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[Electronic Supplementary Information]

Highly Robust and Optimized Conjugation of Antibodies to Nanoparticles using Quantitatively Validated Protocols

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KEYWORDS: Catalyst-Free Click Chemistry, Full-length Antibody Conjugation, Antibody Orientation Control, Experimental Quantification Protocol.

Materials

Tetraethyl orthosilicate (TEOS), 3-mercaptopropyltrimethoxy-silane (MPTS), ethylene glycol (EG), silver nitrate ($AgNO_3$, > 99.99%), 3-aminopropyltriethoxysilane (APTES), dimethyl sulfoxide (DMSO), N-methyl-2-pyrrolidone (NMP), N-hydroxysuccinimide (NHS), N,N′-diisopropylcarbo-diimide (DIC), N,N′-diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP), bovine serum albumin (BSA, > 98%), and phosphate-buffered saline (PBS, tablet) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and used without further purification. 3-azidopropyl-1,4,7-triaza-cyclononane-1,4,7-tri-acetic acid (NOTA-N_3), NOTA-ADIBO, and ADIBO-PEG4-Male-imide (ADIBO-PEG4-Mal) were purchased from FutureChem (Seoul, Korea). Instant thin-layer chromatography-silica gel (ITLC-SG) plates were purchased from Agilent Technologies, Inc. (Santa Clara, CA, USA). PD-10 size-exclusion columns were obtained from GE Healthcare (Buckinghamshire, U.K.). Recombinant human EtbB2 (HER2 antigen) was purchased from Sino Biological Inc. (Beijing, China). Herceptin (Trastuzumab, anti-HER2 monoclonal antibody) was purchased from Roche Pharma Ltd. (Reinach, Switzerland). Fluorescently labeled antibody (Cy5 labeled goat anti-mouse IgG (H+L) secondary antibody) was purchased from the ThermoFisher Scientific (Waltham, MA, USA), and utilized for quantitative determination of the number of antibody on a single silica nanoparticle (SiNP) without further purification. Deionized water (DW) was used for all experiments.

Instruments

Radioactivity was measured using a gamma scintillation counter (Packard Cobra II, GMI, NM, USA). Antibody molecular weight was measured by matrix-assisted laser desorption ionization time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) using a TOF/TOF 5800 system (AB Sciex, Foster City, CA, USA). Antibody concentration was measured spectrophoto-metrically using a NanoDrop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilming-ton, DE, USA). Fluorescence imaging was performed using a Xenogen IVIS 200 small animal imaging system (Xenogen, Alameda, CA, USA). Fluorescence images of cells were obtained by confocal laser scanning microscopy (CLSM; SP8 X, Leica, Wetzlar, Germany). The hydrodynamic diameter and size distribution of nanoparticles were analyzed using the dynamic light scattering (DLS) system, Zetasizer Nano ZS90 (Malvern Instruments Ltd, Worcestershire, UK).

Establishment of cancer cell lines

The human breast cancer cell-line, a HER2-expressing variant of MDA-MB-231 (MDA-MB-231/HER2, ATCC, Manassas, VA), and melanoma cell-line (B16F1, ATCC, Manassas, VA, HER2 negative), were acquired from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C and passaged with 0.125% trypsin. Then, those cells were harvested.

MALDI-TOF analysis of the antibody and ADIBO-conjugated antibody

Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) was performed to evaluate antibody modification. The antibody solution was mixed with sinapinic acid dissolved in 50% acetonitrile/50% water containing 0.1% TFA (10 mg/ml) at a 1:1 ratio. Aliquots (1 µL) of each antibody solution were spotted onto MALDI plates and allowed to air dry completely at room temperature. Measurements were acquired in linear mode with an accelerating voltage of 25 kV. Molecular weights of the antibody alone and the ADIBO-functionalized antibody were calculated as the average from 500-1000 laser shots following the standard MALDI-TOF/MS method used for protein analysis.

Determination of antibody and antigen concentration by Nano-drop

To determine the concentration of each sample, we used a NanoDrop^{TM} (Thermo Fisher Scientific Inc, Wilmington, USA). There were selective modes for the quantification of different types of samples: normal mode for antigen quantification and IgG mode for modified antibody concentration. The reliability of this system was verified using accurately measured standard solutions.

Measurement of confocal laser scanning microscopy

Cells were fixed with 4% paraformaldehyde, followed by nuclear staining using mounting solution containing DAPI (4',6-diamidino-2-phenylindole). Confocal laser scanning microscopy (CLSM; SP8 X, Leica, Wetzlar, Germany) was used to obtain fluorescence images of cells targeted by anti-HER2 fluorescent NPs (anti-HER2-QD^2). Excitation laser-lines for DAPI and anti-HER2-QD^2 were 405 nm. The DAPI signals were collected from 420 nm to 490 nm, and the anti-HER2-QD^2 signals were collected from 610 nm to 650 nm, respectively. All obtained images were analyzed using the imaging process software provided by Leica (Leica Application Suite X, Leica, Germany).

Preparation of silica-encapsulated quantum dot embedded SiNPs

The silica-encapsulated quantum dots embedded SiNPs (QD^2) were synthesized by following previous developed method.[1] SiNPs (*ca.* 180 nm) were prepared by the well-known Stöber method.[2] A 3 mL of ammonium hydroxide (27-30%, NH_4OH) was added to 40 mL of ethanol, which contained 1.6 mL of tetraethylorthosilicate (TEOS), accompanied by vigorous magnetic stirring for 20 h at 45°C. The SiNPs were centrifuged and washed several times with ethanol. In order to functionalize the surface of SiNP with thiol group, the SiNPs (200 mg) were dispersed in 4 mL of ethanol containing 200 µL of MPTS and 40 µL of ammonium hydroxide. The mixture was stirred for 1 h at 50°C. The resulting thiol-functionalized SiNPs were washed several times with ethanol by centrifugation (8500 rpm for 15 min at 4°C) and re-dispersed in ethanol. Thiol-functionalized SiNPs (1 mL, 10 mg/mL in ethanol) were dispersed in 4 mL of dichloromethane solution, and red quantum dots (red-QDs, 0.28 mL, 25 mg/mL in chloroform, CZO-620T, Global ZEUS, Korea) were injected to the mixture solution, and mixture was strongly shaken for 1 min. Then, 50 µL MPTS and 50 µL NH_4OH were injected to the mixture. After incubation

for 30 min with shaking, the mixture was centrifuged and washed with ethanol several times. The mixture was well dispersed in 5 mL of ethanol, and each of 50 µL TEOS and 50 µL NH_4OH were injected to the mixture. After incubation for 12 h, the resulting mixtures were centrifuged and washed with ethanol several times.

