Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Confined Microemulsion Sono-Polymerization of Poly(ethylene glycol) Nanoparticles for Targeted Delivery

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Contents of Supporting Information

EXPERIMENTAL SECTION

Materials. Acrylate-terminal methoxy poly(ethylene glycol) (ACLT-PEG_{2k}, Mw =2 kDa) and 8-arm-PEG_{20k} acrylate (8-arm-PEG-ACLT, Mw = 20 kDa) were purchased from Jenkem Technology Co. Ltd. (Beijing, China). Tween 80, Span 80 and *n*-hexane were bought from Aladdin (Shanghai, China). AEMA was obtained from J&K (Shanghai, China). Bispecific antibody was produced in mammalian cells and purified using affinity chromatography as previously reported. ICG-NHS was purchased from New Joe Biological Technology Co., Ltd. (China). DPBF and (DCFH-DA were supplied by Sigma-Aldrich (Shanghai, China). Fetal bovine serum (FBS) was obtained from Gibco (Germany). Dulbecco's modified Eagle's medium (DMEM, High Glucose) and Dulbecco's Phosphate-Buffered Saline (DPBS) were bought from Beijing Neuronbc Laborataries Co., Ltd. (Beijing, China). Cy5 succinimidyl ester (Cy5-NHS), Hoechst 33342, WGA-AF488 conjugate were obtained from Thermo Fisher Scientific (Shanghai, China). DNA Damage Assay Kit by γ-H2AX Immunofluorescence was bought from Beyotime Biotechnology (Shanghai, China). Water was obtained using Milli-Q ultrapure water apparatus (Integral 5) with a resistivity of 18.2 MΩ cm.

Characterization methods. Sono-microemulsion polymerization processes were performed on a RF generator (AG series amplifier LVG 60-10 produced by T&C Power Conversion Inc.) in combination with a 400 kHz plate transducer (Model 6G12 by Honda Electronics Co. Ltd.). The concentrations of ICG and DPBF were characterized on a Shimadzu UV-vis spectrophotometer (UV-2600, Japan). Zeta potentials and hydrodynamic diameters of NPs were measured using a Malvern Zetasizer (Nano ZS90, UK). AFM measurements were performed on Asylum Research (Cypher ES) with tapping model. CLSM measurements were carried out by using a Leica confocal microscope (TCS SP8 STED 3X, Germany). Quantitatively analysis of cellular association was performed by using an ACEA flow cytometer (NovoCyte 3009). MTT and ROS detections were performed on a plate reader (Tecan, Spark 10M).

Fabrication of PEG NPs. PEG NPs was prepared by the sono-microemulsion polymerization method. Taking the PEG NPs with the size of 25 nm as an example,

Span 80 (0.99 g) and Tween 80 (0.66 g) were dissolved in 13 mL of *n*-hexane, followed by the addition of 0.9165 mL of aqueous solution containing AEMA (3 mg), ACLT-PEG_{2k} (90 mg) and 8-arm-PEG_{20k}-ACLT (10 mg) under sonication (40 kHz, 100 W) for 5 min. The obtained inverse microemulsion was deoxygenated by bubbling N_2 for 30 min, followed by sono-polymerization (40 W) at 412 kHz and 40 °C for 2 h. After polymerization, *n*-hexane and water were removed by rotary evaporation. Subsequently, the crude product was precipitated with *n*-hexane and dialyzed with THF and water for 3 days to remove the surfactants and unreacted monomers. The conversion rate of monomers was 95%. Other samples were prepared by the same procedure via changing the ratio of water, oil or surfactants (Table S2).

Modification of ICG and BsAb. To encapsulate ICG into PEG NPs, ICG-NHS (0.1 mg) was incubated with PEG NPs (1.0 mg) in PBS (1.0 mL) for 4 h. The mixture was purified by ultrafiltration (Sartorius Stedim, Centrisart I, 300000 MWCO) to remove the unreacted ICG. The loading content of ICG was 5.8%. For the preparation of targeted PEG NPs, BsAb (20.0 μg) was incubated with PEG NPs (300 μg) at 4 °C for 12 h. The targeted PEG NPs was purified by ultrafiltration and stored at 4 °C for further use. The loading content of BsAb was 4.7%.

