The arrows show the position of the HisEGFP-GB1-conjugated QDs (GB1-EGFP-QDs).

**Fluorescence spectra, size, and stability of the QD probes**

The fluorescence spectrum of GB1-EGFP-QDs shows dual-color (visible and NIR) emission resulting from EGFP and the QDs (Figure 2A). To confirm FRET from EGFP to the QDs, we examined the NIR fluorescence intensity of GSH-QDs in the absence of HisEGFP-GB1 (37.4 kDa) and EGFP-GB1 (35.3 kDa) with no histidine tags (Figures S1 and S2 in the Supporting Information). Gel electrophoresis shows that the mobility of GSH-QDs is significantly lowered by the addition of HisEGFP-GB1, whereas the mobility of GSH-QDs is not changed by the addition of EGFP-GB1. This result shows that HisEGFP-GB1 can bind to GSH-QDs through its histidine tags, whereas EGFP-GB1 cannot bind to the QDs because of the absence of histidine tags. Notably, EGFP emission (at $\lambda = 515$ nm) in the mixture of GSH-QDs and HisEGFP-GB1 is very weak relative to that in the mixture of GSH-QDs and EGFP-GB1. This finding indicates the FRET from EGFP to the QDs in the HisEGFP-GB1-conjugated QDs (GB1-EGFP-QDs).
and presence of HisEGFP-GB1. The NIR fluorescence of GSH-QDs increased by a factor of 30% in the presence of HisEGFP-GB1 (Figure 2B). In addition, we observed that the fluorescence lifetime (\(\tau\)) for EGFP fluorescence in HisEGFP-GB1 is significantly decreased by the addition of GSH-QDs (Figure 2C). The increase in the NIR emission of the QDs and the decrease in the lifetime of EGFP emission in GB1-EGFP-QDs show FRET from EGFP (donor) to the QDs (acceptor) in GB1-EGFP-QDs.[17]

For comparison, we examined the fluorescence spectra of GSH-QDs in the absence and presence of EGFP-GB1 and its EGFP fluorescence decay in the absence and presence of GSH-QDs. Compared to HisEGFP-GB1, the addition of EGFP-GB1 to GSH-QDs results in almost no difference in the NIR fluorescence intensity of GSH-QDs (Figure S3A). The fluorescence decay curve of EGFP in GB1-EGFP-G1 does not change upon the addition of GSH-QDs (Figure S3B). These results indicate that EGFP-GB1 protein does not bind to GSH-QDs and that FRET cannot occur in the mixture of GSH-QDs and EGFP-GB1.

The FRET efficiency from EGFP to the QDs can be calculated from both the fluorescence lifetimes and the fluorescence intensity of a donor (EGFP) [Eqs. (1) and (2)]:

\[
E = 1 - \frac{\tau_{\text{QDs}}}{\tau_{\text{DA}}} \quad (1)
\]

\[
E = 1 - \frac{F_{\text{QDs}}}{F_{\text{DA}}} \quad (2)
\]

in which \(\tau_{\text{D}}\) and \(\tau_{\text{DA}}\) are the lifetimes of the donor in the absence and presence of the acceptor and \(F_{\text{D}}\) and \(F_{\text{DA}}\) are the relative fluorescence intensities of the donor in the absence and presence of the acceptor.[16] From the fluorescence lifetimes (Figure 2C) and relative fluorescence intensity (Figure 2D) of EGFP emission, FRET efficiency was calculated to be 75 and 90%, respectively. This high FRET efficiency may come from direct binding of HisEGFP-GB1 protein to the surface of the QDs by its histidine tags.

For application of the fluorescent probes for in vivo imaging, their hydrodynamic size and stability are important issues. This is because the hydrodynamic size of the probes affects the efficiency of their clearance from the body. It was previously shown that a hydrodynamic size larger than 15 nm prevented renal clearance.[18] Transmission electron microscopy showed that GB1-EGFP-QDs are monodispersed particles, and the core size of the QDs is \((4.1 \pm 0.8)\) nm (Figure S4). From dynamic light scattering measurements, the hydrodynamic sizes of GB1-EGFP-QDs and GSH-QDs were determined to be approximately 9 and 5 nm, respectively (Figure S5A); this is indicative of renal clearance of the QDs from the body. The values of the zeta potential for GB1-EGFP-QDs and GSH-QDs are also shown in Figure S5B.