Preparation of surface-enhanced Raman scattering (SERS) nanoprobes (SERS dots)

Synthesis of SERS dots were followed by our previous developed method.[3] In briefly, *ca.* 180 nm synthesized SiNPs were functionalized with the thiol group as follows. SiNPs (50 mg) were dispersed in 1 mL of ethanol containing 50 µL of MPTS and 10 µL of aqueous ammonium hydroxide (28-30%) The mixture was shaken for 9 h at 25°C. The resulting MPTS-treated SiNPs were centrifuged and washed with ethanol several times. A 50 mg portion of MPTS-treated SiNPs were mixed with 25 mL ethylene glycol. Then, a 25 mL portion of $AgNO_3$ solution (in ethylene glycol) was added to the SiNPs solution and thoroughly mixed (final concentration of $AgNO_3$ was 3.5 mM). A 41.3 µL portion of octylamine (5 mM) was then rapidly added to the above solution and stirred vigorously for 1 h at 25°C. Afterwards, synthesized Ag-embedded silica (AgSi) were centrifuged and washed with ethanol several times for purification. Then, 1 mL portion of Raman label compound (4-FBT, 10 mM in ethanol) was added to each 10 mg of AgSi. The resulting dispersion was shaken for 1 h at 25°C. The AgSis, bearing adsorbed each Raman label compound at their surfaces, were centrifuged and washed with ethanol 2 times. To encapsulate the AgSis with a silica shell, 10 mg portion of Raman label treated AgSis were dispersed in 15 mL of dilute sodium silicate aqueous solution (0.036 wt% SiO_2). The dispersion was stirred with a magnetic stir bar for 15 h at 25°C. Ethanol (60 mL) was added to the reaction mixture while mixing vigorously with a magnetic stirring bar, and then, the dispersion was stirred for an additional 3 h to form a thin silica shell. Finally, 250 µL of aqueous ammonium hydroxide (28-30%) and 30 µL of TEOS were added to the reaction mixture, and it was stirred for 24 h at 25°C. The resulting surface-enhanced Raman scattering (SERS) nanoprobes (SERS dots) were centrifuged and washed with ethanol several times and 2-propanol 1 times, finally dispersed in 20 mL of 2-propanol.

Preparation of fluorescent SERS dots (F-SERS dots)

Synthesis of Fluorescence-SERS dots (F-SERS dot) were followed by our previous developed method with modifying slightly.[4] In briefly, synthesized SERS-dots were encapsulated by the outer silica layer which contained a fluorescence dye. Firstly, a 50 µL of the APTES (19.2 mM in ethanol) and 5 µL of Rhodamine B isothiocyanate (RITC, 8 mM in DMSO) were mixed to allow the conjugation of between the two molecules. The resulting solution was stirred for 8 h at 25°C. A 10 mg of SERS dots was dispersed in 24 mL of a solution containing 20 mL of 2-propanol and 4 mL of D.I. water. Then, 55 µL of the RITC-APTES conjugated ethanol solution was added to this dispersion. Next, 10 µL of TEOS and 600 µL of aqueous ammonium hydroxide (28-30%) was added to this reaction mixtures. The mixture was stirred for 15 h at 25°C. The resulting F-SERS dots were centrifuged and washed with ethanol several times for purification.

Supplementary Information References:

1. B.-H. Jun, D. W. Hwang, H. S. Jung, J. Jang, H. Kim, H. Kang, T. Kang, S. Kyeong, H. Lee, D. H. Jeong, K. W. Kang, H. Youn, D. S. Lee and Y.-S. Lee, *Adv. Funct. Mater.*, 2012, **22**, 1843-1849.

2. W. Stöber, A. Fink and E. Bohn, *J. Colloid Interface Sci.*, 1968, **26**, 62-69

3. H. Kang, S. Jeong, Y. Koh, M. Geun Cha, J.-K. Yang, S. Kyeong, J. Kim, S.-Y. Kwak, H.-J. Chang, H. Lee, C. Jeong, J.-H. Kim, B.-H. Jun, Y.-K. Kim, D. Hong Jeong and Y.-S. Lee, *Sci. Rep.*, 2015, **5**.

4. S. Jeong, Y. I. Kim, H. Kang, G. Kim, M. G. Cha, H. Chang, K. O. Jung, Y. H. Kim, B. H. Jun, W. Hwang do, Y. S. Lee, H. Youn, Y. S. Lee, K. W. Kang, D. S. Lee and D. H. Jeong, *Sci. Rep.*, 2015, **5**, 9455.

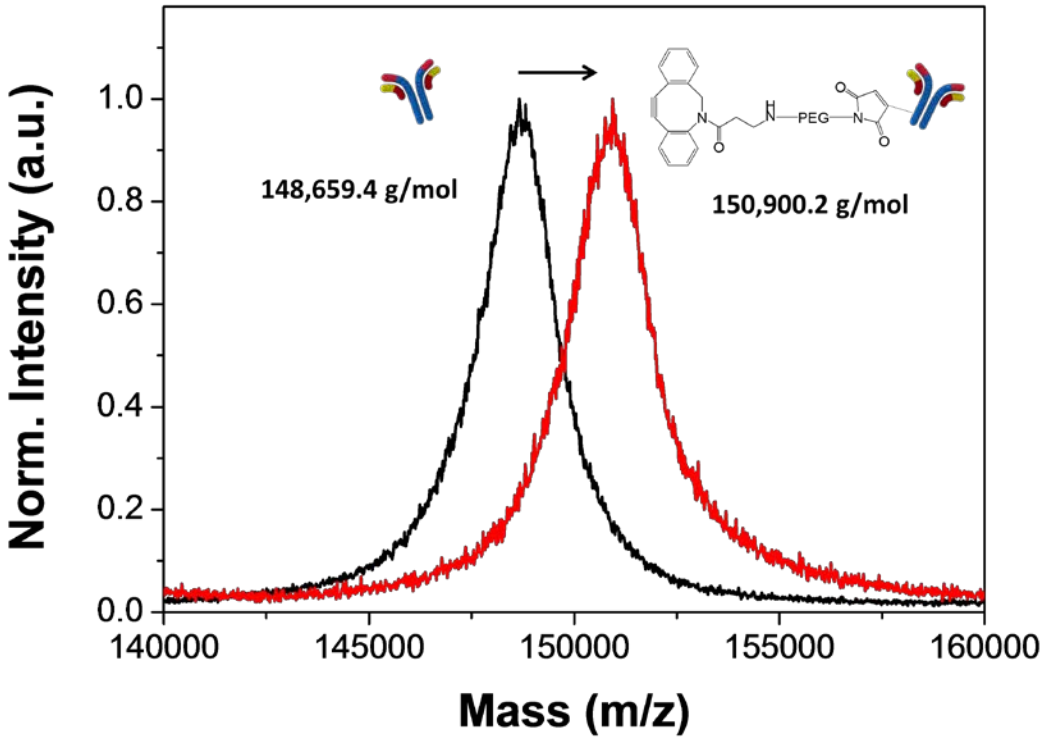

Fig. S1 MALDI-TOF analysis of antibody alone and ADIBO conjugated antibody.

