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Supporting Information

Controlled Biohybrid Nanoprobes with Silver Nanoparticle Clusters for Highly Sensitive Raman Imaging

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conjugated and Ag NC-embedded biohybrid nanoprobes labeled with quantum dot (QD)-conjugated anti-mouse immunoglobulin G (IgG) as a secondary Ab for anti-HER2 mAb. It clearly shows that anti-HER2 mAbs were highly conjugated on the Ag NC-embedded polymer NPs as compared to the control without anti-HER2 mAb conjugates. (A, D) bright-field images, (B, E) fluorescence images and (C, F) merged images of both bright-field and fluorescence images.

8. **Figure S5.** Optically sectioned fluorescence images of QD-labeled SKBR3 cells obtained by CLSM. Red QD-labeled fluorescence images of the SKBR3 cells were measured for comparison with their Raman images. The serial optical sections were taken using 3 μm optical slices.

Materials

Silver nitrate, sodium citrate tribasic dehydrate, sodium borohydride, rhodamine B isothiocyanate (RBITC), RBITC-conjugated dextran (MW 70 kDa), ethylene glycol, sulfo-*N*-hydroxysuccinimide ester (sulfo-NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 4-(4-maleimidophenyl)butyric acid N-succinimide ester, and ethanolamine were purchased from Sigma Aldrich. Poly(acrylamide-co acrylic acid), poly(AAm-co-AA) (MW 200 kDa, 10% acrylic acids) was purchased from Polysciences, US. The human cancer cell line SKBR3 was obtained from the Korean Cell Line Bank. Anti-HER2 mAbs (MAB1129) and anti-human IgG mAbs specific for Fc of anti-HER2 mAbs (I6135) were purchased from R&D Systems, US and Sigma Aldrich. Quantum dot 655 non-targeted QDs with an emission maxima at 655 nm (Qdot® 655 ITK™ carboxyl quantum dots, Q21321MP) and Quantum dot 565 targeted anti-mouse IgG QDs with an emission maxima at 565 nm (Qdot® 565 donkey anti-mouse IgG conjugate, Q22076) were obtained from Invitrogen, US. Ultrapure deionized (DI) water (18.2 MΩ·cm at 25°C) was obtained from a Millipore system and used in all experiments.

Preparation of silver nanoparticles and silver nanoparticle clusters

Silver nanoparticles (Ag NPs) as seeds and silver nanoparticle clusters (Ag NCs) were prepared using a previously reported method with modification.[1, 2] Aqueous solutions of silver nitrate, sodium borohydride, and sodium citrate tribasic dehydrate, all at 0.5 M, were prepared. Two separate solutions were prepared using these aqueous solutions. Solution A with a concentration of 0.6 mM sodium borohydride, 8.0 mM of sodium citrate, and 2.0 mM of sodium hydroxide was added at an equal volume ratio to Solution B with a concentration of 4.0 mM silver nitrate. The reaction was allowed to proceed for ten hours in dark conditions. The colloidal solution of Ag NPs was filtered through a hydrophobic PTFE

Dismic 13Jp syringe filter (ADVANTEC, Japan) with a pore size of 0.2 μm and was stored under dark conditions. To prepare Ag NCs, the colloidal solution of Ag NPs was homogeneously mixed with a 10 mM solution of rhodamine B isothiocyanate (RBITC) as a Raman dye, 0.5 M silver nitrate, and 0.5 M sodium citrate up to a final concentration of 7.5 μM RBITC, 0.5 mM silver nitrate, and 0.25 mM sodium citrate. Importantly, the final concentration of RBITC was optimized at 7.5 μM to prevent severe aggregation of the Ag NPs. The mixture was then stirred thoroughly for two minutes at room temperature and incubated at 95°C for a pre-determined time to prepare silver nanoparticle clusters (Ag NCs). Small aliquots were withdrawn during formation of the Ag NCs with RBITC at regular time intervals, diluted five-fold with 1.0 mM of sodium citrate, and examined via UV-Vis absorption, dynamic light scattering (DLS), and Raman spectroscopy. The reaction was stopped by cooling the mixture to room temperature when maximum Raman intensity and optimal Ag NC size were reached. Finally, the solution of Ag NCs was mixed with 0.5% w/v of bovine serum albumin (BSA) at a final concentration of 0.00125% w/v to stabilize the Ag NCs and prevent any further aggregation. Finally, the Ag NCs were filtered through a hydrophobic PTFE Dismic 13Jp syringe filter with a pore size of 0.2 μm.

