

Electronic Supplementary Information

Size-Controlled Synthesis of Polymeric DNA

Nanoparticles for Targeted Anticancer Drug Delivery

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Experimental section

Materials

DNA oligonucleotides and Tris-EDTA (TE) buffer were purchased from Integrated DNA Technology (Coralville, IA, USA). T4 DNA ligase (cat. No. M1804) was obtained from Promega (Madison, WI, USA) and ϕ 29 DNA polymerase (part No. P7020-LC-L), from Enzymatics Inc. (Beverly, MA, USA). Premixed dNTP solution was procured from Epicentre (Madison, WI, USA). Cyanine 5.5 (Cy5.5) NHS ester was obtained from Lumiprobe (Hunt Valley, MD, USA). Poly-L-lysine was purchased from Sigma Aldrich (St. Louis, MO, USA) and sodium hyaluronate (part No. HA20K-1), from Lifecore (Chaska, MN, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution, and phosphate-buffered saline (PBS) were supplied by Corning, Inc. (Corning, NY, USA). Bovine calf serum and Hoechst-33342 were procured from Thermo Fisher Scientific (Waltham, MA, USA).

Synthesis of size-tuned DNA particles

Circular DNA (300 nM) to be used as a template for RCA was synthesized with 1 μ M of phosphorylated single stranded DNA and single stranded DNA including T7 promoter sequence in TE buffer. These two DNA strands were mixed and hybridized by heating the solution for 2 min at 95 °C and cooling down gradually to 20 °C using a PCR thermal cycler (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA). For the ligation, hybridized DNA was incubated with 0.06 U/ μ L of T4 ligase and the ligation reaction buffer (300 mM Tris-HCl (pH 7.8), 100 mM MgCl₂, 10 mM ATP, 100 mM DTT) at room temperature for 24 h. Size-tuned PDMs were synthesized by incubating circular DNAs at the final concentration of 0.3 μ M with 0.7, 1.5, and 3.0 mM of deoxyribonucleotide triphosphate (dNTP), reaction buffer (50 mM Tris-HCl, 10 mM (Na₄)₂SO₄, 10 mM MgCl₂, 35 mM dithiothreitol, pH 7.4), and 0.75 U/ μ L of phi29 DNA polymerase for 14 h at 24 °C. After the incubation, the DNA particles were sonicated and centrifuged for 5 min at 6,000 rpm. The supernatants were removed and nuclease free water was added. This sonication step was repeated three times to achieve monodisperse particles. For the condensation process, the Cy5.5 conjugated PLL stock solution (Mw 30,000-70,000, 10 mg/mL) was added into the polymerized DNA microparticle (PDM) solutions at 2 mg/mL concentration, followed by gentle mixing for 12 h at room temperature. To remove unreacted PLL molecules, the supernatant was eliminated by centrifuging at 10,000 rpm for 5 min. For the LbL assembly, the condensed PDMs were then resuspended in PBS (-Ca²⁺/-Mg²⁺) that was diluted 100-fold in distilled water. HA stock solution (average Mw of 200,000, 10 mg/mL) was added to the solution at a concentration of 1 mg/mL. After incubating for 12 h at room temperature,

the supernatant was removed by centrifugation at 10,000 rpm for 5 min to remove the unattached HA and purify nanoparticles. Finally, the nanoparticles were resuspended in PBS ($-Ca^{2+}/-Mg^{2+}$) for storage and cell treatment.

