Elementary Supplementary Information

Immunoglobulin binding (B1) domain mediated antibody conjugation to quantum dots for *in vitro* and *in vivo* molecular imaging

Setsuko Tsuboi,[a] Akira Sasaki,[a,b] Takao Sakata,[c] Hidehiro Yasuda[c] and Takashi Jin[a,d]*

[a] *Laboratory for Nano-Bio Probes, Quantitative Biology Center, Riken, Suita, Osaka 565-0874, Japan*

[b] *Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan*

[c] *Research Center for Ultra-High Voltage Electron Microscopy, Osaka University, Ibaraki, Osaka 567-0047, Japan*

[d] *Graduate School of Frontier Biosciences, Osaka University, Yamada-oka 2-1, Suita, Osaka 565-0871, Japan*

Experimental details

1. Materials and Chemicals

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 was purchased from PCI. Tri-octylphosphine oxide (TOPO) trioctylphosphine (TOP), tri-butylphosphine (TBP), hexadecylamine (HDA, 90%), and bis(trimethylsilyl)sulfide (TMS_2S) were purchased from Tokyo Chemical Industry (Japan). Sulfur (S, crystalline, 99.9999%), glutathione (GSH, reduced form) and potassium *t*-butoxide were purchased from Wako Chemicals (Japan). Anti-Her2 monoclonal antibody (Ab) was purchased from Chugai Pharmaceutical Co., Ltd. Other organic solvents were of analytical reagent grades.

A Se-Te 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 as follows: sulfur (40 mg, 1.25 mmol) was added to TBP (10 mL) and heated at 100 ºC. After sulfur was completely dissolved, the solution was cooled to room temperature. Then, cadmium 2,4-pentanedionate (388 mg, 1.25 mmol) was added to the sulfur-TBP solution, and the solution was warmed at 100 ºC to dissolve cadmium 2,4-pentanedionate. The Cd-S stock solution was stored under an argon atmosphere at room temperature.

2. Synthesis of QDs

Visible-emitting QD600 (CdSe/ZnS QDs) was synthesized by the literature method.[1,2] NIR-emitting QD830 (CdSeTe/CdS QDs) was synthesized as follows: a mixture of cadmium 2,4-pentanedionate (150 mg, 0.48 mmol), ODPA (300 mg, 0.90 mmol), TOPO (1 g), HDA (3g), and TOP (0.5 mL) were loaded into a 25 mL three-necked flask and heated to 330 ºC under an argon atmosphere. At this temperature, 1 mL of a Se-Te stock solution was quickly injected by using a syringe, which caused an immediate change in the solution color from colorless to brown. By monitoring the QD fluorescence, the formation of QDs (ca. 790 nm emission) was checked. When desired QDs were formed, the solution was cooled to 60 ºC, and chloroform (10 mL) was added. The QDs were precipitated by the addition of methanol and separated by centrifugation. The resulting QD precipitates and HDA (3 g) were loaded into a 25 mL three-necked flask and heated to 250 ºC. At this temperature, the formation of a CdS shell was performed. The addition of a Cd-S stock solution (ca. 0.25 mL) resulted in the formation of CdSeTe/CdS QDs that emit at ca. 830 nm. The QD solution was cooled to 80 ºC, and chloroform (10 mL) was added. The QDs were precipitated by the addition of methanol and separated by centrifugation. To remove excess 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.

3. Preparation of GSH-coated QDs (GSH-QDs)

One mL of an aqueous solution (100 mg/mL) of GSH was slowly added to a tetrahydrofurn solution (1 μM, 2 mL) of CdSeTe/CdS QDs at room temperature under sonication. The resulting precipitates of QDs were separated by centrifugation. Then, 2 mL of an aqueous solution of potassium *t*-butoxide (20 mg/1 mL) was added to the QD precipitates. The solution was sonicated for 5 min and filtered through a 0.45 μm membrane filter. Excess GSH and potassium *t*-butoxide were removed by dialysis with an aqueous solution (10 mM $Na_2 CO_3$).

4. HisGB1 expression and purification

ProteinG B1 sequence was amplified by PCR from pET His6 ProteinG TEV LIC cloning vector (2P-T), Addgene plasmid 29713. The 6xHistidine sequence was included in the oligonucleotides used as primers in PCR. The PCR fragment was fused with pGEX-6P-1 plasmid (GE Healthcare) by using InFusion HD cloning kit (Clontech). The pGEX-6P-1-His-proteinG B1 plasmid was transformed into *E.coli* KRX competent cells (Promega). For large-scale cultures, the transformed cells were grown in 200 mL of LB