Characterization of Ag NCs by UV-Vis absorption, DLS, and Raman scattering

All UV-Vis absorption measurements were taken in the range of 300 to 850 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) in single scan mode with a medium scan speed at room temperature. The colloidal solution of Ag NCs was examined for NC size and distribution using a Zetasizer Nano ZS90 (Malvern instruments, UK). A He-Ne laser at 633 nm with a maximum power of 5 mW was used as a light source. Temperature was controlled at 25 ± 1°C throughout all DLS experiments. Each sample was examined for at least 12 scan cycles, and the average size of the Ag NCs was determined. Finally, Raman

measurements were obtained using a Raman spectroscope (Renishaw 2000, Renishaw, UK) with a microscope system. A He-Ne laser at 633 nm was used as an excitation source for the Raman label with a laser power of 10 mW. A holographic notch filter was used to remove the Rayleigh lines from the collected Raman spectrum. Samples were loaded into small glass capillaries (Kimble Chase, plain capillary tubes, soda lime glass**,** inner diameter: 1.1 - 1.2 mm, wall: 0.2 ± 0.02 mm, length: 75 mm). An objective lens with a magnification of 20X was used to focus inside the sample capillary. Capillary (KIMBLE CHASE) information, Plain capillary tubes, Soda Lime Glass**,** I.D. : in diameter 1.1 – 1.2 mm, Wall : 0.2 ± 0.02 mm, Length : 75 mm

Encapsulation of Ag NCs within polymeric nanoparticles by EHD jetting

The colloidal solution of Ag NCs was centrifuged at 5,000 rpm for ten minutes and washed three times with 1 mM sodium citrate to remove any excess BSA or other reactants. The Ag NCs were homogeneously suspended in a 4.0% w/v poly(acrylamide-co acrylic acid), poly(AAm-co-AA) (MW 200 kDa, 10% acrylic acids, Polysciences, US) in DI water and ethylene glycol mixture at a 2:1 v/v ratio to generate solutions with concentrations of Ag NCs ranging from 0.2 to 1.2% w/v. The polymer solution having homogeneously dispersed Ag NCs was then loaded into a 1-ml syringe (Becton-Dickinson, New Jersey, US) with a 26-gauge stainless steel capillary. The cathode was connected to a high-voltage power supply NNC HV 30 (Nano NC, Korea) and attached to the steel capillary, while the counter electrode was connected to the aluminum foil collecting substrate (Fisherbrand, US). The distance between the two electrodes was varied over a range of 20-25 cm. A microsyringe pump (KDS-100, Kd Scientific, USA) was used to maintain a constant laminar flow during EHD jetting. The voltage was varied between 13 to 16 kV and the flow rate was maintained in the range of 0.05 to 0.08 ml/hr. Identical pellets of Ag NCs after centrifugation were freeze-

dried using a lyophilizer MCFD8508 (Ilshin Lab, Korea) under vacuum and weighed. All jetting experiments were carried out at ambient conditions. The Ag NC-embedded polymeric NPs situated on the collecting substrate were incubated at 175°C for 80 minutes to ensure thermal cross-linking of the polymer chains[3]. The Ag NC-embedded polymeric NPs after thermal stabilization were harvested from the substrate and dispersed in DI water, followed by sonication for 45 seconds using a tip sonicator (VC 505, Vibra-cell Sonics and Materials, USA) prior to their characterization.