Characterization of size-tuned DNA particles

Morphological and structural analyses

Field emission scanning electron microscopy (FE-SEM; JSM-7001F; JEOL, Tokyo, Japan) was used to obtain high resolution digital images of the size-tuned PDMs for morphological characterization at an accelerating voltage of 5 kV. For SEM observation, PDMs were dried on a silicon wafer and coated with Pt for 180 s. SEM images were analyzed with ImageJ (v1.8, National Institutes of Health, Bethesda, MD, USA) to obtain an average size of size-tuned PDMs. Scanning transmission electron microscope (STEM; JEM-F200; JEOL, Tokyo, Japan) was used for the structural characterization of size-tuned PDMs at an accelerating voltage of 200 kV. For sample preparation, PDMs were deposited onto a Formvar/carbon-coated copper grid (3430C-FA; SPI Supplies, West Chester, PA, USA) and air-dried at room temperature. Each DNA particle type was visualized with confocal laser scanning microscopy (CLSM; LSM 700; Carl Zeiss, Thornwood, NY, USA) after staining with SYBR Green II (Thermo fisher Scientific, Waltham, MA, USA). Powder X-ray diffraction (PXRD) patterns were collected on a Rigaku Ultima IV diffractometer with Cu K α radiation at room temperature. The measurements were obtained at 40 kV and 40 mA with a step size of 0.02 °C. Data points were collected from 5 °C to 40 °C at a scan rate of 1 °C/min. An x-ray diffraction pattern of simulated magnesium pyrophosphate ($Mg_2P_2O_7 \cdot 3.5H_2O$) was acquired from the crystal data using JADE diffraction analysis software (v9.3.2, Materials Data, Inc., Livermore, CA, USA).

Physicochemical properties of size-tuned DNA particles

The size distribution and zeta potential of size-tuned PDMs, condensed PDMs, and PDNs were determined by a particle size and zeta potential analyzer (ELS-2000ZS; Otsuka Electronics Co., Osaka, Japan). All samples were dispersed in nuclease-free water, and measured at 25°C.

Polymerization rates of size-tuned DNA particles

Real-time polymerase chain reaction detection system (RT-PCR; CFX96; Bio-Rad Laboratories Inc., Hercules, CA, USA) was utilized to observe the polymerization rates of different DNA polymerase (dPol) to dNTP ratios. Similar to the synthesis of size-tuned PDMs, mixtures of circular DNAs at the final concentration of 0.3 μ M with 0, 0.7, 1.5, and 3.0 mM of dNTP, reaction buffer (50 mM Tris-HCl, 10 mM $(Na_4)_2SO_4$, 10 mM $MgCl_2$, 35 mM dithiothreitol, pH 7.4), 1000 X SYBR Green II, and 0.75 U/ μ L of phi29 DNA polymerase were incubated

for 4 h at 24 °C. Fluorescence intensities resulting from intercalation of SYBR Green II were measured at an interval of 10 min throughout the incubation until saturation of the fluorescence intensity.

Biostability of size-tuned DNA particles

The biostability of polymerized DNAs of condensed PDMs was evaluated and compared to those of PDMs of all sizes in serum and nuclease. To assess the resistance to serum degradation, 250 ng polymerized DNAs in DNA particles were incubated with 10% fetal bovine serum at 37 °C for 4 h. Nuclease stability tests were performed by treating 250 ng polymerized DNAs in DNA particles with 5 units/mL of DNase I (Sigma Aldrich) at 37 °C for 4 h. After serum and DNase treatments, polymerized DNAs in DNA particles were stained with SYBR Green II. All samples were mixed with loading dye and loaded onto a 4% agarose gel in the presence of the equivalent amounts of untreated samples of all sizes as reference. Gel electrophoresis was performed in TBE buffer at 100 V for 30 min, and gel images were obtained using a gel documentation system (Gel Doc XR+; Bio-Rad Laboratories Inc., Hercules, CA, USA). Gel images were further analyzed with ImageJ software to quantify protected polymerized DNA amount in DNA particles, and compared the DNA band intensities of polymerized DNA before and after serum and DNase treatments.

In vitro experiments of size-tuned DNA particles

Cell Culture

SKOV3 ovarian cancer cells stably expressing firefly luciferase (SKOV3-Luc) (Cell Biolabs Inc., San Diego, CA, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. NIH-3T3 fibroblast cells (Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM supplemented with 10% bovine calf serum at 37 °C in a humidified atmosphere of 5% CO₂.

Intracellular delivery

Size-tuned PDNs were synthesized with Cy5.5-conjugated PLL and used to study the cellular uptake trend. SKOV3 and NIH-3T3 cells were seeded in an eight-chambered culture slide at a concentration of 1×10^5 cells/well. After 24 h of incubation, the cells were treated with each size-tuned PDN at 15 nM equivalent of antisense oligonucleotide (ODN) concentration for 2 h and subjected to nuclear staining using 2 μ M Hoechst-33342 (Thermo fisher Scientific, Waltham, MA, USA), which were visualized with CLSM. The CLSM images were further analyzed with ImageJ to quantify mean fluorescence intensities. To assess endosomal escape, SKOV3 cells were incubated with Cy5.5-labeled PDN-L for 4 h, and sequentially treated with 50 nM of LysoTracker Green (Thermo fisher Scientific, Waltham, MA, USA) for 1 h and 2 μ M Hoechst-33258 for 5 min

to visualize endo-lysosomes and nuclei, respectively. CLSM was used to observe the distribution of Cy5.5-labeled PDN-L.

