Electronic Supplementary Information

Dual targeting RNA nanoparticles for efficient delivery of polymeric siRNA to cancer cells

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Experimental Section

Materials: Phosphorylated linear ssDNA and primer ssDNA were purchased from Integrated DNA Technologies (Coralville, IA, USA). T7 RNA polymerase, T7 RNA polymerase reaction buffer, and ribonucleotide solution mix were purchased from New England Biolabs (Ipswich, MA, USA). T4 DNA ligase and T4 ligase buffer were obtained from Promega Corporation (Madison, WI, USA). Poly-L-lysine was purchased from Sigma-Aldrich (St. Louis, MO, USA) and cyanine 5.5 (Cy 5.5) NHS ester, from Lumiprobe (Hunt Valley, MD, USA). Hyaluronic acid (Mw: 200 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). Methyltetrazine-Amine HCl (MTA) was purchased from Brodpharm (San Diego, CA, USA) and (*E*)-cyclooct-4-enyl 2,5-dioxo-1-pyrrolidinyl carbonate (TCO-NHS ester) was from Click Chemistry Tools (Scottsdale, AZ, USA). The HER2-specific monoclonal antibody, anti-PLK1 antibody, and anti-β-actin antibody were obtained from Sigma Aldrich. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution (PS), and phosphate-buffered saline (PBS) were purchased from Corning, Inc. (Corning, NY, USA).

Synthesis of PSMPs and PSNPs: To synthesize the circular DNA, phosphorylated linear single-stranded DNA including the sequence complementary to the siRNA targeting PLK and single-stranded DNA including T7 promoter sequence were designed. For hybridization, 1 μM of each of the two DNA strands were mixed and hybridized by heating at 95°C for 2 min and cooling down gradually to 20°C using a PCR thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA). The hybridized DNA was then ligated by T4 ligase (0.03 U μL^{-1}) in ligase buffer (300 mM Tris-HCl pH 7.8, 100 mM MgCl2, 10 mM ATP, 100 mM DTT) at 24°C for 24 h. Gel electrophoresis was performed on 3% agarose gel in 1× TBE buffer to observe the formation of circular DNA. The gel was run at 80 V for 60 min, and the gel image was obtained with a gel documentation system (Gel Doc XR+; Bio-Rad Laboratories). PSMPs were synthesized by incubating circular DNA at a final concentration of 0.3 μM in a mixture containing ribonucleotide solution mix (2.5 mM), DTT (6 mM), and T7 polymerase (20 U μL^{-1}) in reaction buffer (40 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM DTT, and 2 mM spermidine, pH 7.9) and this mixture was incubated 37°C for 16 h. After incubation, the mixture was purified by pipetting and sonication, which were followed by centrifugation at 3000×g for 5 min to produce monodisperse particles. The supernatants were removed and resuspended in nuclease-free water. To condense the PSMP (Con-PSNP), Cy5.5-conjugated PLL solution (Mw 30,000–70,000, 10 mg mL^{-1}) was added to the PSMP solution at 1 mg mL^{-1} and reacted on an orbital shaker for 12 h at room temperature. Unreacted Cy5.5-conjugated PLL molecules were removed by centrifuging the solution at 10,000 rpm for 5 min and removing the supernatant. For HA layering, Con-PSNPs were resuspended in 100 nM of sodium phosphate buffer (pH 7.4), and tetrazine (TET) functionalized with the HA stock solution (average Mw of 200,000, 10 mg mL^{-1}) was mixed with the Con-PSNP solution at 0.4 mg mL^{-1}, followed by incubation for 12 h at room temperature. Unreacted HA was removed by centrifuging the samples at 10,000 rpm for 5 min and removing the supernatant. To conjugate HER2-Ab, *trans*-cyclooctene-NHS ester

(TCO)-conjugated HER2-Ab was added to the Single-PSNP solution and gently mixed for 12 h at room temperature. Unreacted HER2-Ab was removed by centrifugation at 10,000 rpm for 5 min. After removing the supernatant, the pellet was re-suspended in phosphate buffer.