media with ampicillin (100 μg/mL) at 37°C on shaking table, until they approached to 0.6 of O.D. 600 (absorbance). To induce production of the targeted protein, isopropyl β-*D*-1-thiogalactopyranoside (0.2 mM) and L-Rhamnose (0.1 %) were added to the LB media, and then incubated with shaking gently for 16 hrs at 18°C. The cells were harvested by centrifugation at 5,000 xg for 10 min. The cells were resuspended with 5 mL of binding buffer (PBS:Phosphate-buffered saline, pH=7.4). Before cell lysis, Complete EDTA-free protease inhibitor cocktail tablets (1X, Roche) were added as a protease inhibitor. The solution was sonicated on ice using 10 s bursts at middle-intensity with a 10 s cooling period between each burst. The lysate was clarified by centrifugation at 20,000 xg for 20 min to eliminate cell debris. The next step was the purification by Glutathione Sepharose 4B (GE Healthcare). Two mililiter of Sepharose media equilibrated with binding buffer was added to each 5 mL of lysed sample, and incubated with gentle agitation at 4°C for 1hr. The Sepharose media was then washed with Turbo 3C Protease buffer, followed by digestion of the GST-tag by overnight incubation at 4℃ with Turbo 3C Protease (Accelagen) in Turbo 3C Protease buffer. Lastly the His-proteinG B1 was buffer-exchanged using a gel filtration column (PD-10 columns, GE Healthcare).

5. Fluorescence measurements

Fluorescence spectra of QDs were measured with a photonic-multichannel analyzer (C10027, Hamamatsu Photonics, Japan) with a 150 W-Xenon lamp (excitation light source at 488 nm). Fluorescence autocorrelation curves of QDs were measured using a compact FCS system (C9413-01MOD, Hamamatsu Photonics, Japan). For the determination of the concentration of GSH-QDs, we measured the number of QD particles in a 20 μL of the QD solution by using FCS, and estimated its concentration using a 20 nM solution of Rhodamine 6G as a reference. For all measurements, pH of the aqueous solution of QDs was set to be 7.4 with PBS buffer.

6. **Transmission Electron Microscope (TEM)**

The morphologies of QDs were observed by TEM using a Hitachi H-800 microscope operating at an acceleration voltage of 200 kV. The TEM sample (1μM QDs in PBS) was prepared by dropping the sample solution onto a copper grid.

7. Gel electrophoresis

SDS-PAGE: HisGB1protein (1 μg/lane) was run on a 5-20 % polyacrylamide gel (Extra PAGE one Precast gel, Nacalai tesque) in Tris-glycine-SDS buffer, applied at 200 V for 40 min and stained with Coomassie Brilliant Blue (CBB Stain One Super, Nacalai tesque). A size marker (Precision Plus Protein Standard, BIO-RAD) was used to compare between His-GB1 protein. The expected size for HisGB1protein was 9.3 kDa. Agarose Gel Electrophoresis: GSH-QDs and the mixture of GSH-QDs and HisGB1protein（50 μg/mL）were run on 1% agarose gel in Tris-Acetate buffer (pH 8.0), applied at 100 V for 15min.

8. Size-exclusion column chromatography

Size-exclusion column chromatography with a HPLC system (ELITE LaChrom, HITACHI) was consisting of a pump system (L-2130), UV detector (L-2400) and FL detector (L-2485), with a TSK-gel G4000SW column (7.8 mm × 30 cm, TOSOH). A mobile phase was 10 mM PBS (pH 7.4) and flow rate was adjusted to 1mL/min. Standard proteins of thyroglobulin (670 kDa), ferritin (450 kDa), BSA (66 kDa) and EGFP (30.8 kDa) were measured to draw a calibration curve. The HPLC chromatograph of GSH-QDs and HisGB1 conjugated QDs was obtained by monitoring absorption at 488 nm.

9. Dynamic light scattering (DLS).

DLS data were obtained using a Zetasizer Nano-ZS (Malvern) with a 633He/Ne laser. Hydrodynamic sizes of GSH-QDs (QD600, QD830), HisGB1-QDs and their Ab conjugates were estimated from the DLS data.

10. Cell viability

Human breast tumor cells (KPL-4) were incubated with HisGB1 conjugated GSH-QDs (0.05–50 nM, PBS) for 6, 24 and 48 hrs. MTT assay was performed according to the procedure of a MTT Cell Count Kit (Nacalai Tesque). The MTT reagent was added to each well and cells were incubated for 2 hrs at 37℃. Then, the STOP solution was added to stop the reaction. According to the instruction of the Kit, the absorbance at 570 nm and 650 nm of solubilized MTT formazan products were measured with a Microplate Spectrophotometer (Multiskan GO;ThermoFisher).

11. Cellular imaging

Cellular imaging was performed using a fluorescence microscope, BZ-X700 (KEYENCE CORPORATION, Japan). KPL-4 and HeLa cells were seeded to Poly-L-Lysine coated glass bottom

dishes (D11531H, Matsunami 35 mm), and incubated in Dulbecco's Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum (FBS) for overnight at 37°C. Anti HER2 antibody and Hoechst33342 were added to the cell and incubated for 20 min at 37°C. Then, the cells were washed with PBS three times. The complex of QD600 (or QD830) and HisGB1 protein was added to the cells, and incubated for 10 min at 37°C. Then, the cells were washed with PBS three times and filled with Opti-MEM (Life technologies). The cells were observed with a TRITC filter (Ex:545 ± 25 nm/Em:605 ± 70 nm) for QD600 and an ICG filter (Ex:769 ± 41 nm /Em:832 ± 37 nm) for QD830.