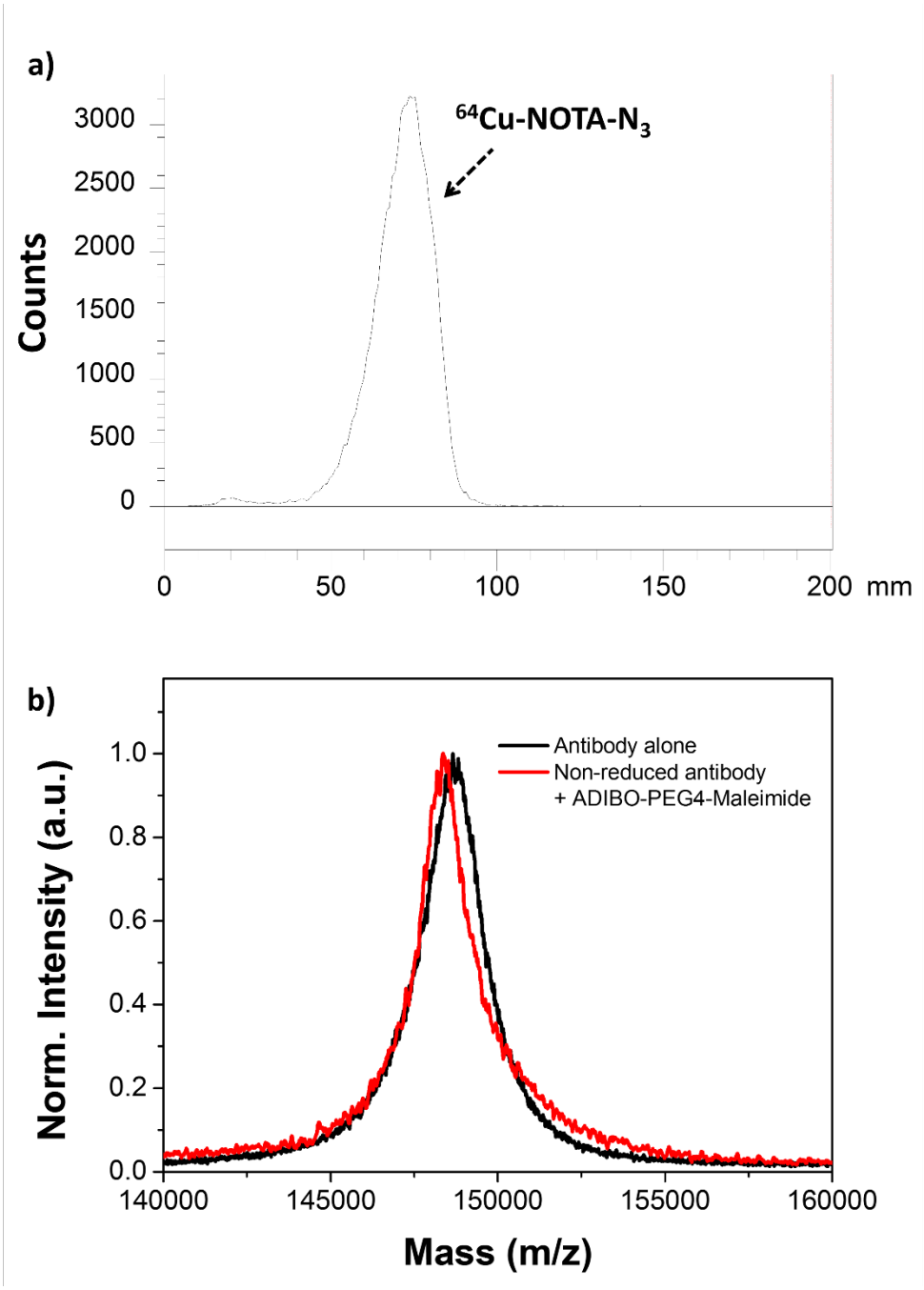

Fig. S2 Radio thin-layer chromatography (TLC) analysis (a) and MALDI-TOF analysis (b) of non-reduced antibody reacted with ADIBO-PEG4-maleimide to evaluate conjugation of maleimide and free thiol groups of native antibodies.

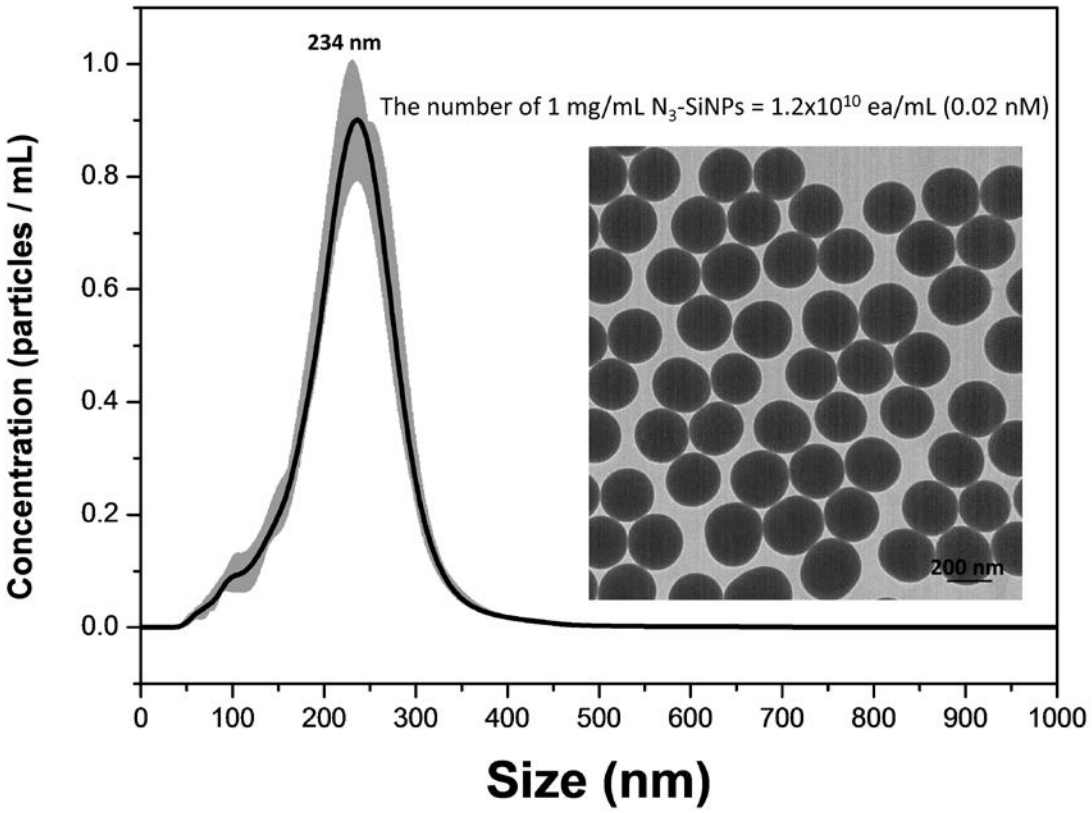

Fig. S3 Characterization of silica nanoparticles (SiNPs) by nanoparticle tracking analysis (NTA, NanoSight Ltd) and transmission electron microscopy (TEM).