Knockdown efficacy

The efficacy of the polymerized ODN in size-tuned PDNs was evaluated by measuring the expression level of firefly luciferase in SKOV3-Luc cells. The cells were seeded in a 96-well plate at 6×10^3 cells/well and incubated for 24 h. Cells were treated with PDNs of different sizes at 30 nM equivalent of ODN concentration in serum-free medium. After a 3 h incubation, the medium was replaced with growth medium supplemented with 10% FBS and 1% penicillin/streptomycin. After 72 h, the expression level of luciferase was measured using a multi-label microplate reader (Victor X5; Perkin Elmer, Waltham, MA, USA) with a luciferase assay system (Promega, Madison, WI, USA). The relative luciferase activity was calculated by comparing luciferase activity of cells treated with anti-luciferase ODN-loaded PDNs with that of cells treated with scrambled ODN-loaded PDNs.

Cytotoxicity assay

The cytotoxicity of size-tuned PDNs was evaluated with Cell Counting Kit (CCK)-8 (Dojindo Molecular Technologies, Kumamoto, Japan). SKOV3 cells were seeded in a 96-well plate at a concentration of 6×10^3 cells/well and treated with each size of PDN at ODN concentration of 30 nM in serum-free medium. After 3 h, the medium was replaced with growth medium. CCK-8 assay was performed as recommended by the manufacturer after 72 h. The amount of water-soluble formazan in the medium was detected by recording the absorbance at 450 nm with a multi-label microplate reader following a 1 h incubation with reaction solution. Cell viability after treatment with various concentrations of size-tuned PDNs was calculated by comparing the absorbance of PDN-treated cells with that of untreated cells.

Statistical analysis

Experimental data are expressed as the mean \pm standard deviation (SD) for three samples per group. Differences between groups were analyzed using one-way ANOVA with a Scheffe test, a function of the SPSS software package version 24.0. The data were marked as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Table S1. DNA sequences for circular DNA synthesis

Strand	DNA Sequences
Linear ssDNA (Anti-luciferase ODN, 126 bp)	5'-Phosphate-ATAGTGAGTCGTATTACGATGCATGCTACCAACCG AAGCCATTCTACCCACTCGAATTCTTCCTTACTATTGCTCACAAT AATTCGAGTGGGTAGAATGGCTTTAGAGGATGCATGCCATATCCCT-3'
Linear ssDNA (Scrambled ODN, 126 bp)	5'-Phosphate-ATAGTGAGTCGTATTACGATGCATGCTACCAACCG AAGCTGAAAGAACACGAACCTTTTCTTCCTTACTATTGCTCACAAT AAAAGTTCGTGTTCTTTCAGCTTTAGAGGATGCATGCCATATCCCT-3'
Primer ssDNA (32bp)	5'-TAATACGACTCACTATAGGGATATGGCATGCA-3'

*Bold indicates complementary sequence to antisense sequence.

Table S2. Average hydrodynamic size and zeta potential of size-tuned polymeric DNA particles.

Type	Size	Hydrodynamic size (nm)	Zeta Potential (mV)
PDM	Small	847.5 ± 102.2	-24.8 ± 0.3
	Medium	1653.7 ± 380.5	-15.9 ± 1.5
	Large	1905.8 ± 421.0	-19.9 ± 2.4
Condensed PDM	Small	88.5 ± 25.0	28.6 ± 0.8
	Medium	114.8 ± 31.5	26.7 ± 4.8
	Large	154.8 ± 40.5	28.0 ± 2.6
PDN	Small	95.8 ± 27.6	-32.8 ± 1.0
	Medium	133.8 ± 37.1	-29.2 ± 0.2
	Large	177.4 ± 48.7	-30.4 ± 0.4