12. *In vivo* NIR fluorescence imaging

A suspension of KPL-4cells (0.5×10^7 cells per mouse) was transplanted to the dorsal skins of 5-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. Two hundred μL of an aqueous solution (200 μg of Ab + 1 nmol of HisGB1-QDs in 1 mL of PBS) was injected into a tumor-bearing mouse through a tail vein. NIR fluorescence images (Ex: 650 nm, Em: 830 ± 20 nm) were taken using an *in vivo* fluorescence imaging system (Bruker, MS FX PRO). Exposure time of the excitation light was 30 s. 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 RIKEN Animal Care and Use Committee.

References

1) Akihito Komatsuzaki, Tatsuya Ohyanagi, Yoshikazu Tsukasaki, Yukihiro Miyanaga, Masahiro Ueda and Takashi Jin, *Small*, 2015, **11**, 1396-1401.

2) Tatsuya Ohyanagi, Tomohiro Shima, Yasushi Okada, Yoshikazu Tsukasaki, Akihito Komatsuzaki, Setsuko Tsuboi and Takashi Jin, *Chem. Commun*., 2015, **51**, 14836-14839.

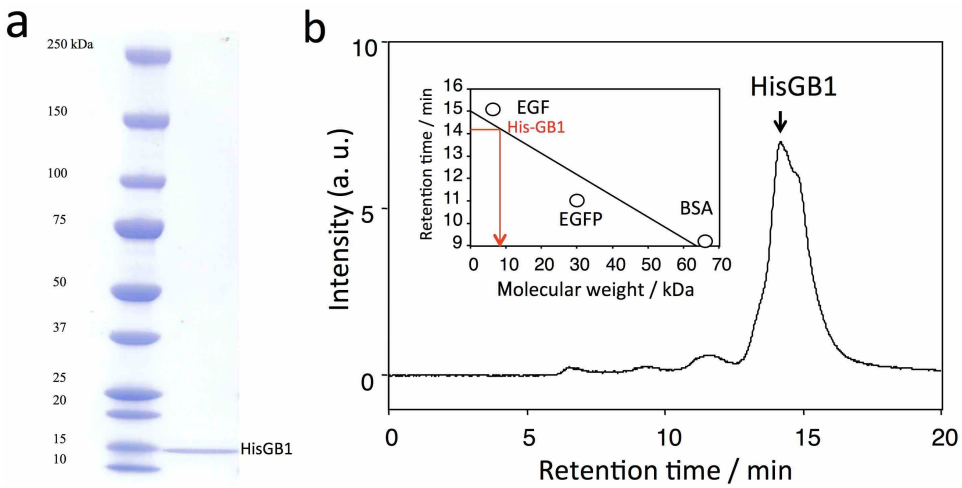

Fig. S1 (a) SDS-PAGE of HisGB1 protein. (b) Size-exclusion HPLC chromatograph of HisGB1 protein (9.3 kDa). Inset shows the relationship between retention times of standard protein and their molecular weights for the HPLC column (TOSOH, TSKgel G4000SW). EGF: epidermal growth factor, 6.2 kDa, EGFP: enhanced green fluorescent protein, 30.8 kDa, BSA: bovine serum albumin, 66 kDa.

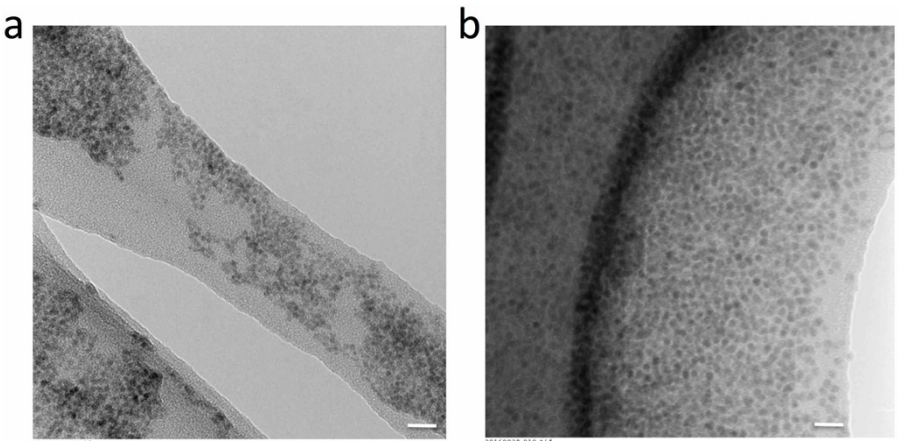

Fig. S2 Transmission electron microscopy images of (a) QD 600 and (b) QD830. Scale bar: 20 nm.

