Supporting Information

In Vitro and In Vivo Fluorescence Imaging of Antibody Drug Conjugate-Induced Tumor Apoptosis Using Annexin V-EGFP Conjugated Quantum Dots

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Experimental

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1. 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, and n-octadecylphosphonic acid (ODPA) was purchased from PCI Synthesis. Tri-octylphosphine oxide (TOPO) trioctylphosphine (TOP), tributylphosphine (TBP), and hexadecylamine (HDA, 90%) 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). Alexa 680-NHS ester was purchased from Thermo Fisher Scientific. Other chemicals were of analytical reagent grades.

2. Synthesis of CdSeTe/CdS QDs

A typical procedure is as follows: 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 by adding sulfur (40 mg, 1.25 mmol) to TBP (10 mL). 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 suffer-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.

A mixture of cadmium 2,4-pentanedionate (150 mg, 0.48 mmol), ODPA (300 mg, 0.90 mmol), TOPO (1 g), HDA (3 g), 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, 0.5 mL of a Se-Te stock solution was quickly injected by using a syringe, which caused an immediate color change in solution from colorless to brown. By monitoring the QD fluorescence spectra, the formation of QDs (ca. 800 nm emission) was checked. When the desired QDs were formed, the solution was cooled to 60 ºC and chloroform (10 mL) was added to the solution. The QDs were precipitated by addition of methanol, and the QD precipitates were 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 the CdS shell was performed. The addition of a Cd-S stock solution (0.25 mL) resulted in the formation of CdSeTe/CdS QDs that emit at ca. 830 nm. Then the QD solution was cooled to 80 ºC, and chloroform (10 mL) was added. The QDs were precipitated by 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.

GSH coating: an aqueous solution (1 mL) of GSH (100 mg/mL,) was slowly added to a tetrahydrofuran solution (1 μM, 2 mL) of CdSeTe/CdS QDs at room temperature under sonication.

The precipitates of QDs were separated by centrifugation. To the QD precipitates, an aqueous solution of potassium *t*-butoxide (20 mg/mL, 2 mL) was added under stirring. 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 using a 10 mM Na_2CO_3 aqueous solution. The resulting GSH-QDs was preserved at 4 ºC.

3. Preparation of recombinant protein (Annexin V-EGFP)

a) Construction of a recombinant plasmid (pRSET-EGFP-Annexin V)

Annexin V sequence was amplified by PCR from pET12a-PAPI, which was a gift from Jonathan Tait (Addgene plasmid #19961, B. L. Wood, D. F. Gibson, and J. F. Tait, Blood 1996, 88, 1873-1880). EGFP sequence was amplified by PCR from pEGFP-C1 plasmid (Clontech). The PCR fragments were fused with pRSET plasmid (ThermoFisher) by using an InFusion HD cloning kit (Clontech).

b) Expression of the recombinant protein in *E. coli.*

The pRSET-EGFP-Annexin V was transformed into *E.coli* KRX competent cells (Promega). Transformed *E.coli* were grown as a preculture (2 mL) in LB medium containing ampicillin (100 μg/mL) at 37 °C overnight. For large-scale cultures, the overnight culture (2 mL) was grown in 200 mL of LB medium containing ampicillin (100 μg/mL) at 37°C, until they approached to 0.6 of OD 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 medium, and then incubated with shaking gently for 16 h at 18 °C.

c) Extraction and purification of the recombinant protein.

The cells were harvested by centrifugation at 5,000 x g for 10 min. The pellet was resuspended in 5mL of Binding Buffer (50 mM Tris-HCl, 500mM 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 suspension was sonicated on ice. Bursts of 10 seconds with intermediate intensity are repeated 7-10 times with a 10 second cooling period between each burst. The lysate was clarified by centrifugation at 20,000 x g for 30 min to eliminate cell debris. The supernatant was then purified by Ni Sepharose 6 Fast Flow (GE Healthcare). 0.5 mL of Sepharose media equilibrated with binding buffer was added to each 5mL of lysed sample, and incubated with gentle agitation at 4 °C for 60 min. After the solution was transferred to an empty column, it was washed with binding buffer five column volumes. Lastly, the recombinant 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 a size-exclusion column (PD-10, GE Healthcare) to exchange buffer.