Photothermal and photodynamic examination. To assess the photothermal behavior, free ICG or ICG@PEG NP dispersion (equivalent ICG concentration of 10 μg mL^{-1}) was irradiated with 808 nm NIR laser (1.5 W cm^{-2}) for 300 s. The temperature was recorded using an infrared camera (type/model). *In vitro* photodynamic performance was evaluated by DPBF probe. Specifically, free ICG or ICG@PEG NP dispersion (equivalent ICG concentration of 10 μg mL^{-1}) was mixed with DPBF (10 μg mL^{-1}), followed by irradiation with NIR laser (1.5 W cm^{-2}). The absorbance of DPBF (410 nm) was detected by UV-spectrometer every 1 min.

Cell association. SKBR-3 and MCF-7 cells were selected to investigate the cell association. SKBR-3 and MCF-7 cells were seeded into 24-well plates at the density of 5×10^4 cells per well and allowed to attach for 12 h. Cy5-labeled PEG and PEG-BsAb NPs with different concentrations (10, 50, 100 μg mL^{-1}) were incubated with cells. After

12 h incubation, the cells were washed twice by DPBS and harvested by trypsinization, followed by analysis with flow cytometer. For cells imaging, SKBR-3 and MCF-7 cells were seeded in confocal dishes and cultured at 37 ºC for 12 h. Cy5-labeled PEG and PEG-BsAb NPs (100 μg mL^{-1}) were incubated with cells for 12 h, respectively. Cells were washed with DPBS and fixed with 4% paraformaldehyde for 15 min, followed by staining with Hoechst 33342 and WGA-AF488 conjugate sequentially. Fluorescence images were obtained by CLSM (Cy5 excitation at 647 nm, AF488 excitation at 495 nm, and Hoechst 33342 excitation at 358 nm).

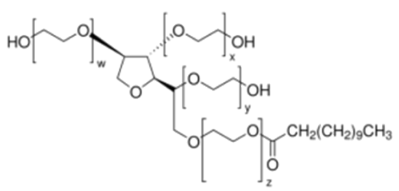
ROS detection. SKBR-3 and MCF-7 cells were seeded into 96-well plates at the density of 2×10^4 cells per well and incubated for 12 h. ICG@PEG NPs and ICG@PEG-BsAb NPs were added into cell media and incubated for 24 h, followed by washing with DPBS and incubation with ROS probe (DCFH-DA, 20 μM) for 30 min. Subsequently, cells were irradiated with 808 nm NIR laser (1.5 W cm^{-2}) for 5 min and the fluorescence intensity were detected by microplate reader after washing with DPBS two times. Cells were also imaged by using a fluorescence microscope (model).

Cell cytotoxicity. MTT assay was used to evaluate the cell cytotoxicity. SKBR-3 and MCF-7 cells were seeded into 24-well plates at a density of 1×10^4 cells per well and incubated for 12 h. Cells were cultured with different concentrations of ICG-loaded PEG NPs (equivalent ICG concentrations of 0.6, 1.3, 2.5, 5.0, 10.0, and 20.0 μg mL^{-1}, respectively). The medium was replaced with fresh medium after 24 h, and irradiated with 808 nm laser (1.5 W cm^{-2}) for 300 s. After another 24 h incubation, 10 μL of MTT stock solution (5 mg mL^{-1}) was added to each well and incubated for 4 h. The supernatant was removed and 100 μL of DMSO was added to dissolve formazan crystals, followed by characterization of the absorbance at 570 nm.

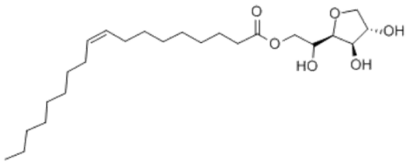
Live/dead assay. SKBR-3 and MCF-7 cells were seeded into 24-well plates at a density of 1×10^5 cells per well and cultured for 12 h. After treatment with ICG-loaded PEG NPs (equivalent ICG concentrations of 5.0 μg mL^{-1}) and NIR irradiation sequentially, Calcein-AM (2 μM) and PI (4 μM) solutions were added to stain live and dead cells, respectively. Cells were imaged by using a fluorescence microscope (Lecia, DMi8).

Cell apoptosis. SKBR-3 cells were seeded into 12-well plates at a density of 2×10^5 cells per well and cultured for 12 h. After treatment with ICG-loaded PEG NPs (equivalent ICG concentrations of 5 μg mL^{-1}) for 24 h, the medium was replaced with fresh medium and irradiated with 808 nm laser (1.5 W cm^{-2}) for 5 min. The cells were further incubated for another 24 h, followed by staining with Annexin V and PI according to manufacturer's instructions for flow cytometry.