Characterization of the Ag NC-embedded polymeric NPs

Scanning electron microscopy (SEM) and field emission (FE) scanning transmission electron microscopy (STEM) were used to characterize the Ag NC-embedded polymeric NPs with respect to size and size distribution as a function of the densities of the Ag NCs within the polymeric NPs. These polymeric NPs were coated with platinum using a K575X Turbo Sputter Coater (EMITECH, UK) and imaged using a VEGA-SB3 (TESCAN, USA) SEM operated at an accelerating voltage of 0.5 to 30 kV. The Ag NCs within the polymeric NPs were examined by generating an EDX point-spectrum. The Ag NC-embedded polymeric NPs were deposited on 400-mesh copper grids with an ultrathin carbon coating (Ted Pella, Inc. USA) and imaged using a JEM-2100F FE-STEM (JEOL, Germany) operated at an accelerating voltage of 80 to 200 kV. An energy-dispersive X-ray spectroscopic (EDS) spectrum of the Ag NCs was also collected using the same FE-STEM for elemental analysis.

Conjugation of the Ag NC-embedded polymer NPs with anti-HER2 mAbs

The polymeric NPs with the Ag NCs were chemically conjugated with a monoclonal antibody (mAb) against the HER2 receptor as a cancer marker of SKBR3 cells. Conjugation was achieved using the residual -COOH groups of the polymer chains after

thermal stabilization, as previously reported.[3, 4] The HER2 receptor is a membrane glycoprotein and a member of the ErbB family of tyrosine kinase receptors. The HER2 receptor is unique because there are no identified ligands for this protein among the ErbB family members. Briefly, 1.25 mg of Ag NC-embedded polymer NPs suspended in 2.0 ml of PBS buffer was sonicated for one minute using a tip sonicator at an amplitude of 30.0 %. These polymeric NPs were filtered through 30 µm cell strainers (BD Falcons, US) to remove any larger aggregates and contaminants. The homogenously dispersed polymeric NPs were mixed with 35 µl of 0.03 M 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and 35 µl of 0.025 M sulfo-*N*-hydroxysuccinimide (sulfo-NHS) in PBS buffer at pH 7.4 to activate residual -COOH groups on the polymer chains. The 0.5 mg/ml solution of anti-HER2 mAbs was diluted in 100 µl of PBS buffer and added to the activated polymeric NPs dropwise to a final concentration of 2.0 nM anti-HER2 mAbs, followed by continuous stirring for two hours. Finally, Ab-conjugated and Ag NC-embedded biohybrid nanoprobes were centrifuged at 3,000 rpm and washed with PBS buffer three times. Furthermore, to ensure anti-HER2 mAb conjugation, Anti-HER2 mAbs were covalently bonded to these SERS nanoprobes using carbodiimide coupling reaction as mentioned above, and then anti-HER2 mAb-conjugated and Ag NC-embedded biohybrid nanoprobes were labeled with quantum dot (QD)-conjugated anti-mouse immunoglobulin G (IgG) as a secondary Ab for anti-HER2 mAb to have a final concentration of 1.0 nM QD-conjugated anti-mouse IgG, followed by continuous stirring for 30 minutes, centrifugation at 5000 rpm, washing with PBS three times. Their fluorescence images were obtained using confocal laser scanning microscopy (CLSM, Leica TCS SP, Leica, Germany) with a 100x objective lens.

Conjugation of quantum dots with anti-HER2 mAbs

Anti-HER2 mAb-conjugated quantum dots (QDs) with an emission maxima at 655 nm

were prepared as a control experiment. Anti-HER2 mAbs at a concentration of 0.1 mg/ml were chemically conjugated to the surface of activated QDs using 0.1 mM EDC and 0.1 mM 4-(4-maleimidophenyl) butyric acid N-succinimide ester. Finally, the activated succinimidyl groups of the QDs were deactivated by 0.1 mM ethanolamine for three hours and removed by ultrafiltration using an Amicon Ultra-4 with a molecular weight cut-off of 100 kDa.