The stability of these probes in aqueous solutions was checked at different pH values. Figure 3 shows the time course of fluorescence intensity of GB1-EGFP-QDs at pH 4, 6.8, 7.4, and 9. Under basic and acidic conditions (pH 4, 6.8, and 9), the fluorescence intensity of GB1-EGFP-QDs decreases with time, whereas their fluorescence intensity under physiological pH conditions (phosphate-buffered saline, PBS, pH 7.4) is stable within 3 h. At pH 4, the fluorescence intensity of GB1-EGFP-QDs immediately diminishes by aggregation of the QDs. We also checked the fluorescence intensity of GB1-EGFP-QDs in a cell-culture medium (Figure S6). Their fluorescence intensity is very stable in the culture medium over 3 h, showing the capability of GB1-EGFP-QDs for cellular imaging.

To confirm the binding ability of HisEGFP-GB1 protein for antibody, we performed agarose gel electrophoresis. The mobility...
of HisEGFP-GB1 significantly decreases by the addition of anti-HER2 antibody (Ab), whereas the mobility of HisEGFP with no B1 domain does not change upon the addition of Ab (Figure 4A). This result shows the binding ability of HisEGFP-G81 toward the antibody.

Next, we examined the antibody-binding ability of HisEGFP-GB1-conjugated QDs (GB1-EGFP-QDs) by agarose gel electrophoresis for the mixture of GSH-QDs and HisEGFP-GB1 (1:30) and the mixture of GSH-QDs and HisEGFP (1:30) in the absence and presence of Ab (Figure 4B). This shows the binding of these proteins to the surface of GSH-QDs through the histidine tags to form HisEGFP-GB1-conjugated QDs (GB1-EGFP-QDs) and HisEGFP-conjugated QDs (EGFP-QDs). The successive addition of Ab to GB1-EGFP-QDs or EGFP-QDs gives different results. The mobility of GB1-EGFP-QDs is lowered by the addition of antibody, whereas the mobility of EGFP-QDs does not change after the addition of Ab (lanes 3 and 6 in Figure 4B). This finding shows the antibody-binding ability for GB1-EGFP-QDs, but not for EGFP-QDs.

To evaluate the binding affinity of HisEGFP-GB1 for Ab, we examined the effect of Ab on the diffusion time of HisEGFP-GB1 protein by using fluorescence correlation spectroscopy (FCS).

FCS can measure fluctuations in the fluorescence intensity of fluorophores at the single-molecule level and gives the diffusion time of the fluorophores in solution. As shown in Figure 5, the binding of antibody to HisEGFP-GB1 is confirmed by changes in the FCS curve of HisEGFP-GB1 after the addition of Ab. Upon increasing the concentration of Ab, the FCS curve shifts to the right side; this is indicative of an increase in the diffusion time of HisEGFP-GB1 by complexing with Ab. The inset shows a relationship between the diffusion time of HisEGFP-GB1 and the concentration of Ab.

The number of HisEGFP-GB1 molecules that can bind to one particle of GB1-EGFP-QDs was determined by using size-exclusion liquid chromatography (Figure 6A). From a relationship between the retention times of standard proteins and their molecular weights of HisEGFP-GB1 and IgG complex (33 nm). The number of HisEGFP-GB1 molecules that can bind to one particle of GB1-EGFP-QDs was determined by using size-exclusion liquid chromatography (Figure 6A). From a relationship between the retention times of standard proteins and their molecular weights...
(inset in Figure 6A), the apparent molecular weights of GSH-QDs and GB1-EGFP-QDs were determined to be 250 and 750 kDa, respectively. As the molecular weight of HisEGFP-G1 is 37.4 kDa, the number of HisEGFP-G1 molecules bound to one particle of GB1-EGFP-QD was estimated to be 13. This finding is comparable to the result of agarose gel electrophoresis for the addition of HisEGFP-G1 to GSH-QDs (Figure 6B).