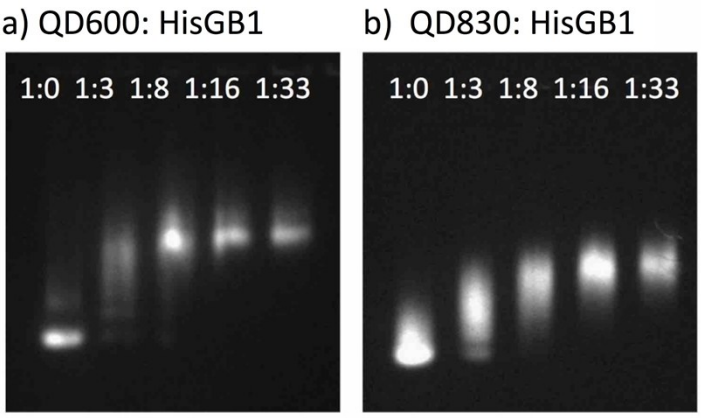

Fig. S3 Agarose gel electrophoresis of QD600 (a) and QD830 (b) in the absence and presence of HisGB1 protein. The values in the gel images are shown the molar ratios of QD600: HisGB1 and QD830: HisGB1. The bands were observed at the fluorescence emission at 600 ± 25 nm for QD600 and at 830 ± 25 nm QD830, respectively.

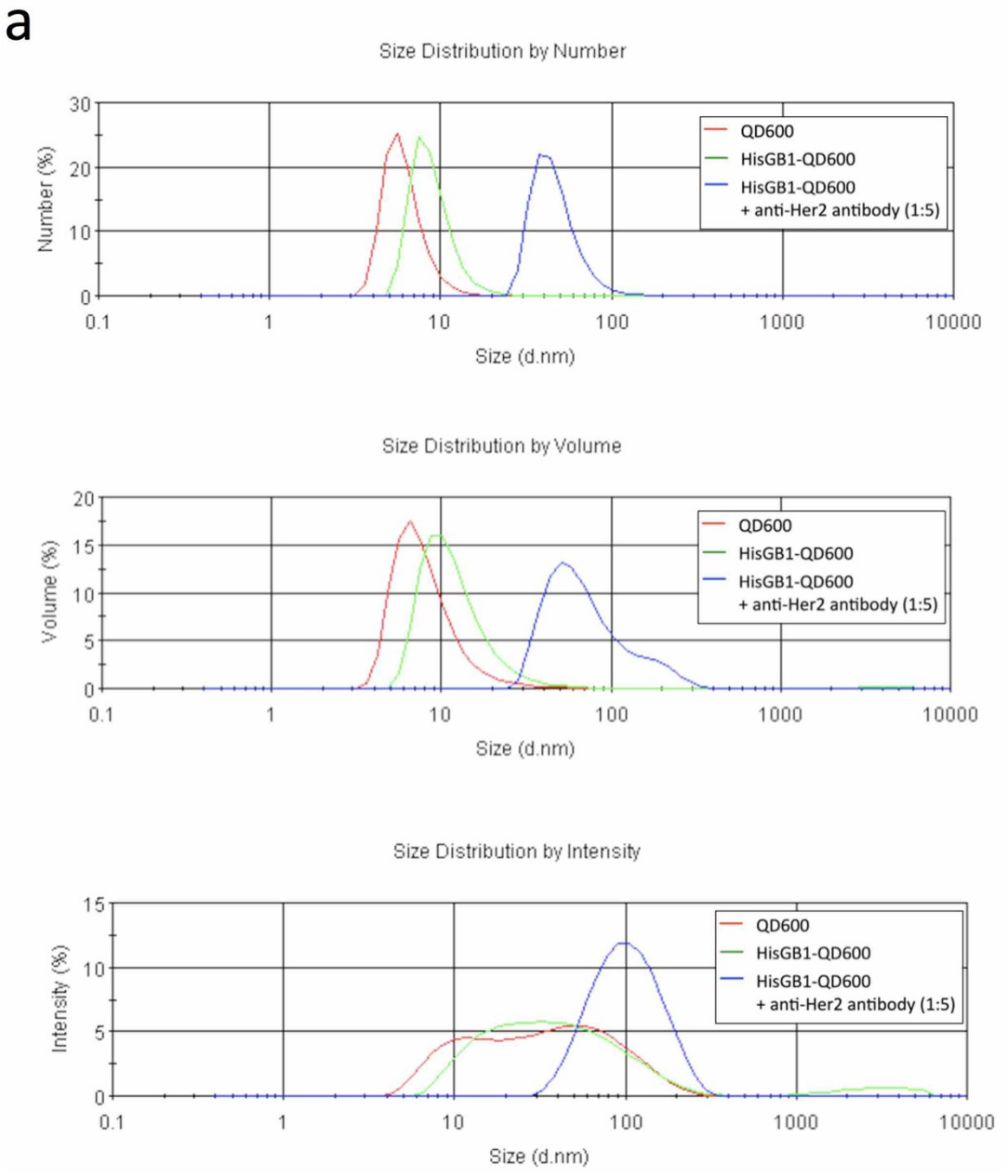

Fig. S4 Distribution of hydrodynamic diameter of QDs, HisGB-QDs, and HisGB-QDs + anti-Her2 antibody (1:5 molar ratio), HisGB1, and anti-Her2 antibody, determined by using DLS. (a) QD600, HisGB1-QD600, and HisGB1-QD600+anti-Her2 antibody (1:5), (b) QD830, HisGB1-QD830, and HisGB1-QD830 + anti-Her2 antibody (1:5), (c) HisGB1 and anti-Her2 antibody