4. Preparation of Alexa 680-conjugated Kadcyla

Alexa 680-NHS ester (1 mg) was resolved to 1 mL of anhydrous dimethyl sulfoxide. To 1 mL aqueous solution (0.01 M Na_2CO_3) of Kadcyla (1 mg), a dimethyl sulfoxide solution of Alexa 680-NHS ester was added. The coupling reaction was performed for 30 min at room temperature. The Alexa 680-conjugated Kadcyla was purified by using a size-exclusion column (PD-10, GE Healthcare) with PBS to remove unreacted dyes.

5. Calculation of FRET efficiency

The FRET efficiency from EGFP to QD can be calculated from both the fluorescence lifetimes and the fluorescence intensity of a donor by the following equations [(1) & (2)]:

$$E = 1 - \tau_{DA}/\tau_D \quad (1)$$

$$E = 1 - F_{DA}/F_D \quad (2)$$

in which τ_D and τ_{DA} are the lifetimes of the donor in the absence and presence of the acceptor, and F_D and F_{DA} are the fluorescence intensities of the donor in the absence and presence of the acceptor, respectively.

6. Hydrodynamic size measurements

The distributions of hydrodynamic size of liposomes were determined from dynamic light scattering (DLS). DLS measurements were performed using a Zeta sizer Nano-ZS (Malvern) with a 633He/Ne laser.

7. Cell viability test

Cell viability was measured using the MTT assay: Human breast tumour cells (KPL-4) were seeded to 96 well plate (6×10^3 cells per well) and incubated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) for overnight at 37 °C. Then, the cells were incubated with GSH-QDs (0-100 nM), Annexin V-EGFP (0-1000 nM) and Annexin V-EGFP-QDs (0-100 nM) for 6, 24, 48 h. The MTT assay was performed according to the procedure provided by the MTT Cell Count Kit (Nacalai Tesque). MTT reagent was added to each well, and the cells were incubated at 37 °C for 2 h. Next, the STOP solution was added to terminate the reaction, and the absorbance of the solubilized MTT formazan product at 570 nm was measured with a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific). Absorbance at 650 nm was subtracted as the background.

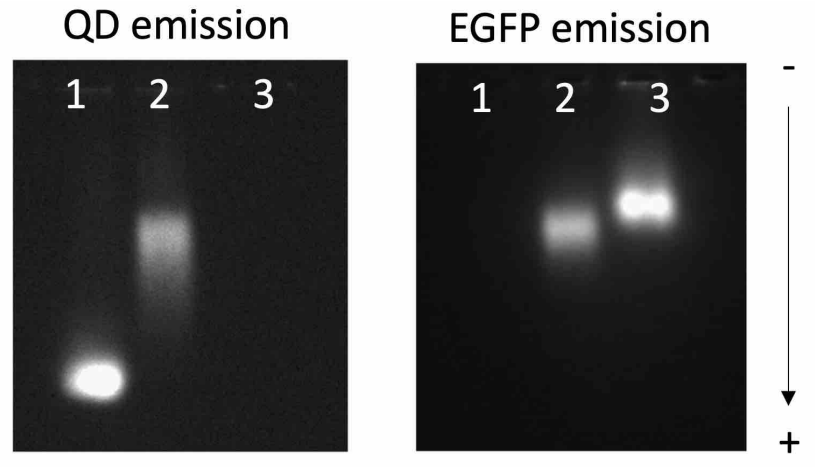

Figure S1. Agarose gel electrophoresis of GSH-QDs and a mixture of GSH-QDs and Annexin V-EGFP. 1: GSH-QDs, 2: A mixture of GSH-QDs and Annexin V-EGFP (1:5 molar ratio), 3: Annexin V-EGFP. The fluorescence bands of QD and EGFP emissions were detected at the wavelength of 830 nm and 525 nm, respectively.