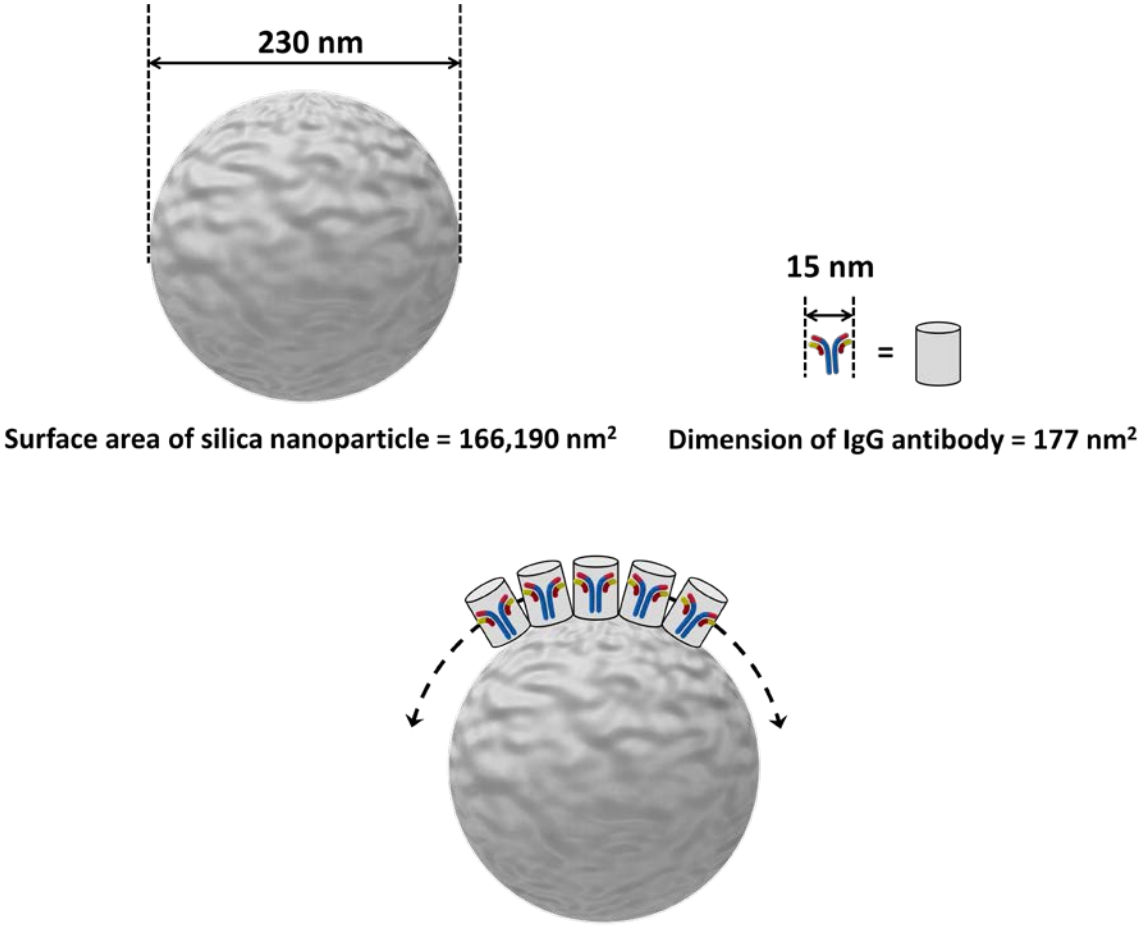

Fig. S4 Theoretical calculation of the maximum number of antibody conjugated with the surface of a single SiNP according to dimension of antibody and surface area of a single SiNP having 230 nm diameter.

Table 1. Coupling efficiency of click chemistry based antibody conjugation method at various conditions of ADIBO-fluorescent antibody.

ADIBO-FL antibody (nM)	Conjugated FL-antibody (nM)	The number of FL antibody on a single SiNP	Coupling efficiency
2	0.99	25	0.50
10	5.40	135	0.54
20	14.53	363	0.73
60	18.22	455	0.30

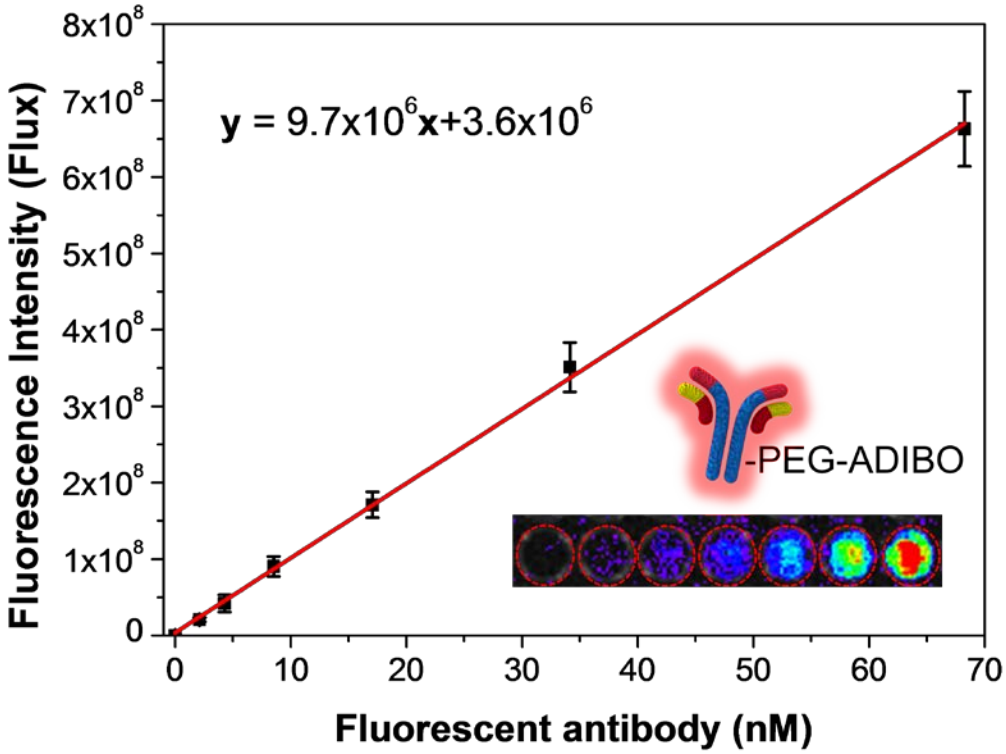

Fig. S5 Standard curve according to the concentration of ADIBO conjugated fluorescent antibody. The inset image was obtained by IVIS with 2 s-acquisition time.