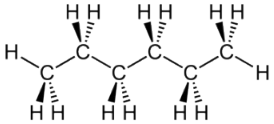
DNA damage. SKBR-3 cells were seeded in confocal dishes at a density of 1×10^5 cells per well and cultured at 37 ºC for 12 h. ICG@PEG-BsAB NPs (equivalent ICG concentrations of 5 μg mL^{-1}) were incubated with cells for 24 h. The cell medium was replaced with fresh medium and irradiated with 808 nm laser (1.5 W cm^{-2}) for 5 min. The cells were further incubated for another 24 h, followed by labelling with primary and secondary antibodies according to manufacturer's instructions for CLSM imaging.



Tween 80 HLB: 15



Span 80 HLB: 4.3



Hexane HLB: 8.7

Fig. S1 Structures and HLB values of Tween 80, Span 80 and hexane.

Table S1 Theoretical HLB values of Tween 80 and Span 80 mixtures with different ratios.

	1	**2**	**3**	**4**	**5**	**6**	**7**
Span 80/%	45	50	55	60	65	70	75
Tween 80/%	55	50	45	40	35	30	25
HLB	10.18	9.65	9.12	8.58	8.05	7.51	6.98

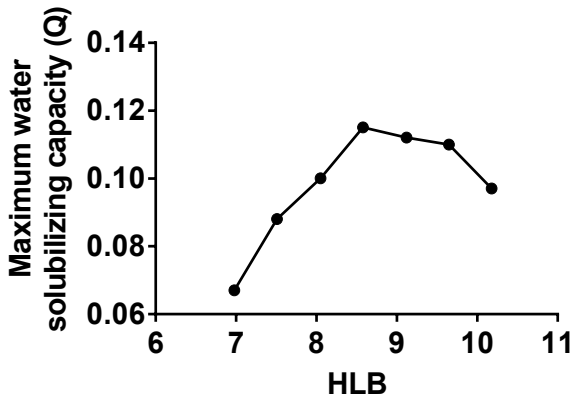


Fig. S2 Maximum water solubilizing capacity of microemulsion system at different HLB.

Table S2 Conditions for the preparation of PEG NPs with different sizes.

W_{oil} : $W_{surfactants}$	W_{water}	Size	PDI
4:1	2%	420 nm	0.283±0.041
4:1	4%	270 nm	0.176±0.011
9:1	1%	120 nm	0.157±0.011
4:1	10%	25 nm	0.187±0.017

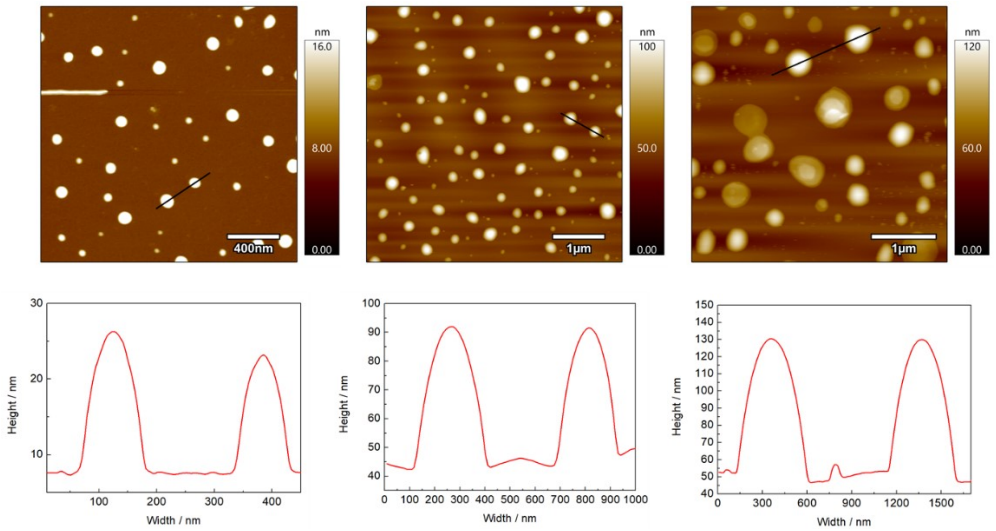
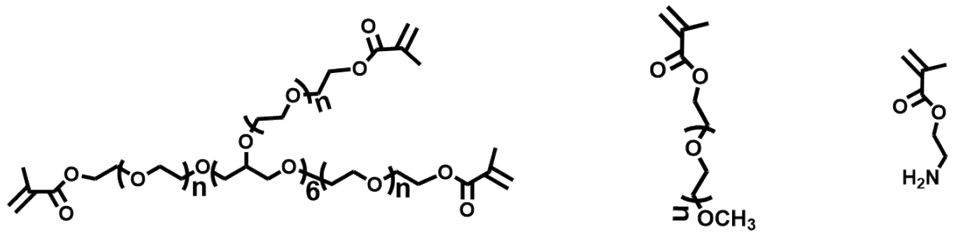