Cellular imaging

To check the probe activity of anti-HER2 antibody + GB1-EGFP-QDs, we performed fluorescence imaging of human breast tumor (KPL-4) cells,[21] for which the HER2 receptors are overexpressed on the cell membrane. HeLa cells were used as a negative control, as the expression level of HER2 is very low relative to that of KPL-4 cells (Figure S7).[22]

For cellular imaging, we first added anti-HER2 antibody to KPL-4 cells. The cells were washed with PBS, and then GB1-EGFP-QDs were added. After incubation of the cells with GB1-EGFP-QDs for 10 min, we performed cellular imaging. Figure 7A shows the fluorescence images for HeLa and KPL-4 cells after staining with antibody (human normal IgG or anti-HER2 antibody), followed with GB1-EGFP-QDs. Human normal IgG was used as a negative antibody. In the case of KPL-4 cells, their plasma membranes were strongly stained by anti-HER2 antibody and GB1-EGFP-QDs, showing the intense fluorescence emission of EGFP and the QDs from the membrane. In contrast, HeLa cells were not stained by anti-HER2 antibody + GB1-EGFP-QDs, and they showed very weak emission from EGFP and QDs. This result shows that anti-HER2 antibody + GB1-EGFP-QDs specifically bind to the plasma membrane of HER2-overexpressing KPL-4 cells. In contrast, the incubation of HeLa and KPL-4 cells with human normal IgG and GB1-EGFP-QDs did not show significant EGFP and QD fluorescence emission from the plasma membranes. Flow cytometric analysis (Figure 7B) also confirmed the specific binding of the anti-HER2 antibody and GB1-EGFP-QDs to KPL-4 cells.

The effect of HisEGFP-G1 and GB1-EGFP-QDs on cell viability was checked by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure S8). Although HisEGFP-G1 did not show cytotoxicity, GB1-EGFP-QDs showed dose-dependent cytotoxicity in HeLa cells.

Whole-body imaging

Finally, we achieved in vivo fluorescence imaging of breast-tumor-bearing mice by using anti-HER2 antibody and GB1-EGFP-QDs. Breast-tumor (≈10 mm in diameter) bearing mice were prepared by transplantation of a suspension of KPL-4 cells (≈10⁶ cells) to the dorsal skins of nude mice. First, we injected GB1-EGFP-QDs to breast-tumor-bearing mice to examine whether the QDs could accumulate in the tumor tissue by the enhanced permeability and retention (EPR) effect.[23] Although intense NIR fluorescence emission of the QDs was observed from a liver, significant NIR emission was not observed from a breast tumor (Figure S9). Most of the QDs were excreted from the body 1 day after postinjection of the QDs (Figure S9).

Figure 7. A) Fluorescence images of HeLa and KPL-4 cells that were incubated by antibody, followed by GB1-EGFP-QDs. Left) Fluorescence images of EGFP (green) and Hoechst 33342 (blue). Right) Fluorescence images of QDs (purple) and Hoechst 33342 (blue). B) Flow cytometric analysis of HeLa and KPL-4 cells treated with normal human IgG + GB1-EGFP-QDs (green) or anti-HER2 antibody + GB1-EGFP-QDs (red).
Next, we injected the mixture of anti-HER2 antibody and GB1-EGFP-QDs to breast-tumor-bearing mice to check the specific accumulation of QDs in the breast tumor. At 22 h postinjection of the antibody–QD conjugates, we observed intense NIR emission from the breast tumor as well as liver (Figure 8A). After 48 h, we observed that most of the QDs were accumulated in the tumor. To confirm the specific accumulation of QDs in the breast tumor, we also performed ex vivo fluorescence imaging for isolated tumor and major organs (Figure 8B). We observed intense NIR fluorescence emission of the QDs from the isolated tumor (top image in Figure 8B). Visible (EGFP) fluorescence was also observed from the tumor (bottom image in Figure 8B). Notably, the EGFP fluorescence emissions from a liver and spleen were much weaker than QD emission (Figure 8B). This may have resulted from protease-mediated degradation of EGFP protein in liver and spleen tissue.\textsuperscript{24} By using anti-HER2 antibody and GB1-EGFP-QDs, we could achieve visible and NIR dual-color fluorescence imaging to confirm the specific tumor accumulation of the QD probe.