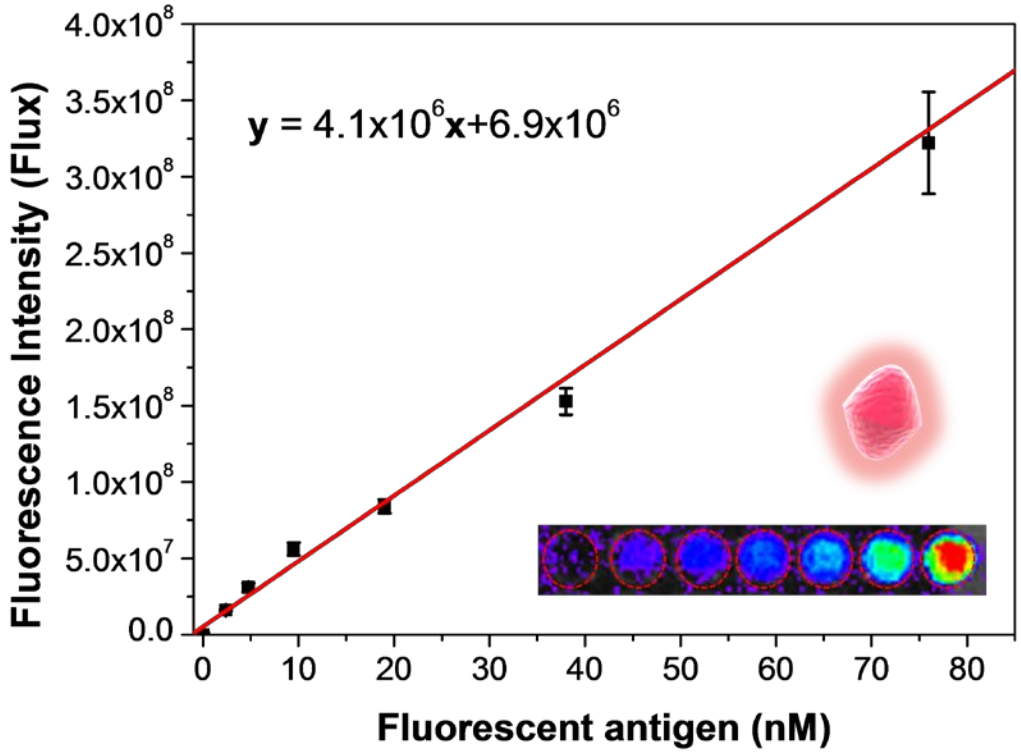

Fig. S6 Standard curve according to the concentration of fluorescent HER2 antigen labeled by FNR-648. The inset image was obtained by IVIS with 2 s-acquisition time.

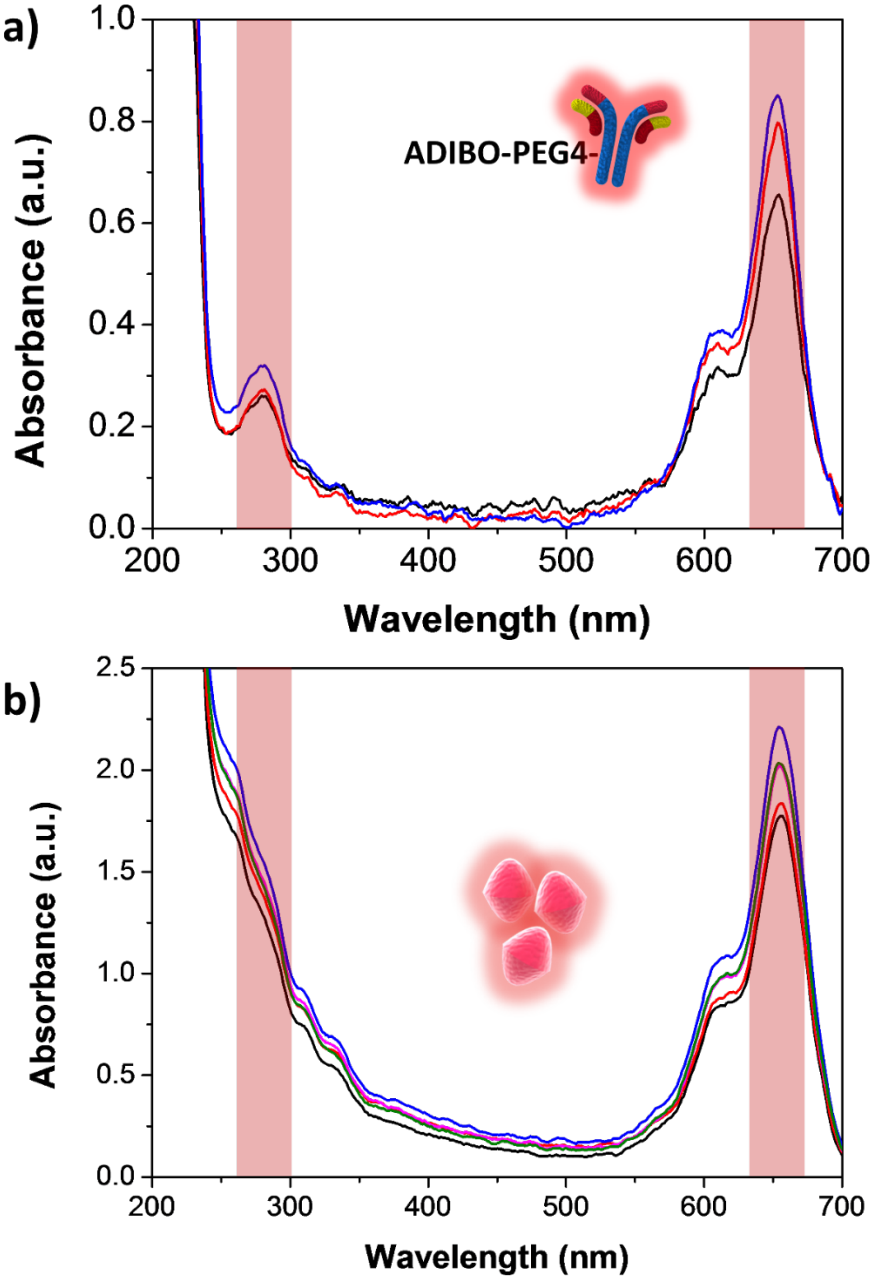

Fig. S7 Determination of ADIBO conjugated fluorescent antibody and fluorescence labeled antigen by Nanodrop. The biomolecules were quantified by measuring the absorption at 280 nm corresponding to amino acids with aromatic rings. The absorption at 648 nm is correlated with conjugated fluorescence dye (FNR-648, λ_{abs} = 648)