Characterization of PSMPs and PSNPs: To investigate the morphologies of the PSMPs and PSNPs, we performed scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM). The surfaces of PSMP and PSNP were analyzed at a voltage of 5 kV using a field emission SEM (JSM-7001F; JEOL, Ltd., Tokyo, Japan). The internal structures of the polymeric RNA nanoparticles were observed by TEM (JEM-F200; JEOL Ltd.) operating in scanning TEM (STEM) mode at a voltage of 200 kV. The powder X-ray diffraction patterns of PSMP and Con-PSNP were analyzed using the Rigaku Ultima IV diffractometer (Tokyo, Japan) with Cu Kα radiation at room temperature. The diffractions were collected from 5° to 45°, at 40 kV and 40 mA with a step size of 0.02° at a scan rate of 1° min^{-1}. Additionally, for CLSM analysis, PSMPs or PSNPs were stained with SYBR green I (Thermo Fisher Scientific, Waltham, MA, USA), Cy5.5-conjugated PLL was used during the condensation step, and Cy3 was conjugated to HA. The fluorescence-labeled PSMP and PSNPs were observed with a confocal laser scanning microscope (LSM 700; Carl Zeiss, Jena, Germany). In addition, the hydrodynamic size and zeta potential of PSMPs, Con-PSNPs, Single-PSNPs, and Dual-PSNPs were measured with a particle size and zeta potential analyzer (ELS-2000ZS; Otsuka Electronics Co., Osaka, Japan). The particles were dispersed in nuclease-free water and measured at 25°C. Energy dispersive spectrometry (EDS) analysis was conducted to analyze the elemental composition of PSMPs at a voltage of 200 kV using STEM-based EDS mapping.

Biostability of PSNPs: The biostability of PSMPs and Dual-PSNPs was analyzed and compared. To assess biostability, 300 ng of polymeric siRNAs in PSMPs and Dual-PSNPs were incubated in PBS buffer containing 10% FBS (v/v) for different times (0, 12, 24, 48 h) at 37°C. After serum incubation, polymeric siRNAs from the treated samples along with equal amount of polymeric siRNA from untreated samples (control) were stained with SYBR Green 1. After the loading dye was added, all samples were loaded onto 4% agarose gel. Gel electrophoresis was performed in TBE buffer at 100 V for 30 min and gel images were obtained with a gel documentation system. Gel images were further analyzed using ImageJ software (v1.8, National Institutes of Health, Bethesda, MD, USA) to quantify the remaining polymeric siRNA in the PSMPs and Dual-PSNPs.

Cell culture: The SKOV3 or MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). The HepG2 cells were grown in EMEM supplemented with 10% FBS and 1% PS. The NIH-3T3 cells were cultured in DMEM supplemented with 10% bovine calf serum (Thermo Fisher Scientific).

In vitro cellular uptake analysis: To analyze the cellular uptake of PSNPs, SKOV3, MCF-7, HepG2, and NIH-3T3 cells were seeded into a 24-well culture plate (SPL Life Sciences, Gyeonggi, South Korea) at a density of 10×10^4 cells/well, and then incubated for 24 h at

37°C. The seeded cells present in either serum-free DMEM or EMEM were then treated with Cy5.5 labeled PSNPs at an siRNA concentration of 20 nM for 2 h at 37°C. For the competitive inhibition experiment, SKOV3 cells were pre-treated with either free HA (40 mg mL^{-1}) or free HER2-Ab (20 μg/well) or both for 1 h. After incubation, the PSNP-containing media were removed, and the cells were washed with PBS and detached using 0.25% trypsin/EDTA for flow cytometry analysis. The detached cells were resuspended in cold PBS and analyzed by flow cytometry (BD™ LSR II; BD Biosciences, San Jose, CA, USA). Data collection involved 10,000 counts per sample. CLSM was used to visualize the internalization of PSNPs in SKOV3 or NIH-3T3 cells. The cells were seeded into 24-well culture plates at a density of 10×10^4 cells/well. After 24 h incubation at 37°C, Cy5.5-labeled PSNPs were added to the seeded cells with serum-free DMEM at an siRNA concentration of 20 nM for 2 h at 37°C. Subsequently, the PSNP-containing media were removed, and the cells were washed with cold PBS to perform nuclear staining using 2 μM Hoechst 33342 (Thermo Fisher Scientific). After staining the cell nuclei, the internalization of PSNPs was observed by CLSM.