**Conclusion**

In summary, we presented a fluorescent, recombinant protein (HisEGFP-GB1)-conjugated, NIR-emitting QD probe for in vitro and in vivo molecular imaging. The present probe (GB1-EGFP-QDs) showed dual-color (visible and NIR) fluorescence emission resulting from EGFP and the QDs. This dual-color probe has the ability to bind antibody through the immunoglobulin binding (B1) domain present at the QD surface. Thus, the probe could be used for fluorescence molecular imaging at the cellular and whole-body levels. As the preparation of antibody (IgG) conjugates with GB1-EGFP-QDs is facile and rapid, this dual-color probe should be very useful for a variety of fluorescent molecular imaging in vitro and in vivo.

**Experimental Section**

**Materials:** Selenium (Se, powder, 99.999\% ) and tellurium (Te, shot, 1–2 mm, 99.99\% ) were purchased from Sigma–Aldrich. Cadmium 2,4-pentanedionate was purchased from Alfa Aesar. n-Octadecylphosphonic acid (ODPA) was purchased from PCI. Trioctylphosphine oxide (TOPO), tributylphosphine (TBP), and hexadecylamine (HDA, 90\% ) were purchased from Tokyo Chemical Industry (Japan). Sulfur (S, crystalline, 99.9999\% ), glutathione (GSH, reduced form), and potassium tert-butoxide were purchased from Wako Chemicals (Japan). Anti-HER2 monoclonal antibody (Ab) was purchased from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Other organic solvents were of analytical-reagent grades.

**Synthesis of GSH-QDs:** The synthesis of GSH-QDs is reported elsewhere.\textsuperscript{14} A typical procedure is as follows. A Se-Tes stock solution was prepared by dissolving Se (24 mg, 0.3 mmol) and Te (13 mg, 0.1 mmol) in TBP (1 mL) at room temperature. A Cd-S stock solution was prepared by adding sulfur (40 mg, 1.25 mmol) to TBP (10 mL). After sulfur was completely dissolved, a sulfur solution was cooled to room temperature. Then, cadmium 2,4-pentanedionate (388 mg, 1.25 mmol) was added to the sulfur solution in TBP, and the solution was warmed at 100\degree C to dissolve cadmium 2,4-pentanedionate. The Cd-S stock solution was stored under argon at room temperature.

A mixture of cadmium 2,4-pentanedionate (150 mg, 0.48 mmol), QDPA (300 mg, 0.90 mmol), TOPO (1 g), HDA (3 g), and TOP (0.5 mL) was loaded into a 25 mL, three-necked flask and heated to 330\degree C under argon. At this temperature, a Se-Tes stock solution (0.5 mL) was quickly injected by using a syringe, and this caused an immediate color change in the solution from colorless to brown. By monitoring the QD fluorescence spectra, the formation of QDs (λ = 800 nm emission) was checked. When the desired QDs were formed, the solution was cooled to 60\degree C and chloroform (10 mL) was added. The QDs were precipitated by the addition of methanol, and the QDs precipitate was separated by centrifugation. The resulting QDs precipitate and HDA (3 g) were loaded into a 25 mL, three-necked flask and heated to 250\degree C. At this temperature, the formation of the CdS shell was performed. The addition

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Figure 8. A) Bright-field and NIR fluorescence images of a breast-tumor-bearing mouse. The images were taken at 0, 22, and 48 h after injection of the mixture of anti-HER2 antibody and GB1-EGFP-QDs to a tail vein of the mouse. NIR fluorescence was detected at λ = (830 ± 20) nm. B) Ex vivo fluorescence images of an isolated tumor and main organs of the mouse. QD emission was detected at λ = (830 ± 20) nm, and EGFP emission was detected at λ = (515 ± 20) nm. Exposure time for detecting QD and EGFP emission was set to 30 s.
of a CdS stock solution (0.25 mL) resulted in the formation of CdSeTe/CdS QDs that emit at λ = 850 nm. Then, the QD solution was cooled to 80 °C and chloroform (10 mL) was added. The QDs were precipitated by the addition of methanol and were separated by centrifugation (10000 g, 10 min). To remove excess amounts of TOPO and HDA, the QDs were dissolved in chloroform again and precipitated by the addition of methanol. This procedure was repeated three times. The resulting QDs were dissolved in chloroform (20 mL) and stored in the dark.