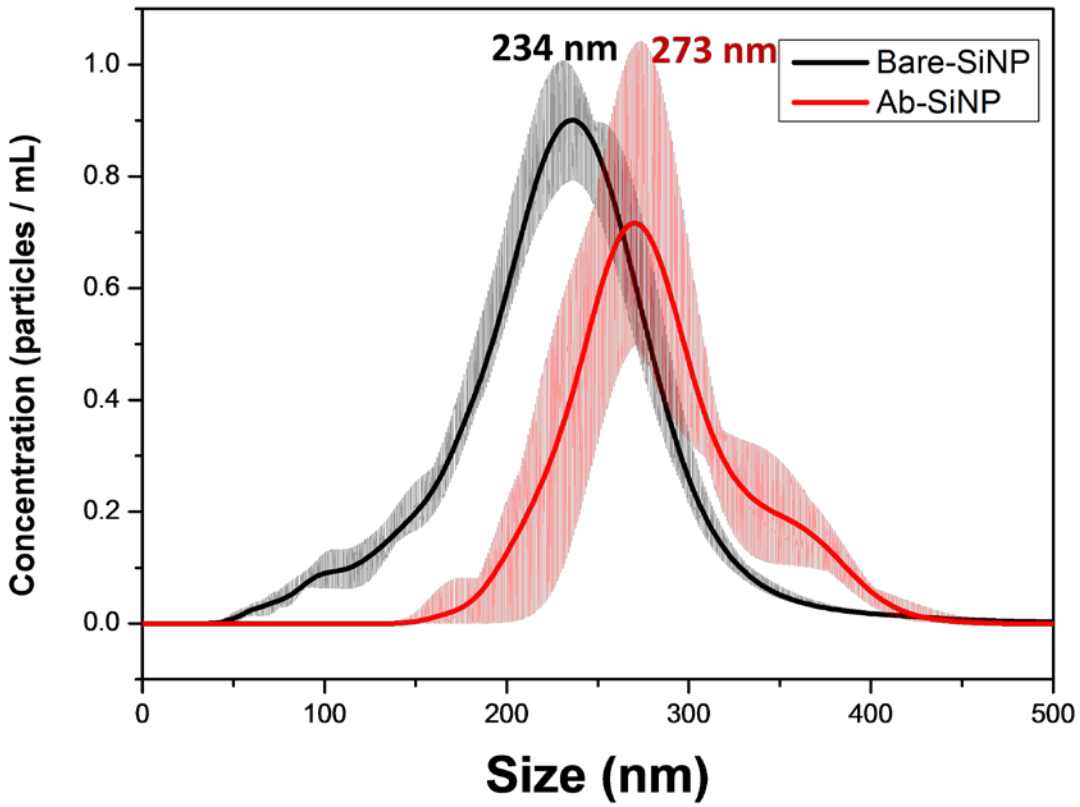

Fig. S8 Characterization of silica nanoparticles (SiNPs) and anti-HER2 antibody conjugated SiNPs by nanoparticle tracking analysis (NTA, NanoSight Ltd).

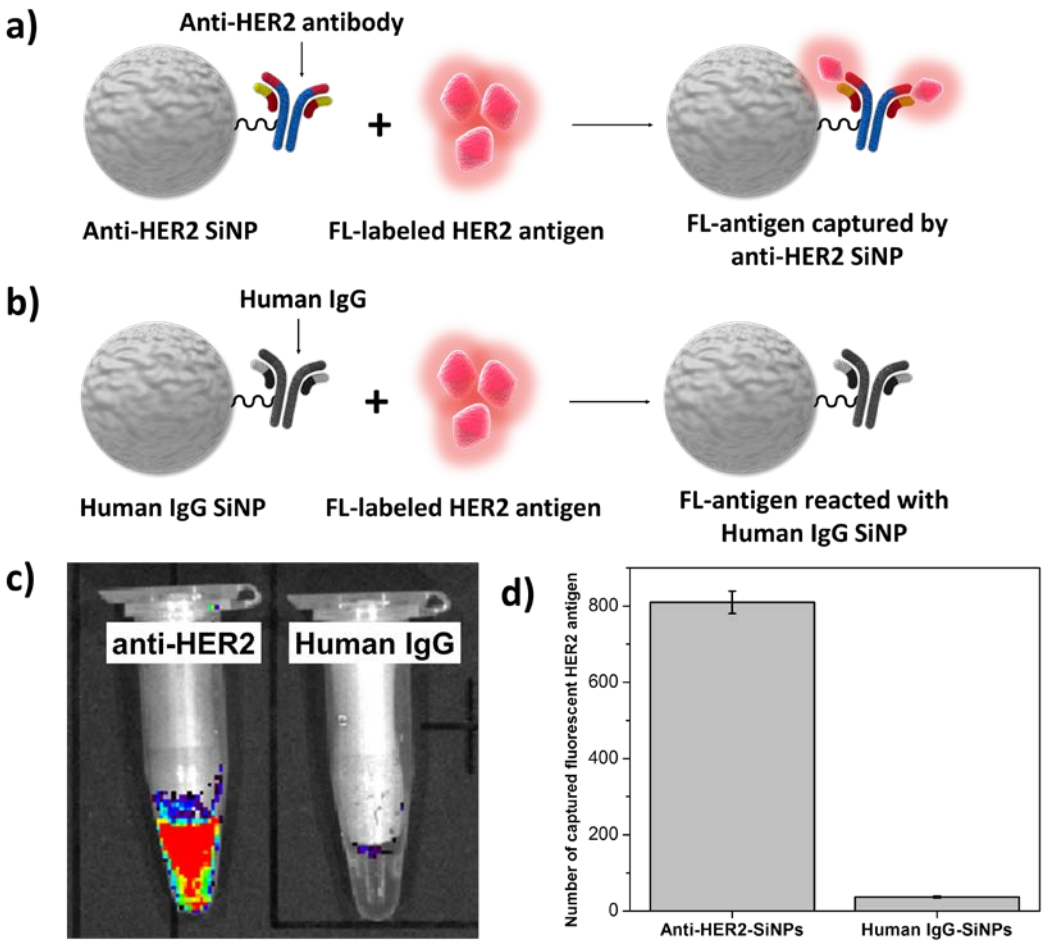

Fig. S9 Quantitative evaluation of the antigen-binding ability of (a) anti-HER2 antibody and (b) Human IgG conjugated SiNP prepared by the click chemistry. (c) Fluorescence image and (d) quantitative analysis of the number of antigens bound to a single SiNP using the fluorescence signal. The fluorescence images were obtained using an IVIS with 2 s-acquisition time.