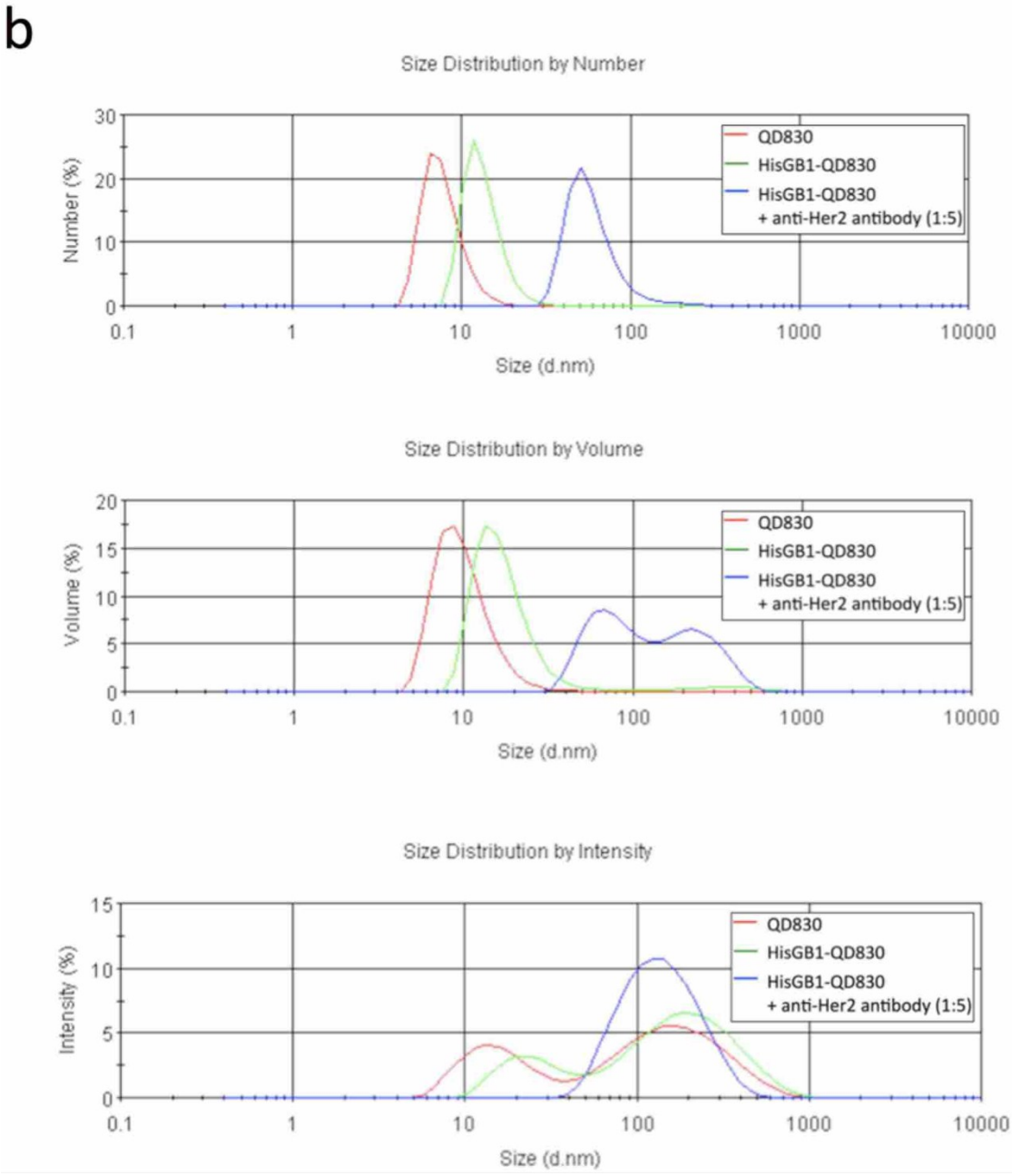
b
Size Distribution by Number
Number (%)
Size (d.nm)
QD830
HisGB1-QD830
HisGB1-QD830
+ anti-Her2 antibody (1:5)
Size Distribution by Volume
Volume (%)
Size (d.nm)
QD830
HisGB1-QD830
HisGB1-QD830
+ anti-Her2 antibody (1:5)
Size Distribution by Intensity
Intensity (%)
Size (d.nm)
QD830
HisGB1-QD830
HisGB1-QD830
+ anti-Her2 antibody (1:5)

Fig. S4 Cont.