Western blotting: SKOV3 cells were seeded into a 6-well culture plate (SPL Life Sciences) at a density of 1×10^5 cells/well and incubated for 24 h at 37°C. Lipofectamine (Thermo Fisher Scientific) was utilized for formulation of complex with siPLK1 (siPLK1/Lipo) according to manufacturer protocol. The seeded cells in serum-free DMEM PSMP, PSNPs were treated with siPLK1/Lipo at a siRNA concentration of 20 nM for 4 h at 37°C. After treatment, the media were replaced with DMEM and incubated for 48 h at 37°C. The cells were lysed using 100 μL of ice-cold lysis buffer, and centrifuged at 14,000 rpm for 30 min. Western blotting analysis was performed with the aliquots of lysates by using anti-PLK1 or anti-β-actin antibody.

In vitro cytotoxicity analysis: The in vitro cytotoxicity of PSMP, PSNPs, and siRNA/Lipo was evaluated using the MTT assay (Roche Diagnostics, Mannheim, Baden-Wurttemberg, Germany). SKOV3 cells were seeded into 96-well culture plates at a density of 6×10^3 cells/well and incubated at 37°C for 24 h. The SKOV3 cells were then treated with PSMP, PSNPs, and siRNA/Lipo at siRNA concentrations ranging from 5 to 40 nM for 4 h at 37°C. After 48 h of incubation, 10 μL of MTT reagent per well was directly added to the medium following the manufacturer's instructions and incubated for 4 h at 37°C. The supernatant was removed, and the resultant formazan salts were dissolved by adding 100 μL dimethyl sulfoxide per well. Absorbance of the sample was recorded at a wavelength of 570 nm using a microplate reader (Victor X5; PerkinElmer, Waltham, MA, USA). All analyses were performed in triplicate.

Statistical analysis: The experimental data were expressed as the mean ± standard deviation (SD) for three samples per group. Differences between groups were analyzed by one-way analysis of variance with a Scheffe test, a function of the SPSS software package version 24.0. The data were marked as $*p < 0.05$; $**p < 0.01$; $***p < 0.005$.

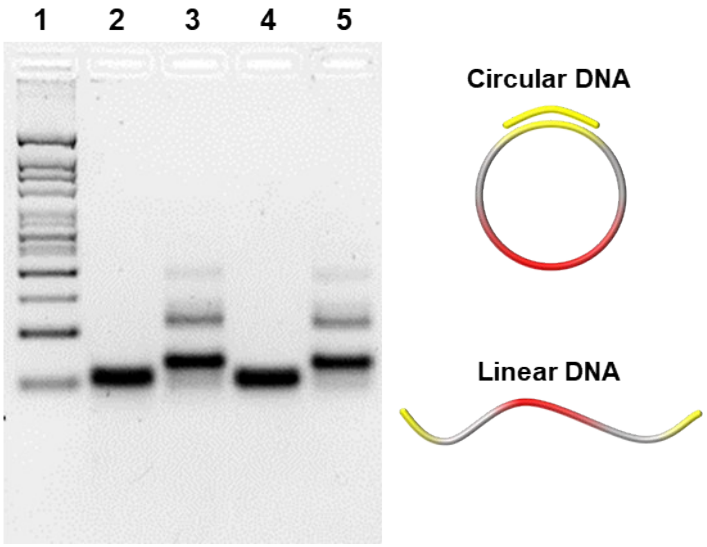

Fig. S1 Gel electrophoresis image of linear and circular DNAs. (1) dsDNA ladder, (2) linear DNA for PLK1 siRNA, (3) circular DNA for PLK1 siRNA, (4) linear DNA for scrambled siRNA, (5) circular DNA for scrambled siRNA. A band shift was observed when the DNA changed from the linear form to the circular form.

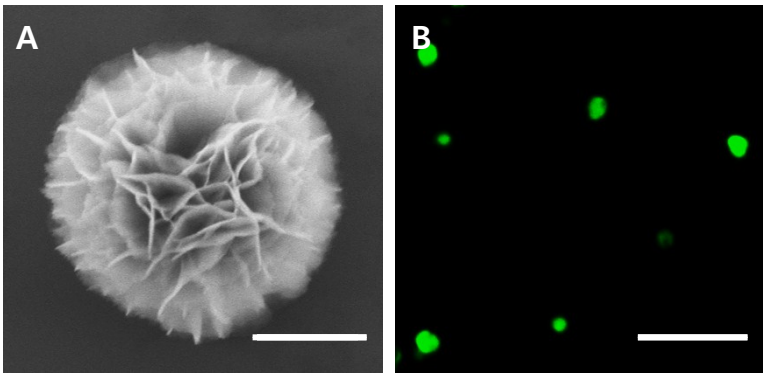

Fig. S2 A) Morphological characterization of PSMP by SEM. Scale bar: 1µm. B) CLSM image of PSMPs after SYBR green staining. Scale bar: 10 µm.