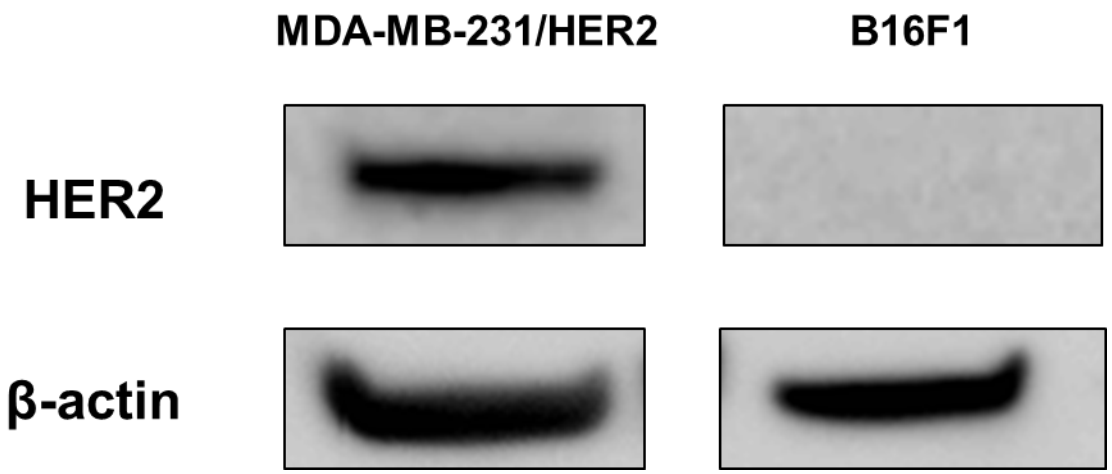

Fig. S10 Western blot analysis for evaluating expression of HER2 biotarget in the breast cancer cell-line (MDA-MB231/HER2) and the melanoma cell-line (B16F1).

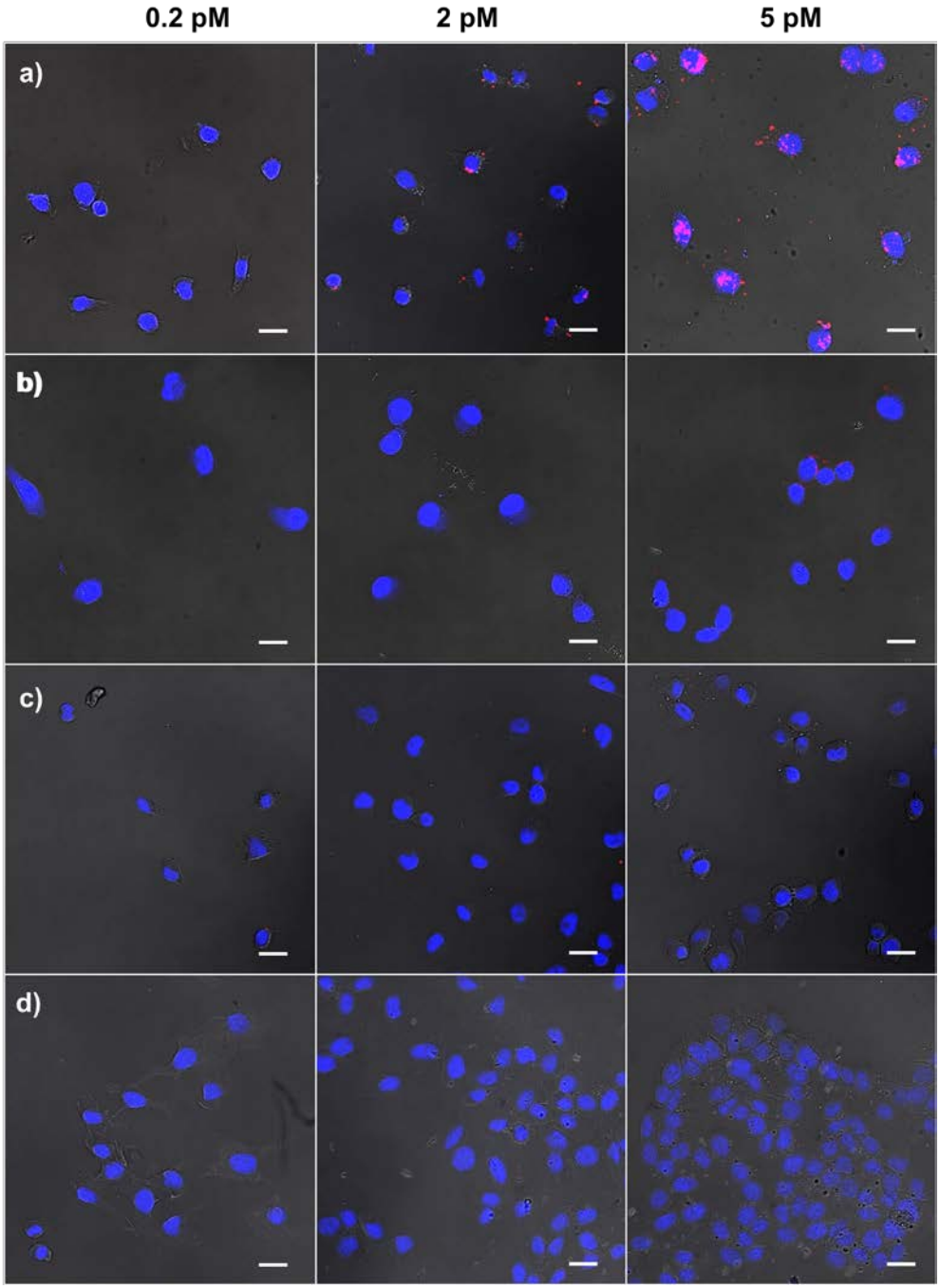

Fig. S11 *In vitro* immunoassay using breast cancer cells (MDA-MB-231/HER2) for comparison of the targeting ability of antibodies on fluorescent NPs (anti-HER2-QD^2) conjugated by click chemistry or EDC/NHC coupling. Confocal laser scanning microscopy images of cancer cells targeted by anti-HER2-QD^2 conjugated by the click chemistry (a) or the EDC/NHS coupling (b); as a negative control, a free anti-HER2 antibody was pre-treated prior to anti-HER2-QD^2_{click} treatment (c), and the HER2 negative melanoma cells (B16F1) were also targeted by the anti-HER2-QD^2_{click} (d). The scale bar represents 20 µm. The anti-HER2-QD^2 are represented by a red color, and the nuclei are stained by DAPI, indicated by a blue color.