C

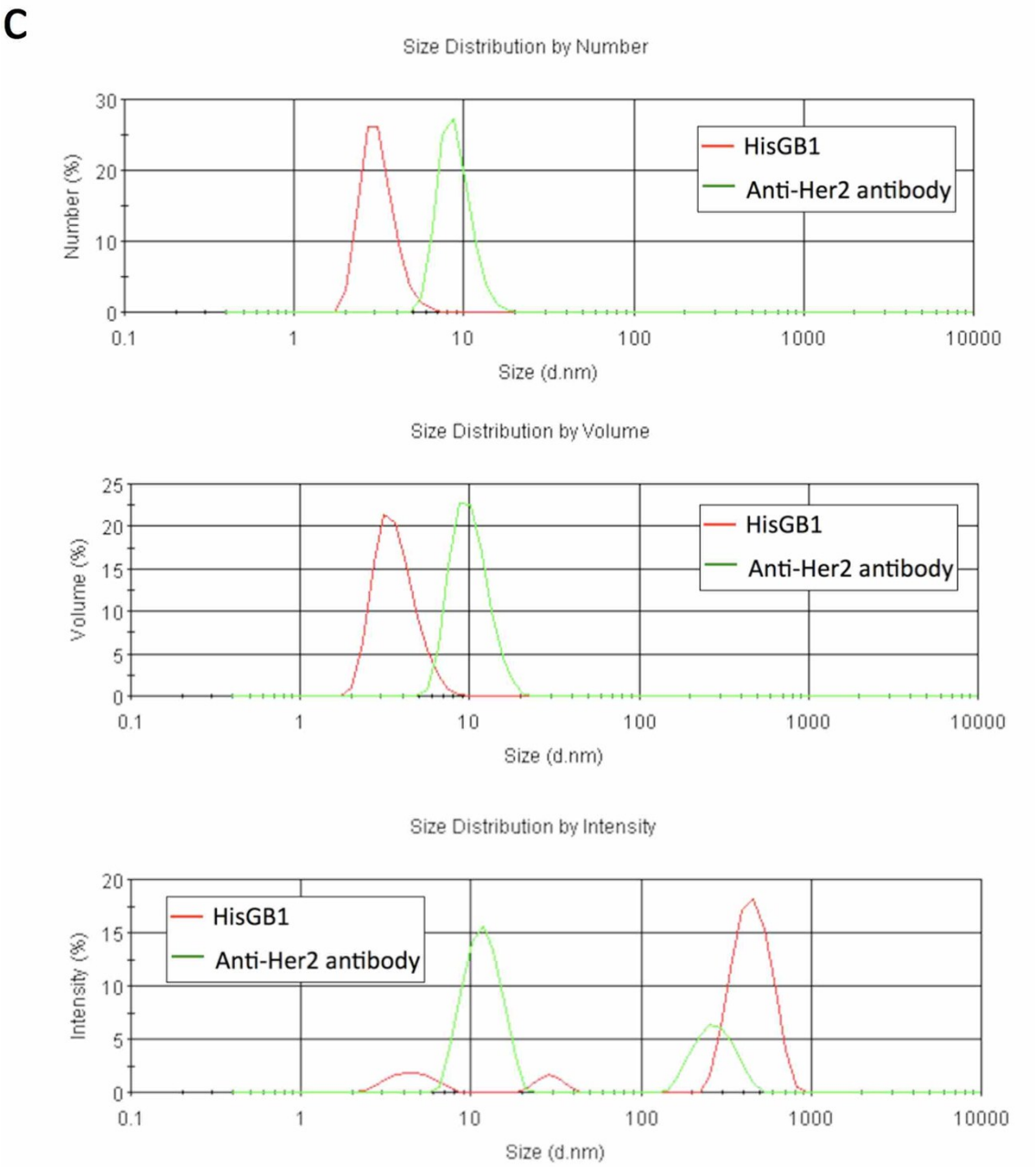
Size Distribution by Number
Number (%)
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)
HisGB1
Anti-Her2 antibody
Size Distribution by Volume
Volume (%)
25
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
HisGB1
Anti-Her2 antibody
Size Distribution by Intensity
Intensity (%)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
HisGB1
Anti-Her2 antibody

Fig. S4 Cont.