Fig. S3 AFM images of PEG NPs with different size and their height profiles along the marked lines.



8-arm-PEG-ACLT ACLT-PEG$_{2k}$ AEMA

Fig. S4 Structures of 8-arm-PEG-ACLT, ACLT-PEG$_{2k}$ and AEMA.

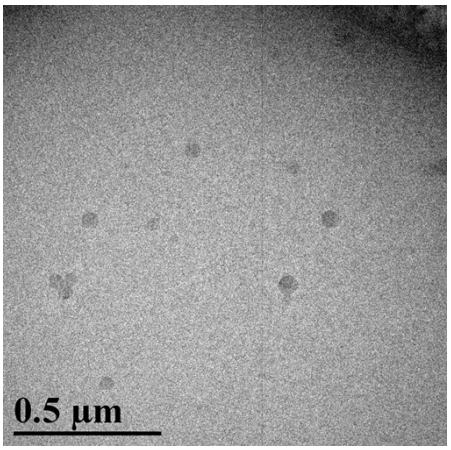


Fig. S5 Cryo-TEM image of PEG NPs (~50 nm).

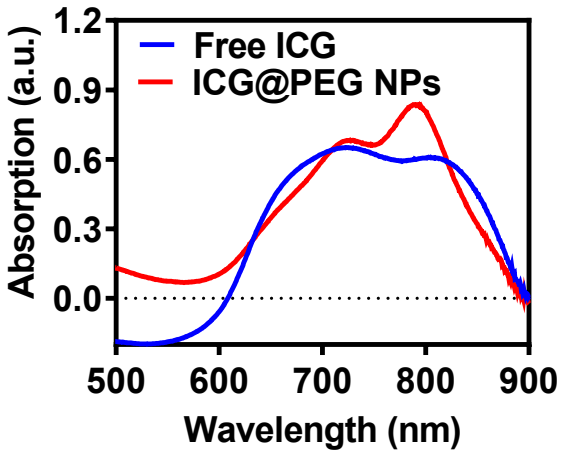


Fig. S6 UV-vis absorption spectra of free ICG and ICG@PEG NPs.

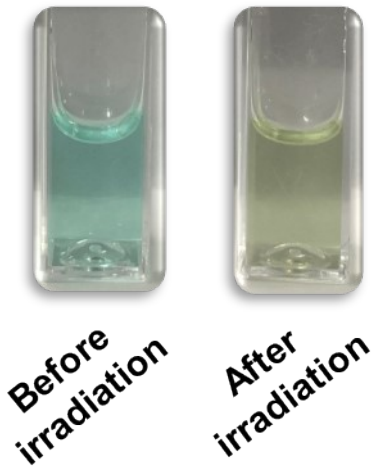


Fig. S7 Photographs of ICG@PEG NP suspensions before and after NIR laser irradiation.

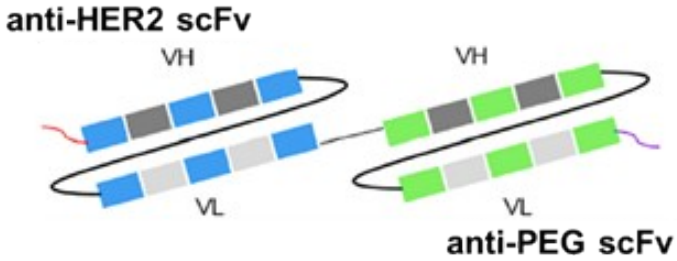


Fig. S8 Scheme of BsAb containing anti-HER2 scFv and anti-PEG scFv.