For the GSH coating, an aqueous solution (1 mL) of GSH (100 mg mL⁻¹) was slowly added to a tetrahydrofuran solution (1 mM, 2 mL) of CdSeTe/CdS QDs at room temperature under sonication. The QDs precipitate was separated by centrifugation. An aqueous solution of potassium tert-butoxide (20 mg mL⁻¹, 2 mL) was added to the QDs precipitate under stirring. The solution was sonicated for 5 min and filtered through a 0.45 mm membrane filter. The excess amounts of GSH and potassium tert-butoxide were removed by dialysis by using a 10 kDa Na₂CO₃ aqueous solution. The resulting GSH-QDs were preserved at 4 °C.

**Protein synthesis:** For the HisEGFP-G81 and HisEGFP proteins, the EGFP sequence was amplified by PCR from pEGFP-C1 plasmid (Clontech). The protein G B1 sequence was amplified by PCR from pET HisG protein G TEV LIC cloning vector (2P-T), Addgene plasmid 29713. The PCR fragments were fused with pRelease plasmid (ThemoFisher) by using the InFusion HD cloning kit (Clontech). Next, the pRelease-EGFP-G81 plasmid was transformed into E. coli KRX competent cells (Promega). For large-scale cultures, the transformed cells were grown in lysogeny broth (LB; 200 mL) with ampicillin (100 μg mL⁻¹) at 37 °C on a shaking table, until the OD₆₀₀ approached 0.6. To induce production of the targeted protein, isopropyl-β-D-thiogalactopyranoside (0.2 mM) and L-rhamnose (0.1%) were added to the LB medium, and the cells were incubated with shaking gently for 16 h at 18 °C. The cells were collected by centrifugation at 5000 g for 10 min. The pellet was resuspended in binding buffer (5 mL, 50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0). Before cell lysis, complete EDTA-free protease inhibitor cocktail tablets (1 x, Roche) were added as a protease inhibitor. The solution was sonicated on ice by using 10 s bursts at medium intensity with a 10 s cooling period between each burst. The lysate was clarified by centrifugation at 20 000 g for 30 min to eliminate cell debris. The next step was purification by Ni Sepharose 6 Fast Flow (GE Healthcare). Sepharose medium equilibrated with binding buffer (3 mL) was added to each 5 mL of lysed sample, and the samples were incubated with gentle agitation at room temperature for 5 min. After the solution was transferred to an empty column, the column was washed with five column volumes of binding buffer. Lastly, the HisEGFP-G81 and HisEGFP proteins were drained from the column by the addition of elution buffer (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0). The eluted fractions were further purified by gel filtration column (PD-10 columns, GE Healthcare).

For the EGF-G81 protein, the first GST-EGF-G81 protein was expressed and lysed following the protocol previously reported by Sasaki et al. The lysate was clarified by centrifugation at 20 000 g for 30 min to eliminate cell debris. The next step was purification by Glutathione Sepharose 4B (GE Healthcare). Sepharose medium equilibrated with PBS (2 mL) was added to each 5 mL of lysed sample, and the samples were incubated with gentle agitation at 4 °C for 60 min. The Sepharose medium was then washed twice with Turbo3C Protease buffer, followed by digestion of the GST-tag by overnight incubation at 4 °C with 80 U of Turbo3C Protease (Accelagen) in Turbo3C Protease buffer. The EGF-G81 protein was further purified by gel filtration column (PD-10 columns, GE Healthcare).

**Preparation of HisEGFP-G81-conjugated QDs (G81-EGFP-QDs):** HisEGFP-G81 (1 mg mL⁻¹, PBS, 200 μL) was added to an aqueous solution of GSH-QDs (1 μM, 10 mM Na₂CO₃ solution, 0.4 mL). Then, the excess amount of HisEGFP-G81 protein was removed by using dialysis or ultrafiltration with a 100 kDa membrane filter.