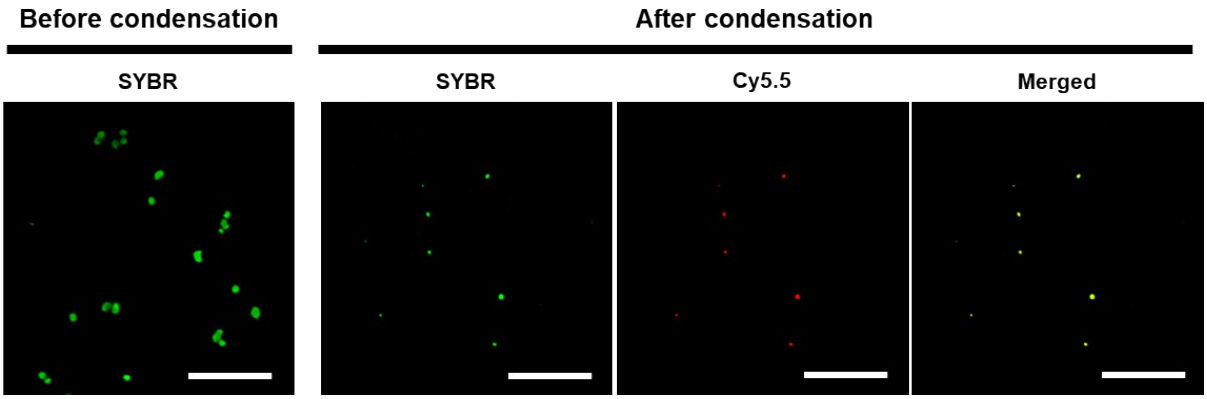

Fig. S3 CLSM images of PSMPs and Con-PSNPs after condensation with Cy5.5-labeled PLL (red). Polymeric siRNA was stained with SYBR Green I (green). Scale bars: 20 µm.