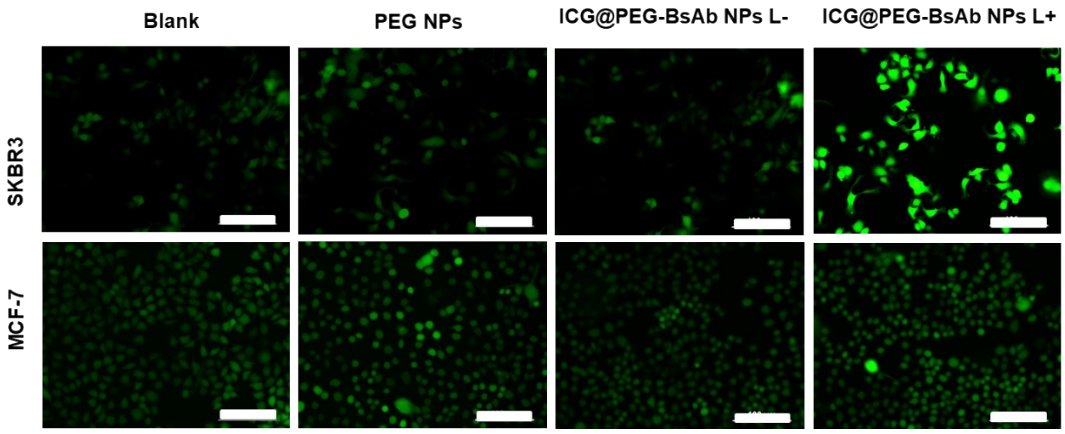


Fig. S9 Fluorescence images of SKBR-3 and MCF-7 cells to show the ROS generation after incubation with PEG and ICG@PEG-BsAb NPs in the presence (L+) and absence (L-) of laser irradiation. Equivalent ICG concentration in each group was 10 μg mL^{-1}. Scale bars are 100 μm.

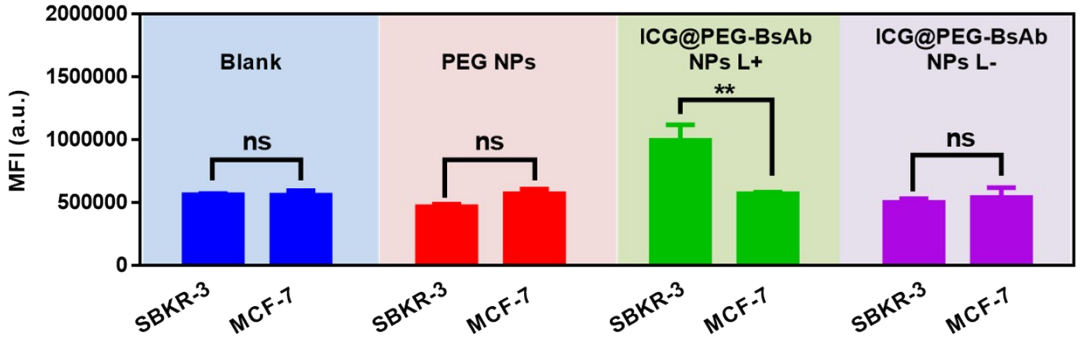


Fig. S10 MFI of SKBR-3 and MCF-7 cells to show the ROS generation after incubation with PEG and ICG@PEG-BsAb NPs in the presence (L+) and absence (L-) of laser irradiation. Equivalent ICG concentration in each group was 10 μg mL^{-1}.

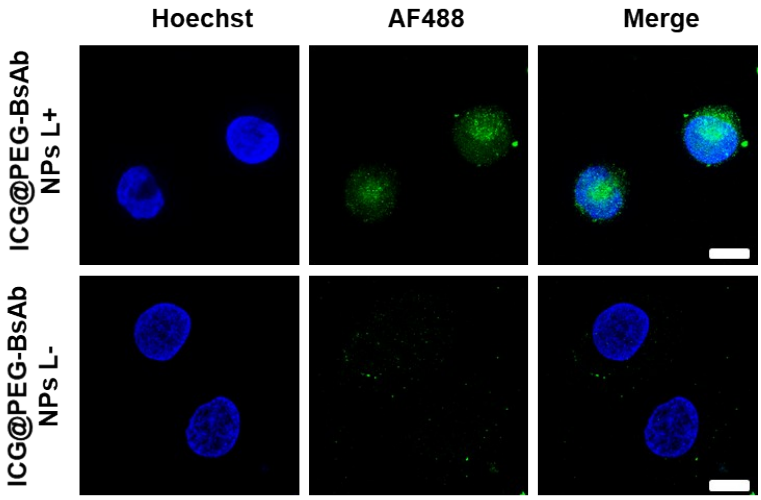


Fig. S11 CLSM images to show the DNA damage after incubation of SKBR-3 cells with ICG@PEG-BsAb NPs in the presence and absence of laser irradiation, scale bars are 10 μm.