Raman imaging of cancer cells

HER2-overexpressing SKBR3 human cancer cells were used for Raman imaging. SKBR3 cells seeded on cover glasses were grown without doxycycline for two days, followed by fixation with 3.7% paraformaldehyde for 15 min. After washing with PBS buffer three times, the SKBR3 cells were incubated with the Ab-conjugated and Ag NC-embedded biohybrid nanoprobes for two hours at room temperature. Non-specifically bound nanoprobes were removed by washing with PBS buffer three times. Finally, Raman mapping images were obtained using a Renishaw 2000 Raman microscope system as previously reported.[5, 6] A He-Ne laser operated at 633 nm was used as the excitation source with a power of 15 mW. All Raman spectra were obtained with a 10 sec exposure time and the Rayleigh line was removed from the collected Raman scattering via a holographic edge filter in the collection path. A charge-coupled device camera was coupled to a spectrograph, which provided 2 cm^{-1} spectral resolution. Raman images were obtained using a Raman point-mapping method with a 50x objective lens, and the exposure time was 1 sec for each pixel. The Raman image of the cells was decoded using the intensity of the 1,645 cm^{-1} Raman peak.

Confocal laser scanning microscopy (CLSM) imaging

Fluorescence images of QD-labeled SKBR3 cells were collected using CLSM with a 100x

objective lens. Cells were viewed using a laser with a 488 nm wavelength, and the emitted fluorescence light was detected between 630 and 670 nm for the red QDs. Red QD-labeled fluorescence images of the cells were compared using their Raman images. Serial optical sections were taken using 3 μm optical slices for the optical measurement of the cells in three dimensions. In addition, Ag NC-embedded polymeric NPs in dry and swollen states were imaged directly on a cover glass using a CLSM Leica TCS SP with a 100x objective lens. When the Ag NC-embedded polymeric NPs were prepared by EHD jetting, RBITC-conjugated dextran with a molecular weight of 70 kDa was introduced into the polymer solution at a final concentration of 0.5 w/v% because the RBITC-conjugated dextrans were chemically crosslinked with the poly(AAm-co-AA) chains during thermal crosslinking and entrapped within the the Ag NC-embedded polymeric NPs for CLSM imaging.

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Table S1. (A) Zeta potential (ζ) of (a) Ag NPs, (b) RBITC-coated Ag NPs, (c) Ag NCs with RBITC, and (d) BSA-stabilized Ag NCs with RBITC by using three different batches (N = 3). The zeta potential value of Ag NPs became less negative when positively charged RBITC was adsorbed.

	Zeta potential, ζ (mV)	Δ ζ (mV)
(a) Ag NPs	-44.4	NA
(b) RBITC-coated Ag NPs	-33.7	10.7
(c) Ag NCs with RBITC	-44.8	-11.1
(d) BSA-stabilized Ag NCs with RBITC	-30.0	14.8

Table S2. Surface ζ potential values of (a) Ag NC-embedded polymeric NPs and (b) anti-HER2 mAb-conjugated and Ag NC-embedded biohybrid nanoprobes (N=3).

	Zeta potential, ζ (mV)	Δ ζ (mV)
(a) Ag NPs embedded polymeric NPs	-30.2	NA
(b) Anti-HER2 mAb-conjugated and Ag NC-embedded biohybrid nanoprobes	-18.3	11.9