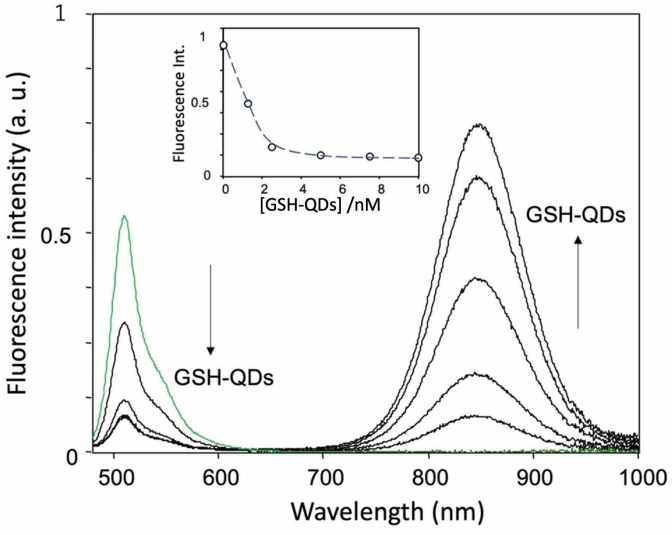

Figure S2. Titration of the fluorescence spectrum of Annexin V-EGFP (30 nM) with GSH-QDs in 10 mM Na_2CO_3. [GSH-QDs] = 0, 1.25, 2.5, 5, 7.5, and 10 nM. Inset shows the fluorescence intensity of EGFP at 515 nm vs. [GSH-QDs].

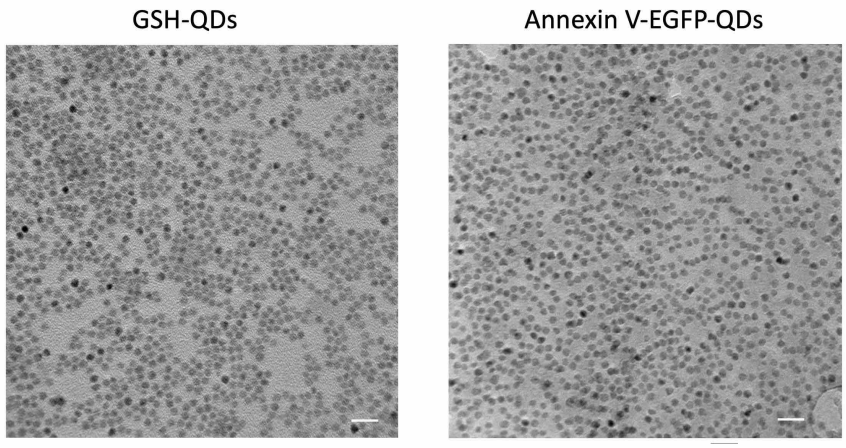

Figure S3. TEM images of GSH-QDs and Annexin V-EGFP-QDs. The diameters of GSH-QDs and Annexin V-EGFP-QDs are 4.3 ± 0.61 nm and 4.4 ± 0.68 nm, respectively. Scale bar: 20 nm.

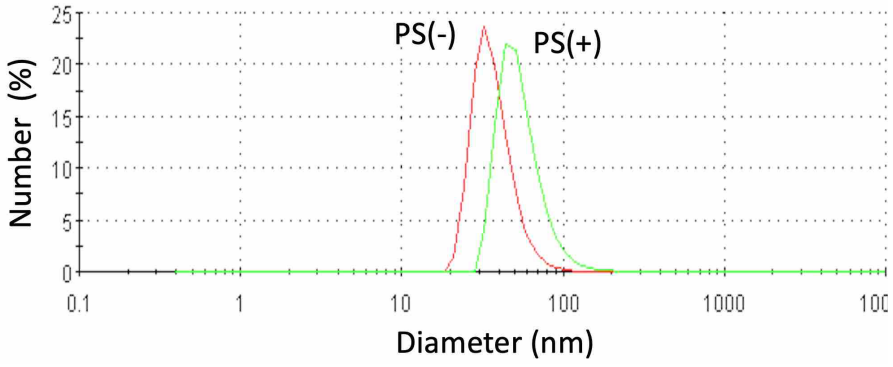

Figure S4. The distribution of hydrodynamic diameters of 0% PS containing liposomes PS(-) and 10% PS containing liposomes, PS(+). The diameters are evaluated from the dynamic light scattering experiment.