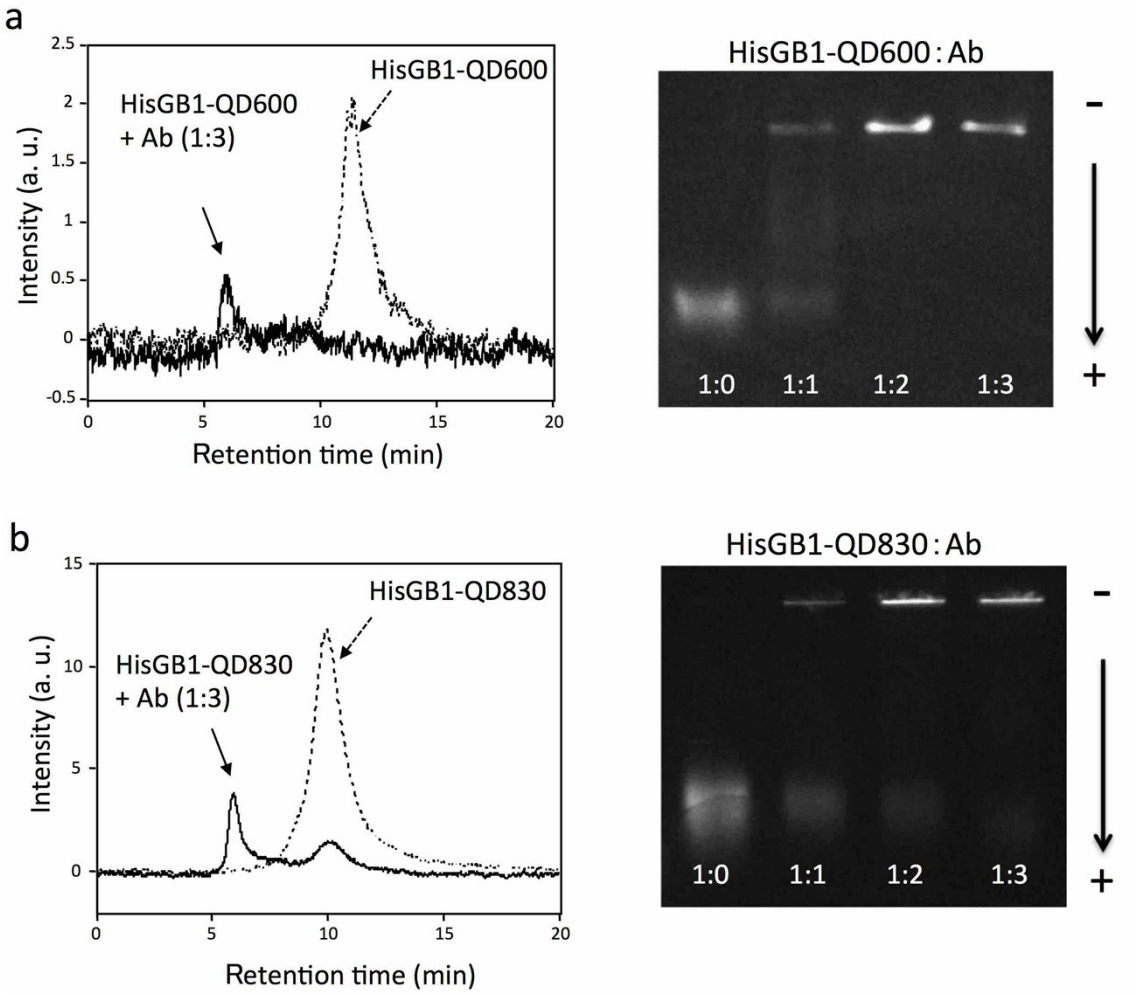

Fig. S5 （a）Size-exclusion HPLC chromatograph and agarose gel electrophoresis for HisGB1-QD600 and HisGB1-QD600 + Ab, (b) Size-exclusion HPLC chromatograph and agarose gel electrophoresis for HisGB1-QD830 and HisGB1-QD830 + Ab. Ab: anti-Her2 antibody. The values mean the molecular ratios of QD: Ab.

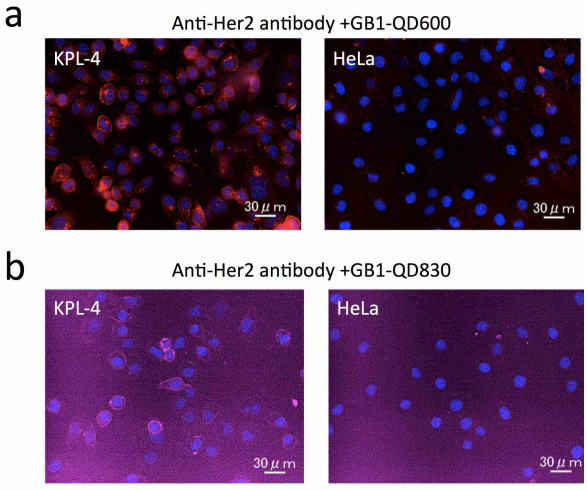

Fig. S6 Fluorescence microscopy images of KPL-4 and HeLa cells incubated with (a) anti-Her2 antibody + HisGB1-QDs with a 600 nm emission (red) and (b) anti-Her2 antibody + HisGB1-QDs with an 830 nm emission (purple). Blue color shows the fluorescence from Hoechst33342.

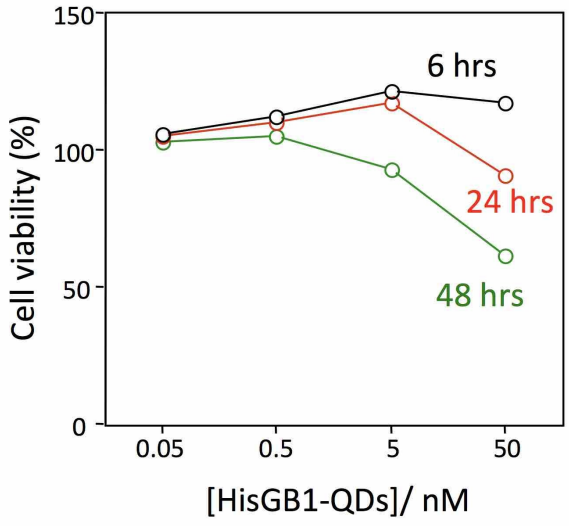

Fig. S7 Viability of KPL-4 cells incubated with HisGB1-QDs.