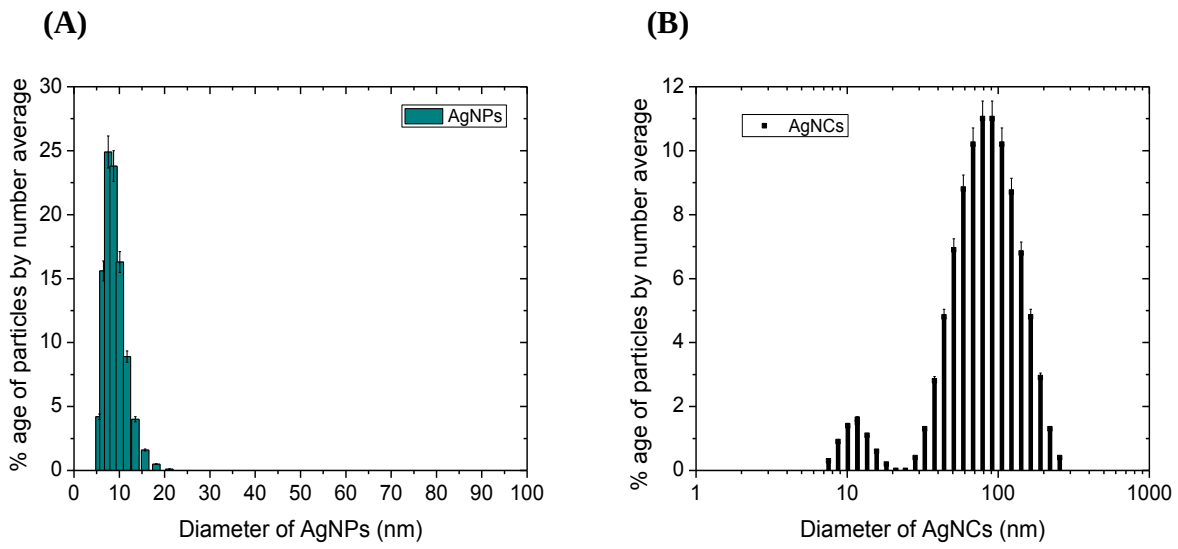

Figure S1. Diameter and size distribution of (A) discrete Ag NPs and (B) Ag NCs, determined by dynamic light scattering (DLS).

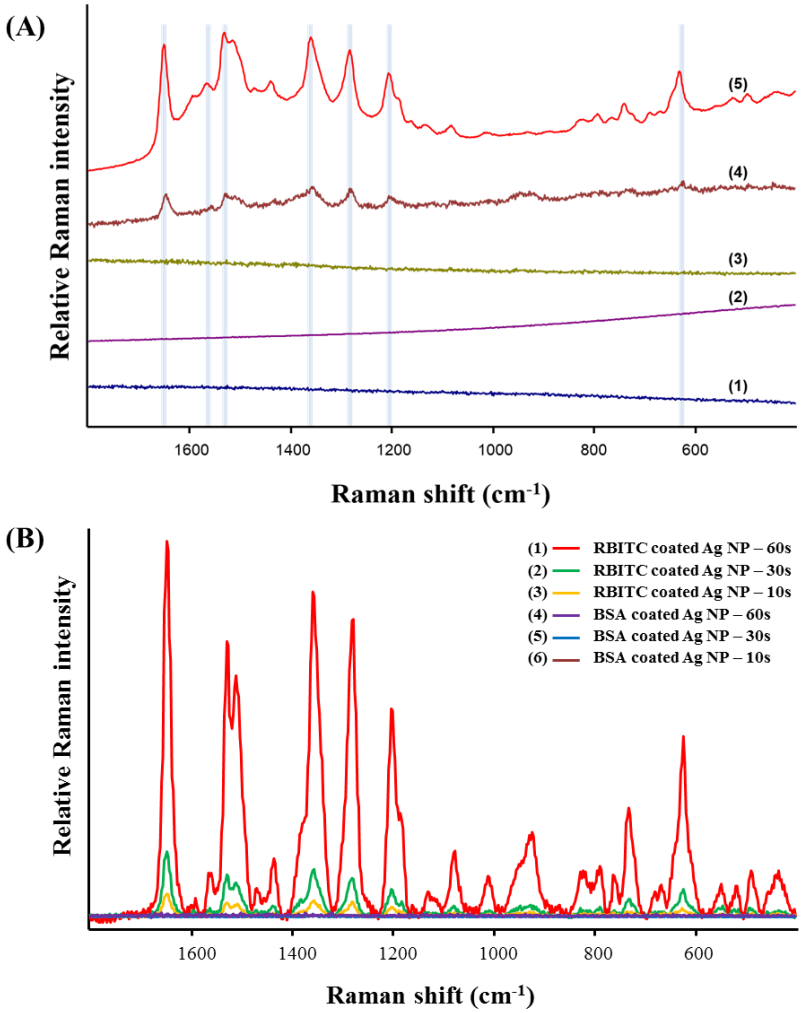

Figure S2. (A) Raman spectra of (1) BSA (powder), (2) RBITC (powder), (3) BSA coated Ag NPs, (4) RBITC coated Ag NPs, and (5) Ag NC-embedded polymeric NPs with RBITC, showing that absolute Raman intensity at 1646 cm^{-1} arises exclusively from RBITC reporter dye under these conditions because there is no detectable signal of the 1646 cm^{-1} band of BSA. (B) Raman spectra of (1-3) RBITC coated Ag NPs and (4-6) BSA coated Ag NPs at different exposure times ((1, 4) 60 sec, (2, 5) 30 sec and (3, 6) 10 sec) using 632.8 nm laser wave length.