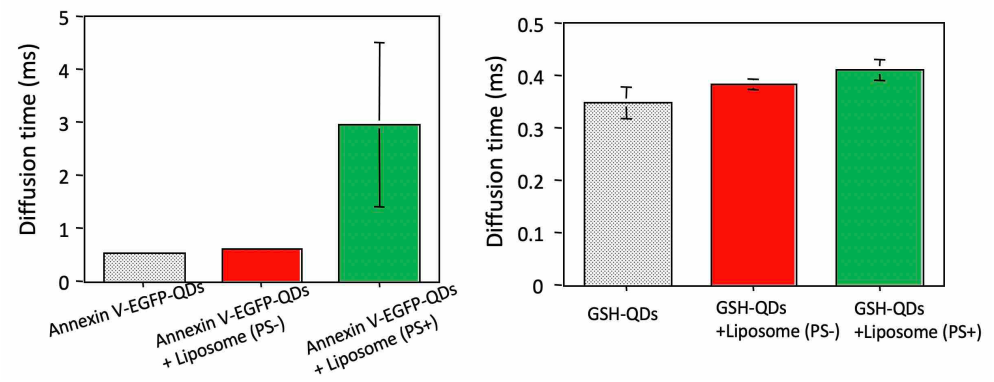

Figure S5. Diffusion time of Annexin V-EGFP-QDs and GSH-QDs in water.

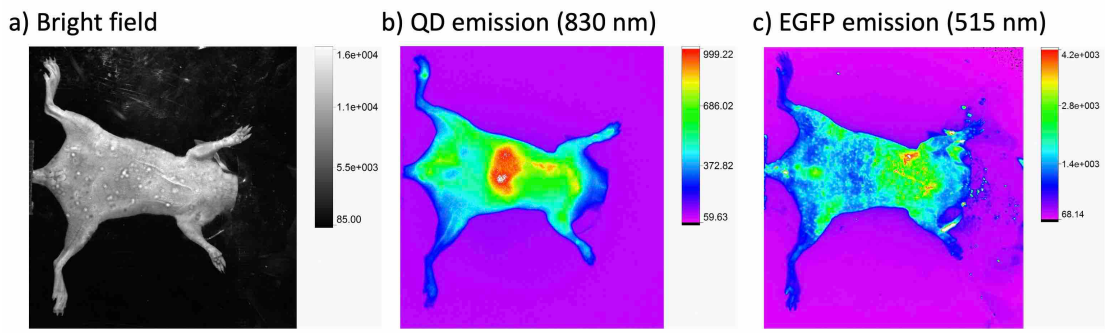

Figure S6. NIR and VIS dual fluorescence imaging of a breast tumor-bearing mouse immediately after the with Annexin V-EGFP-QDs (100 μL of 1 μM solution). a) bright filed image. b) NIR fluorescence image was taken through an 830 nm band-path filter. Intense NIR fluorescence results form Annexin V-EGFP-QDs accumulated to the liver. c) VIS fluorescence image was taken through a 515 nm band-path filter. Most of VIS fluorescence results from the autofluorescence of tissue. EGFP emission could not be observed from Annexin V-EGFP-QDs accumulated to the liver. This is due to the strong absorption of EGFP emission by the liver and the intense back-ground signals of autofluorescence.