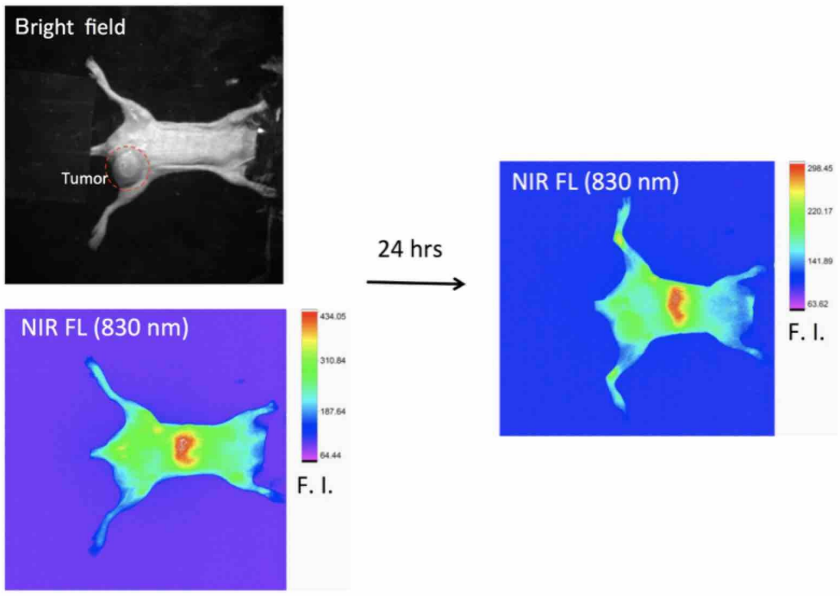

Fig. S8 NIR fluorescence imaging of a breast tumor-bearing mouse injected by 200 μL of an aqueous solution (200 μg of Ab + 1 nmol of GSH-QDs in 1 mL of PBS).

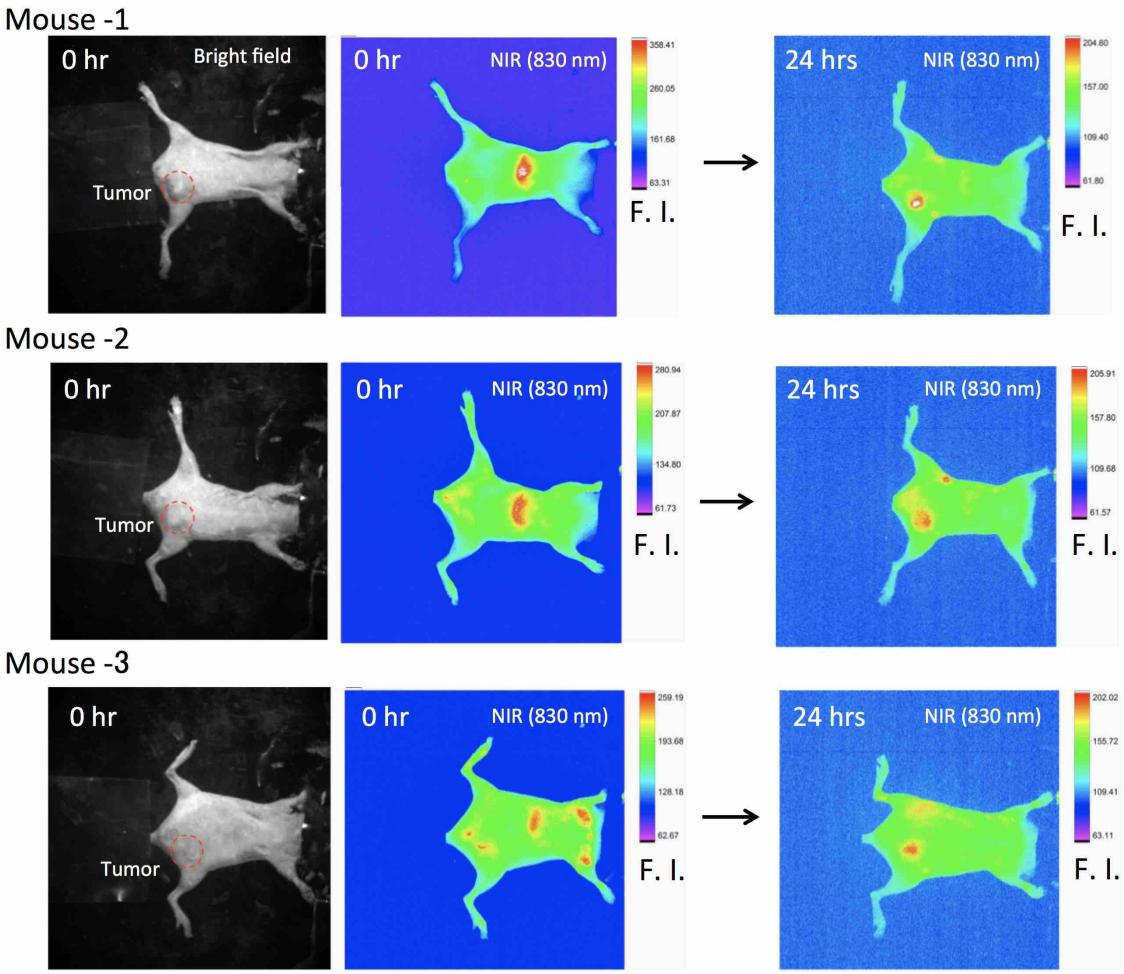

Fig. S9 NIR fluorescence imaging of breast tumor-bearing mice injected by 200 μL of an aqueous solution (200 μg of Ab + 1 nmol of HisGB1-QDs in 1 mL of PBS)