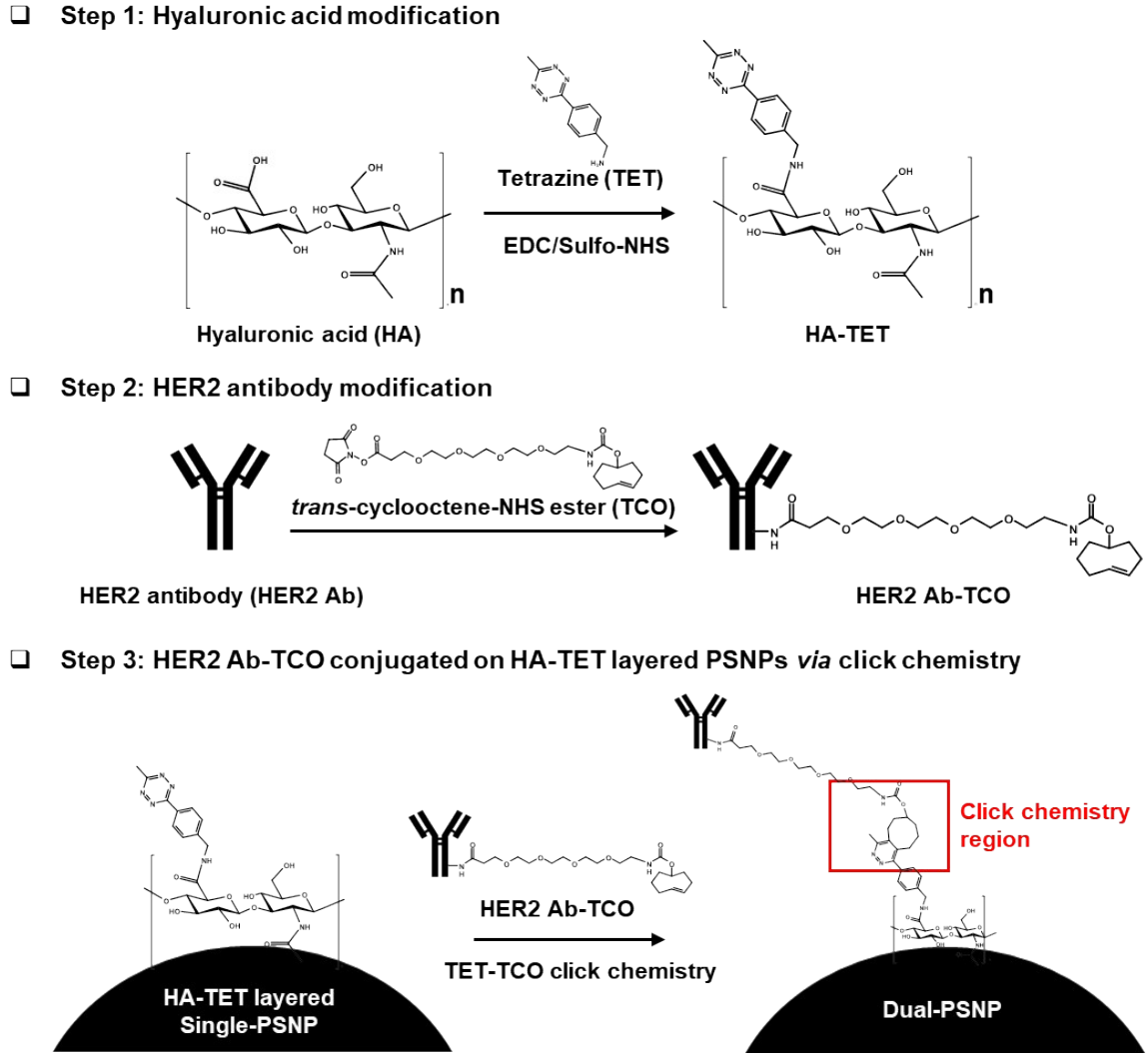

Fig. S4 Schematic illustration of the process of tetrazine (TET) modification of HA (Step 1), modification of HER2 antibody by *trans*-cyclooctene-NHS ester (TCO) (Step 2), and conjugation of HER2-Ab-TCO to HA-TET layered PSNPs (Step 3).

Mass ratio of HER2-Ab/NP	Conjugated HER2-Ab (ng)	Conjugation efficiency (%)
0.025	3.1	7.1
0.1	39.7	22.6
0.4	170.7	24.2

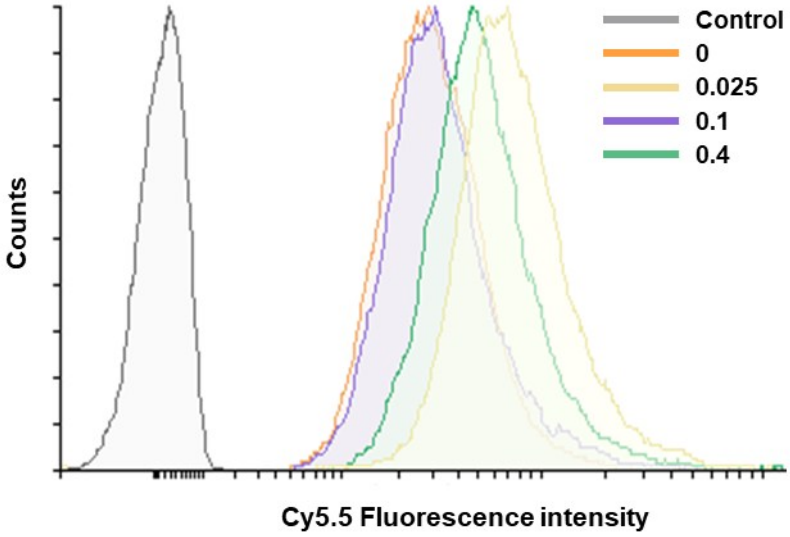

Fig. S5 Optimization of HER2-Ab ligand density on Dual-PSNPs to enhance intracellular uptake. Flow cytometry analysis of SKOV3 cells after treatment with Dual-PSNPs with respect to the mass ratio of HER2-Ab and Dual-PSNP (0, 0.025, 0.1, and 0.4 denote the ng per ng ratios of HER2-Ab/NP).