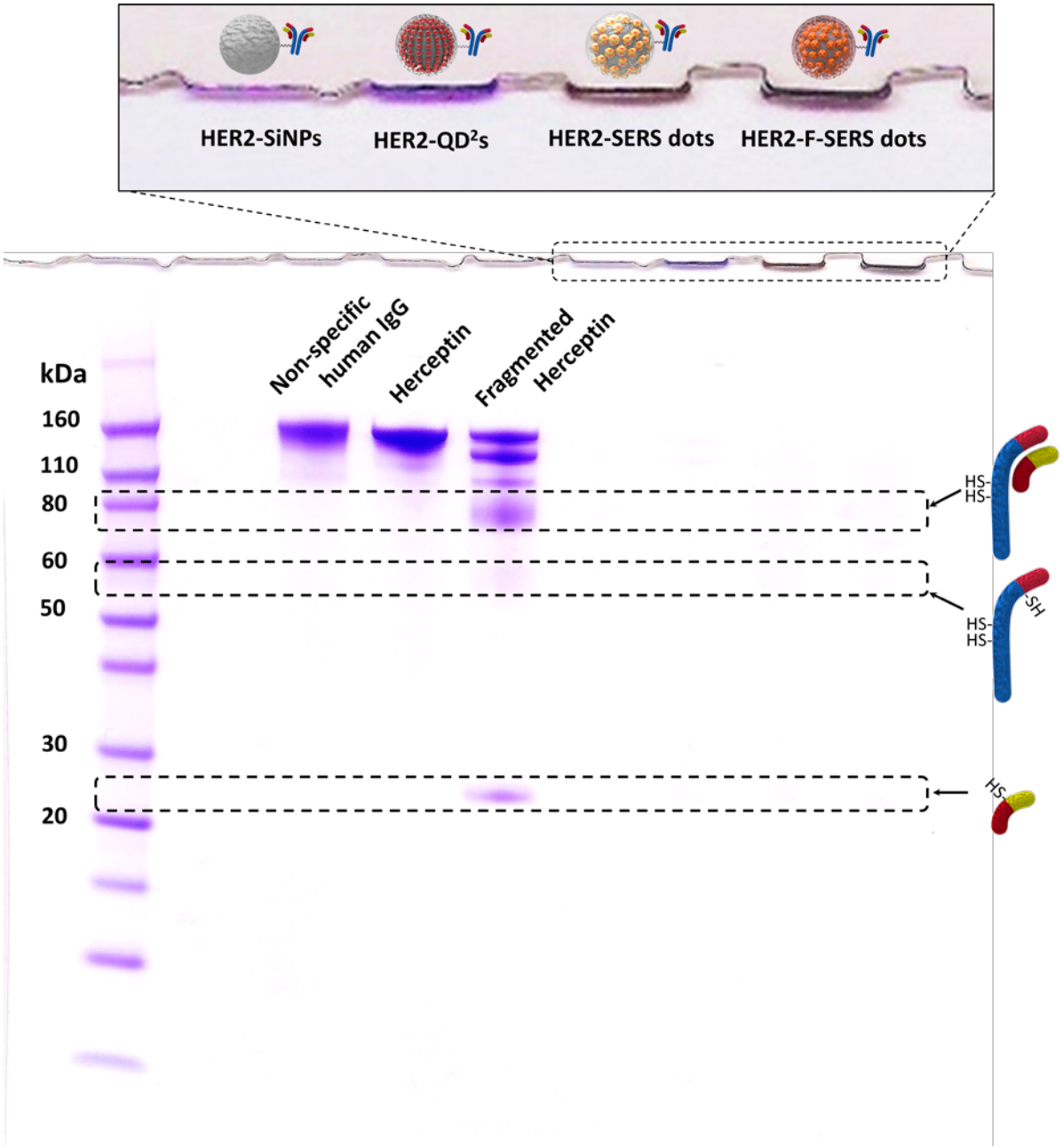

Fig. S12 SDS-PAGE analysis of various antibody conjugated NPs: HER2-SiNPs, HER2-QD^2, HER2-SERS dots, and HER2-F-SERS dots. All azido-NPs were treated with same concentration of ADIBO conjugated antibody, 1 mg/mL.

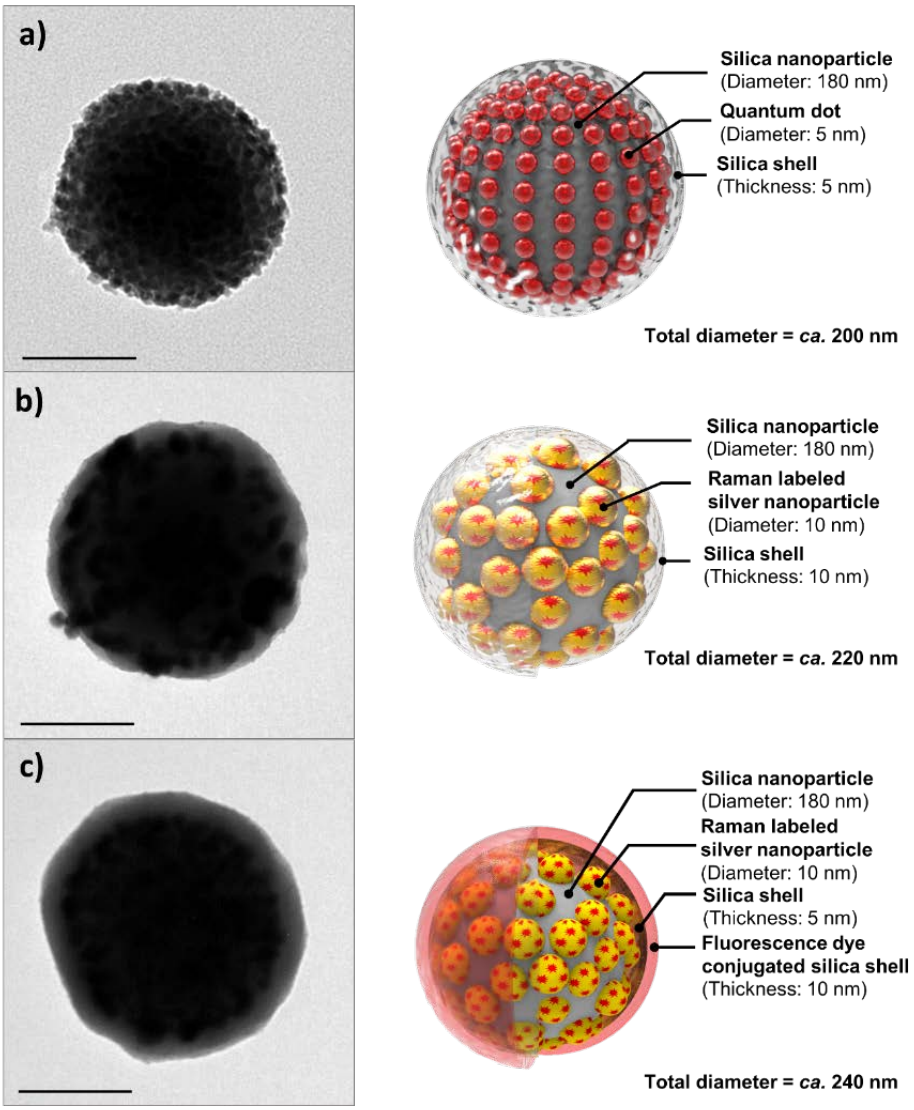

Fig. S13 Transmission electronic microscope (TEM) images and schematic illustration of the various kinds of silica-encapsulated nanoprobes: (a) quantum dot embedded silica nanoparticles (QD^2), (b) surface-enhanced Raman scattering (SERS) nanoprobes (SERS dots), and (c) fluorescence-SERS dual modal nanoprobes (F-SERS dots). The scale bar in the TEM image is 100 nm.