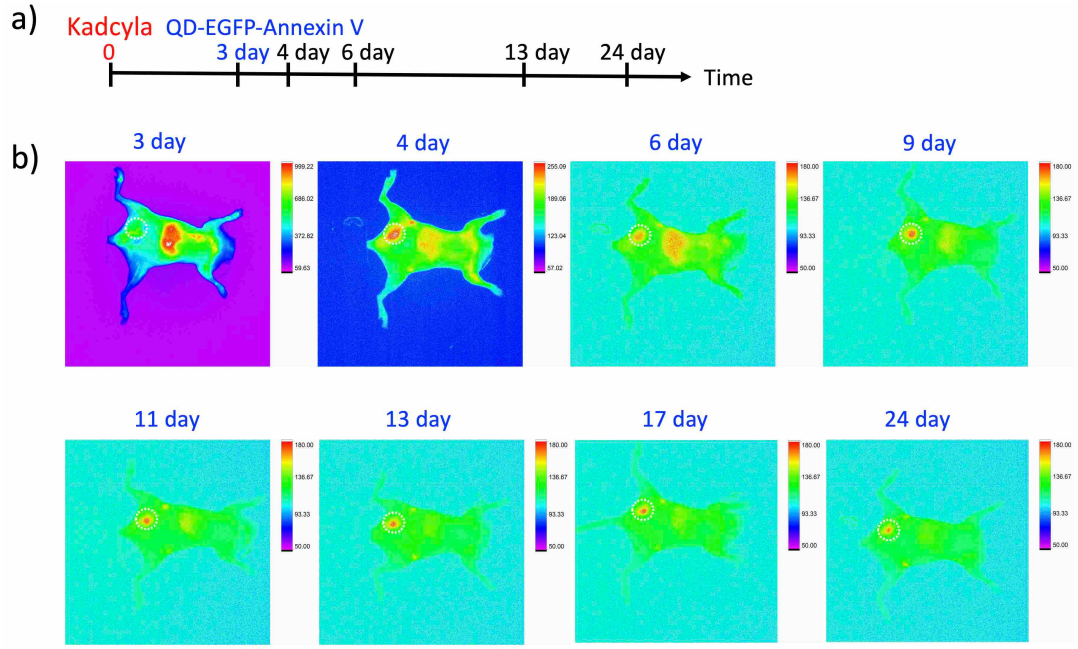

Figure S7. Long-term NIR fluorescence imaging of a breast-tumor bearing mouse. a) Experimental for the administration of Kadcyla (100 μL, 1mg/mL) and Annexin V-EGFP-QDs (100 μL of 1 μM solution). b) Fluorescence images of a breast-tumor bearing mouse. The images were taken 3-24 days after the injection of Kadcyla. White dotted circles show the position of the tumor. Scale bar: 10 mm.

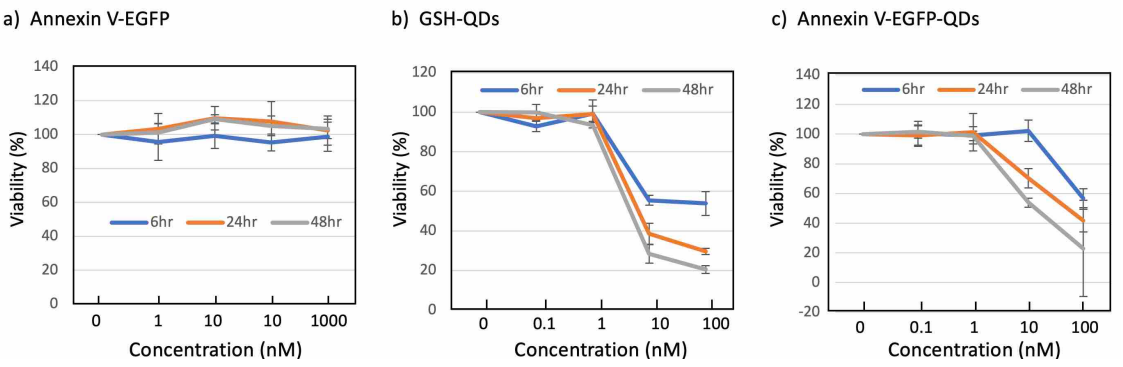

Figure S8. Viability of KPL-4 cells in the presence of (a) Annexin V-EGFP, (b) GSH-QDs, and (c) Annexin V-EGFP-QDs.

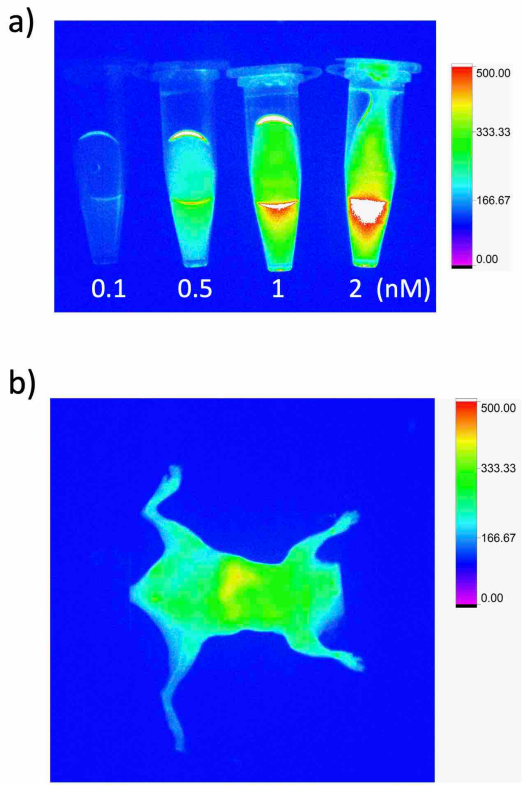

Figure S9. Comparison of NIR fluorescence images (830 nm) of Annexin V-EGFP-QDs in water (a) and in a mouse (b) injected by 100 μL of Annexin V-EGFP-QDs (1 μM, 10 mM Na_2CO_3). The experimental condition of fluorescence measurements was the same with that of *in vivo* fluorescence measurements in Figure 7 in the text.