**Fluorescence measurements:** The fluorescence spectra of the QDs were measured with a photonic-multiplex channel analyzer (C10027, Hamamatsu Photonics, Japan) with a 150 W xenon lamp (excitation light source at λ = 488 nm). Fluorescence autocorrelation curves of the QDs were measured by using a compact FCS system (C9413-01MOD, Hamamatsu Photonics, Japan). For determination of the concentration of GSH-QDs, we measured the number of QD particles in 20 μL of the QD solution by using FCS and estimated its concentration by using a solution of rhodamine 6G (20 nm) as a reference. For all measurements, the pH of the aqueous solution of the QDs was set to 7.4 with PBS buffer. Fluorescence decay curves for EGFP were measured by excitation at λ = 480 nm by using time-correlated single-photon counting (Horiba Fluoro Cube).

**Size-exclusion HPLC:** Size-exclusion column chromatography with a HPLC system (ELITE LaChrom, HITACHI) was performed by using a TSK-gel G4000SW column (7.8 mm x 30 cm, TOSOH). The mobile phase was 10 mM PBS (pH 7.2–7.4), and the flow rate was adjusted to 1 mL min⁻¹. Standard proteins of thyroglobulin (670 kDa), ferritin (450 kDa), bovine serum albumin (850, 66 kDa), and HisEGFP (30.8 kDa) were measured to draw a calibration curve. The HPLC chromatographs of GSH-QDs and HisEGFP-G81-conjugated QDs were obtained by monitoring absorption at λ = 488 nm.

**Agarose gel electrophoresis:** These proteins were run on 1% agarose gel in Tris-acetate buffer (pH 8.0), 100 V for 15 min. Fluorescence emissions of the QDs and EGFP were monitored at λ = 830 and 515 nm, respectively.

**Cellular imaging:** KPL-4 and HeLa cells were seeded to collagen-coated glass-bottomed dishes (D11134H, Matsunami 35 mm) and were incubated in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) for overnight at 37 °C. Anti-HER2 antibody or normal human IgG (0.1 μM) and Hoechst 33342 (5 μg mL⁻¹) were added to the cells, which were incubated for 20 min at 37 °C. Then, the cells were washed with PBS (3 x). Next, the complex of GSH-QDs (5 nm) and HisEGFP-G81 (0.1 μM) was added, and the cells were incubated for 10 min at 37 °C. Then, the cells were washed with PBS (3 x) and filled with Opti-MEM (Life Technologies). Fluorescence images were acquired with a fluorescence microscope (BX-X700, Keyence Corp., Japan) by using an objective lens Nikon PlanApo 40 x. The excitation and emission filters were λₜₗₑ (470 ± 20) nm and λₐₜₑ (525 ± 25) nm (for EGFP signal), λₜₗₑ (560 ± 20) nm and λₐₗₑ (590 nm long pass (QD830), and λₜₗₑ (360 ± 20) nm and λₐₗₑ (460 ± 25) nm (Hoechst).

**Flow cytometric analysis:** After cellular imaging, the cells were collected by trypsinization and were resuspended in PBS. Then, the cell suspension was analyzed by a flow cytometer (MACSVQuant Analyzer, Miltenyi Biotec Inc.). Fluorescence of EGFP was collected through a FL2 (FITC) filter (λₜₗₑ = 488 nm, λₐₗₑ = 525 ± 25 nm). Fluorescence of the QDs was collected through a FL7 (APC-Cy7) filter (λₜₗₑ = 635 nm, λₐₗₑ = 750 nm long pass). In vivo imaging: A suspension of KPL-4 cells (0.5 x 10⁶ cells per mouse) was transplanted to the dorsal skins of five-week-old female BALB/c nu/nu mice (Japan SLC, Inc.). After several weeks, we selected a mouse bearing a tumor less than 10 mm in diameter.
for imaging. An aliquot (200 µl) of an aqueous solution (500 µg of Ab + 0.5 nmol of HisEGFPB1-QDs in Na2CO3 (1 mL, 10 mm) was injected into a xenografted mouse through a tail vein. NIR fluorescence images (λex = 650 nm, λem = 830 ± 20 nm) were taken by using an in vivo fluorescence imaging system (Bruker, MS FX PRO). Exposure time of the excitation light was 30 s. All animal experiments were approved by the RIKEN Animal Care and Use Committee.

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Conflict of Interest

The authors declare no conflict of interest.

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