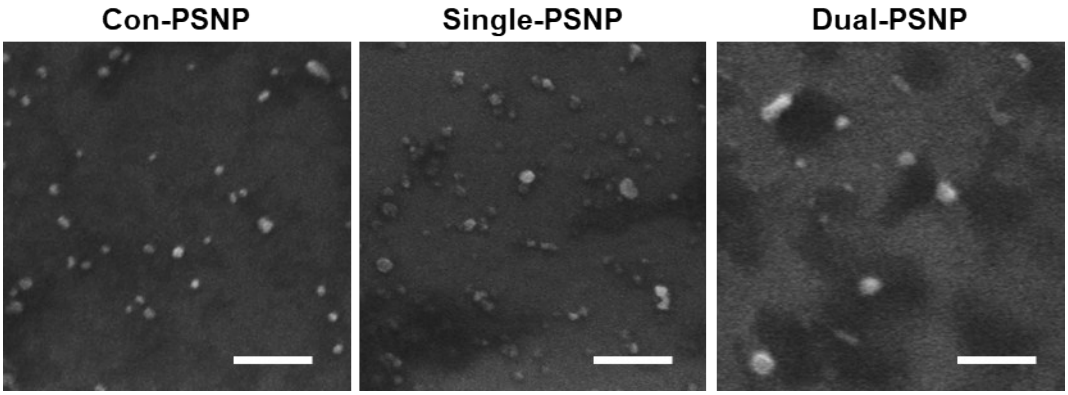

Fig. S6 SEM images of PSNPs. Scale bars: 500 nm.

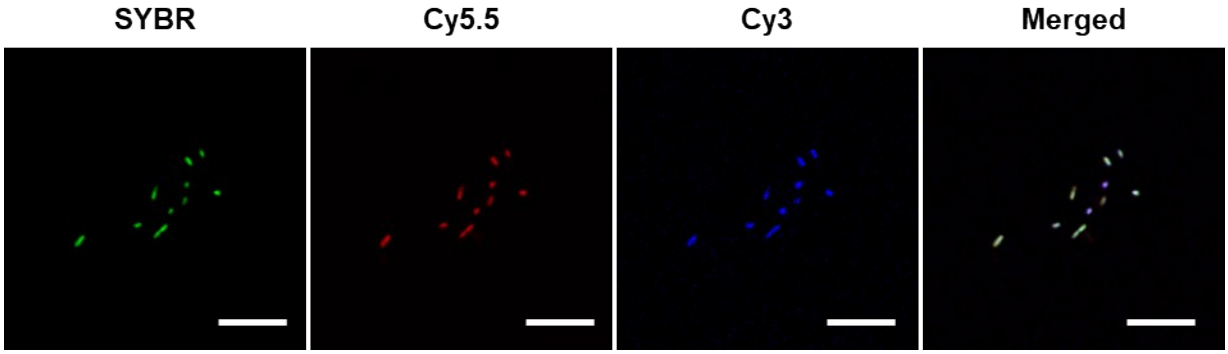

Fig. S7 CLSM images of fluorescence-labeled Dual-PSNPs. Polymeric siRNA was stained with SYBR Green I (green), PLL was labeled with Cy5.5 (red), and HA was labeled with Cy3 (blue). Scale bars: 10 μm.