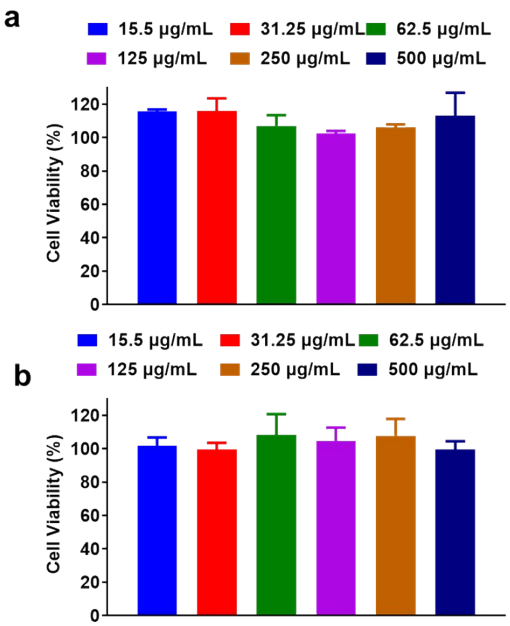


Fig. S12 (a) SKBR-3 and (b) MCF-7 cell viability of PEG-BsAb NPs after 48 h incubation.

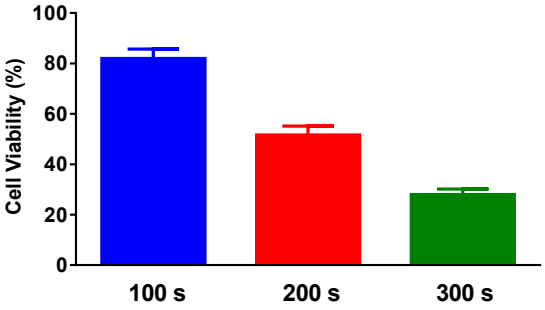


Fig. S13 Irradiation time-dependent SKBR-3 cell viability after 48 h incubation with ICG@PEG-BsAb NPs (equivalent ICG concentrations of 10 μg mL^{-1}). Iirradiation times are 100, 200 and 300 s, respectively.

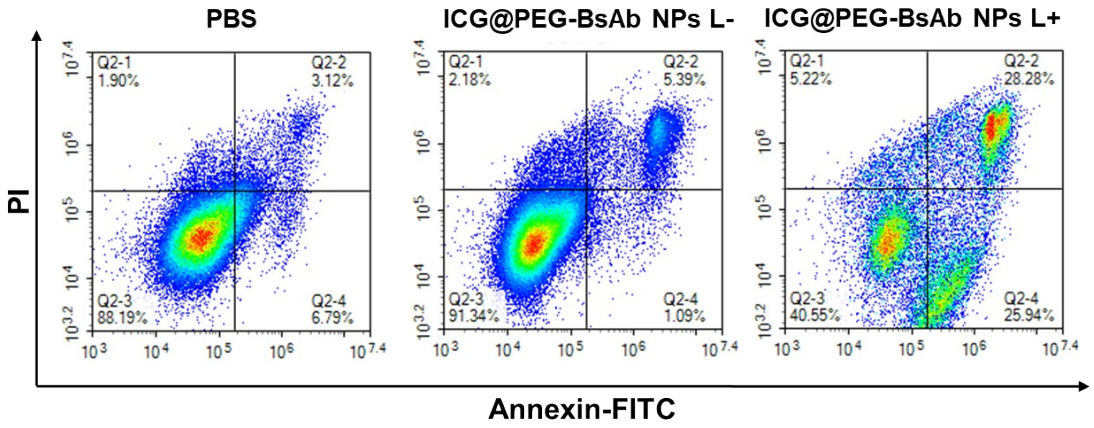


Fig. S14 SKBR-3 cell apoptosis after 48 h incubation with ICG@PEG-BsAb NPs in the presence (L+) and absence (L-) of laser irradiation. Equivalent ICG concentration was 5 μg mL^{-1}.