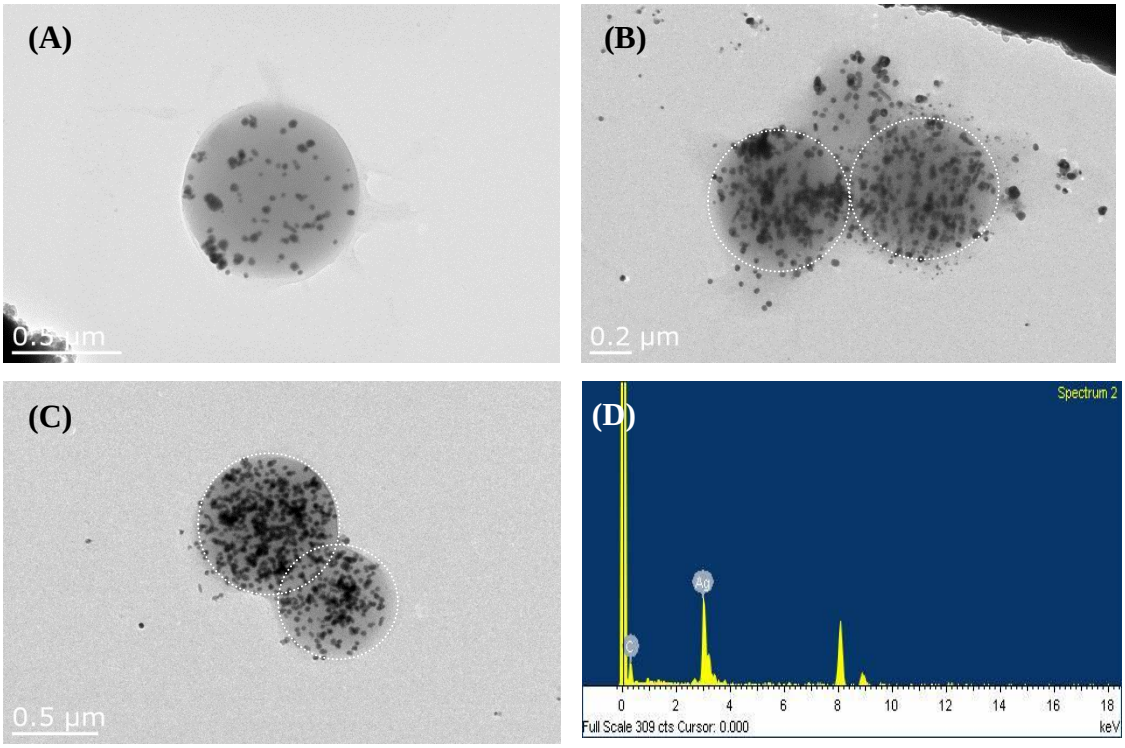

Figure S3. TEM images of Ag NC-embedded polymer NPs at Ag NC concentration of (A) 0.2 w/v%, (B) 0.6 w/v% and (C) 0.8 w/v% in the jetting solution. (D) Energy dispersive x-ray spectrum (EDS) of Ag NCs, indicating the presence of Ag.