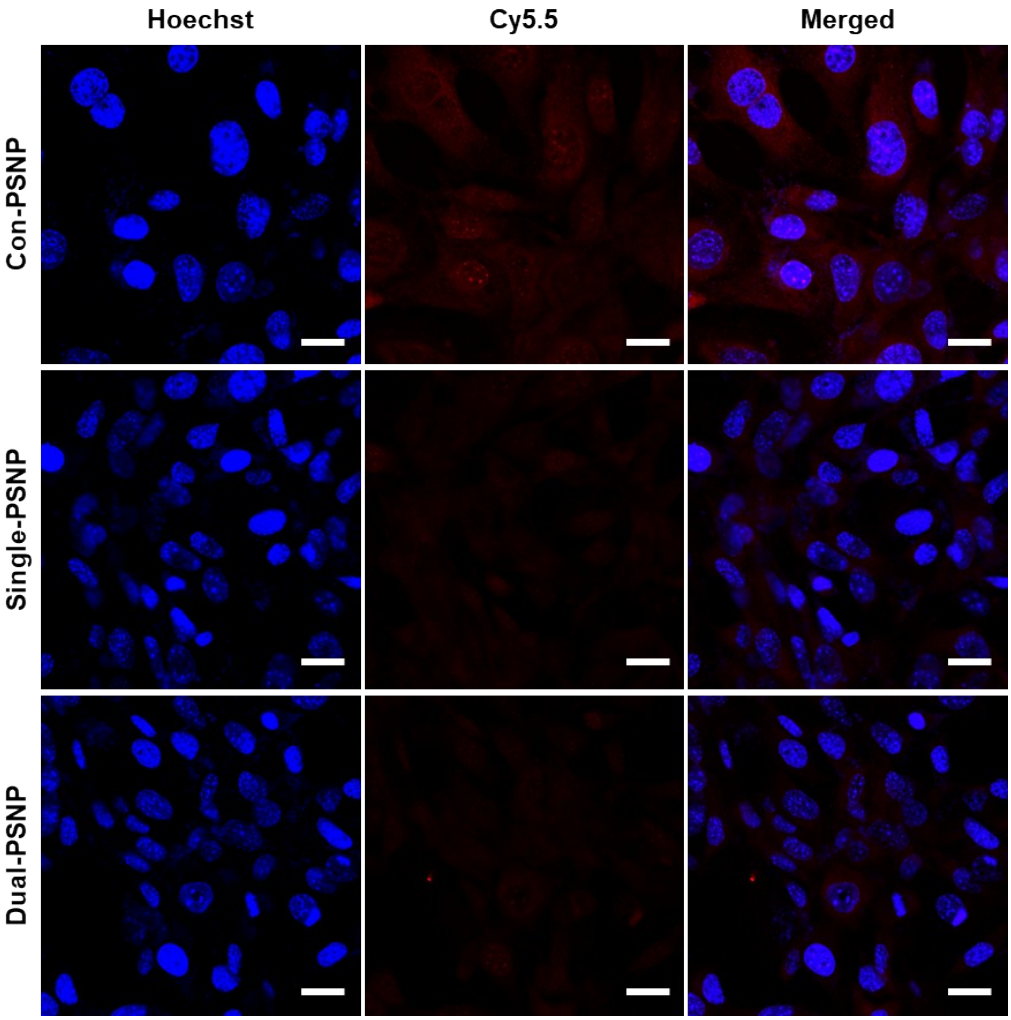

Fig. S8 CLSM images of NIH-3T3 cells after treatment with Cy5.5-labeled PSNPs (red). The cells were stained with Hoechst-33342 to identify the nuclei (blue). Scale bars: 20 μm.