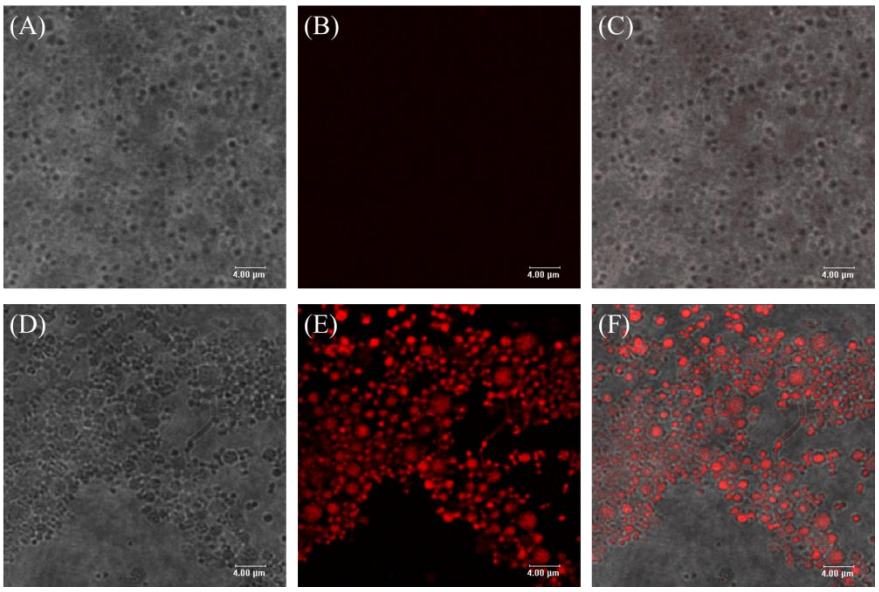

Figure S4. Confocal laser scanning microscopy images (CLSM) of (A-C) Ag NC-embedded polymeric NPs when physically mixed with quantum dot (QD)-conjugated anti-mouse immunoglobulin G (IgG). The CLSM images of (D-F) anti-HER2 mAb-conjugated and Ag NC-embedded biohybrid nanoprobes labeled with quantum dot (QD)-conjugated anti-mouse immunoglobulin G (IgG) as a secondary Ab for anti-HER2 mAb. It clearly shows that anti-HER2 mAbs were highly conjugated on the Ag NC-embedded polymer NPs as compared to the control without anti-HER2 mAb conjugates. (A, D) bright-field images, (B, E) fluorescence images and (C, F) merged images of both bright-field and fluorescence images.

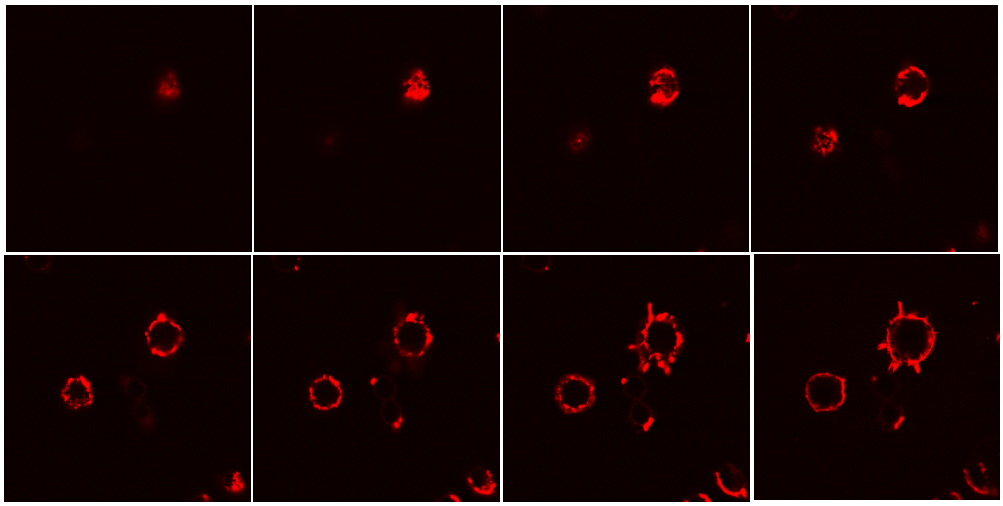

Figure S5. Optically sectioned fluorescence images of QD-labeled SKBR3 cells obtained by CLSM. Red QD-labeled fluorescence images of the SKBR3 cells were measured for comparison with their Raman images. The serial optical sections were taken using 3 μm optical slices.