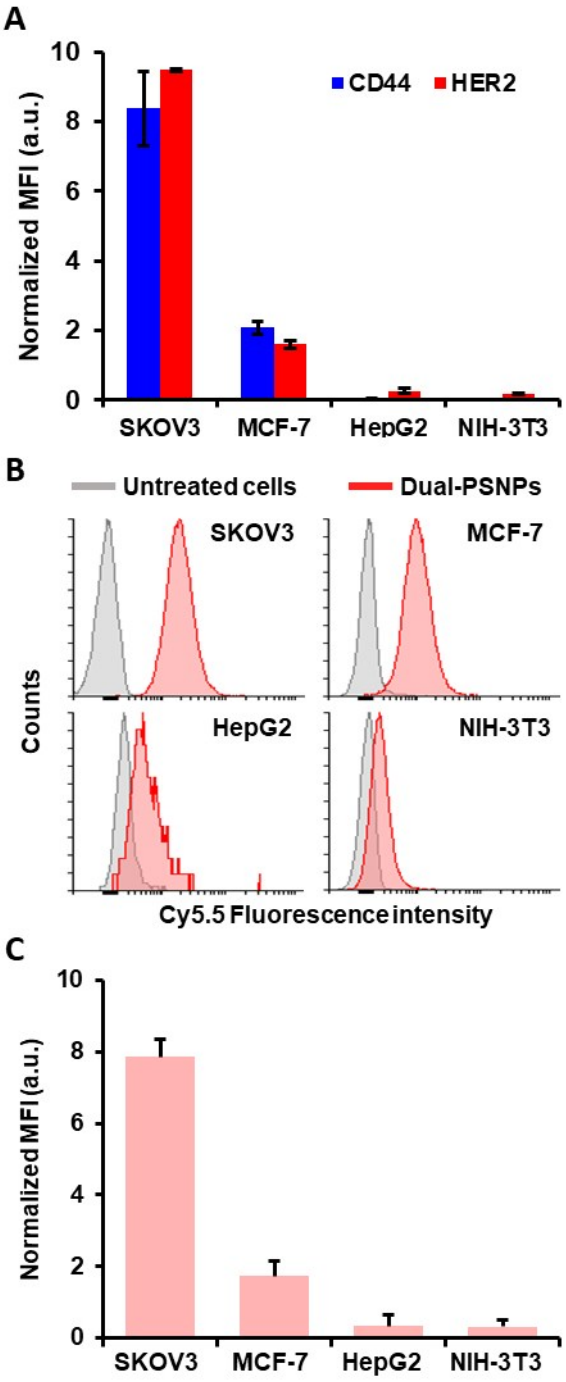

Fig. S9 (A) Flow cytometry analysis after immunostaining of CD44 and HER2 receptor in SKOV3, MCF-7, HepG2, and NIH-3T3 cells. (B) Flow cytometry analysis after treatment with Dual-PSNPs in SKOV3, MCF-7, HepG2, and NIH-3T3 cells. (C) Normalized MFI (mean fluorescence intensity) based on flow cytometry analysis on SKOV3, MCF-7, HepG2, and NIH-3T3 cells.

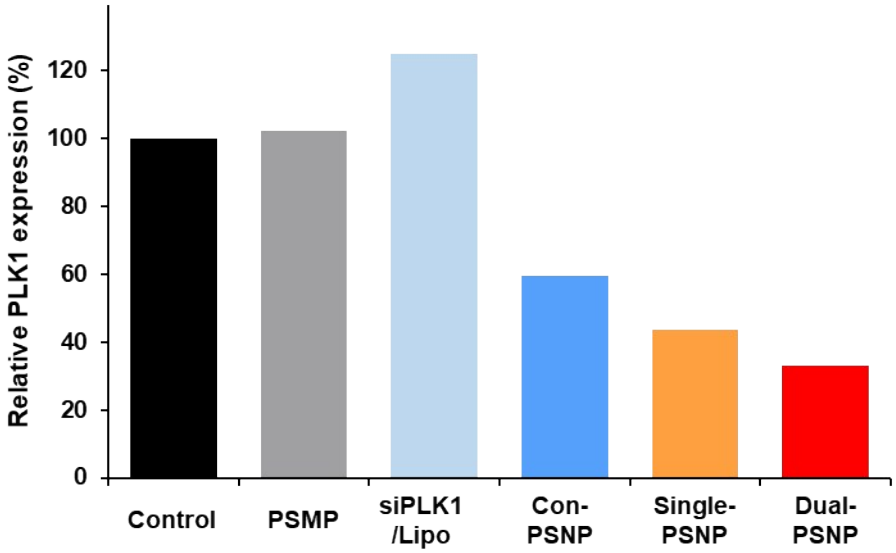

Fig. S10 Quantitative analysis from the image of western blotting of PLK1 protein expression in SKOV3 cells treated with the PSMPs, siPLK1/Lipo, and PSNPs after 48 h of incubation.

Table S1. Linear ssDNA and primer ssDNA sequences used for PSMP synthesis. Bold indicates complementary to siRNA sequence.

Strand	Sequence
Linear ssDNA for scrambled siRNA	5'- /5Phos/ ATA GTG AGT CGT ATT AAC GTA CCA **ACA AGC TGA AAG AAC ACG AAC TTT T**AC TTG **AAA AGT TCG TGT TCT TTC AGC TT**T AGA GGC ATA TCC CT -3'
Linear ssDNA for PLK1 siRNA	5'- /5Phos/ ATA GTG AGT CGT ATT AAC GTA CCA **ACA AAG ATC ACC CTC CTT AAA TAT TTT** ACT TGA **AAA TAT TTA AGG AGG GTG ATC TT**T TAG AGG CAT ATC CCT -3'
Primer ssDNA	5'- TAA TAC GAC TCA CTA TAG GGA T -3'

Table S2. Physicochemical characterization of polymeric siRNA particles.

Sample	Terminal layer	Particle size (nm)	PDI	Zeta potential (mV)
PSMP	**Polymeric siRNA**	**1704.8 ± 280.6**	**0.272**	**- 25.5 ± 1.9**
Con-PSNP	**PLL**	**121.2 ± 33.3**	**0.228**	**29.0 ± 1.7**
Single-PSNP	**HA**	**130.1 ± 36.7**	**0.226**	**- 30.9 ± 0.9**
Dual-PSNP	**HER2-Ab + HA**	**133.8 ± 37.2**	**0.246**	**- 28.1 ± 0.8**