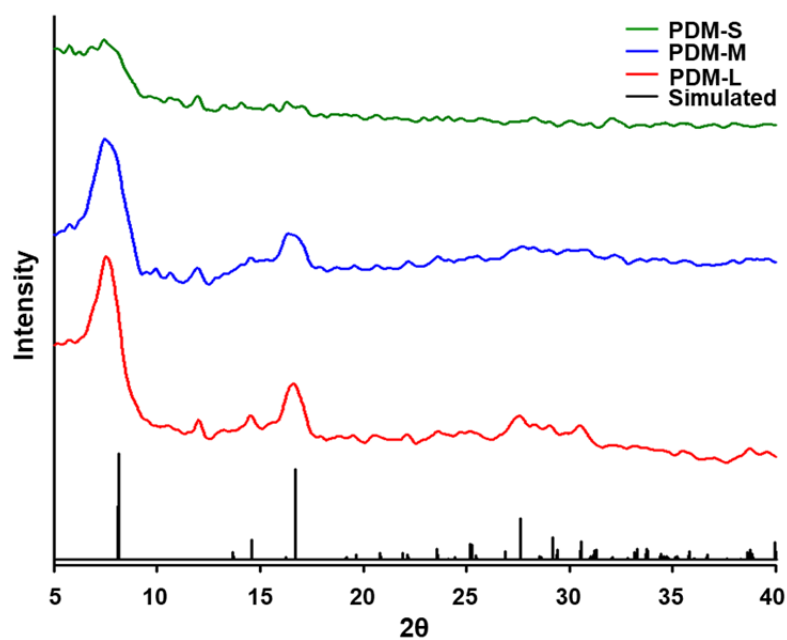


Fig. S1 PXRD patterns of PDMs and simulated magnesium pyrophosphate ($\text{Mg}_2\text{P}_2\text{O}_7 \cdot 3.5\text{H}_2\text{O}$).

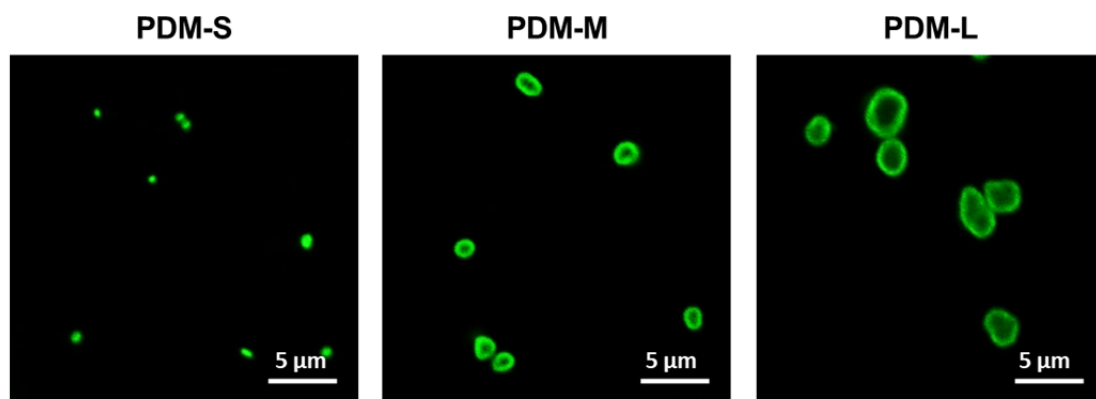


Fig. S2 CLSM images of SYBR green II-stained PDMs. The green fluorescence represents polymerized DNA, representing localization of polymerized DNA within PDMs.

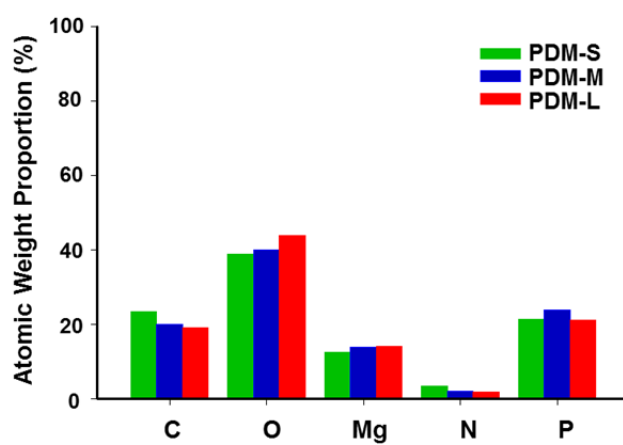
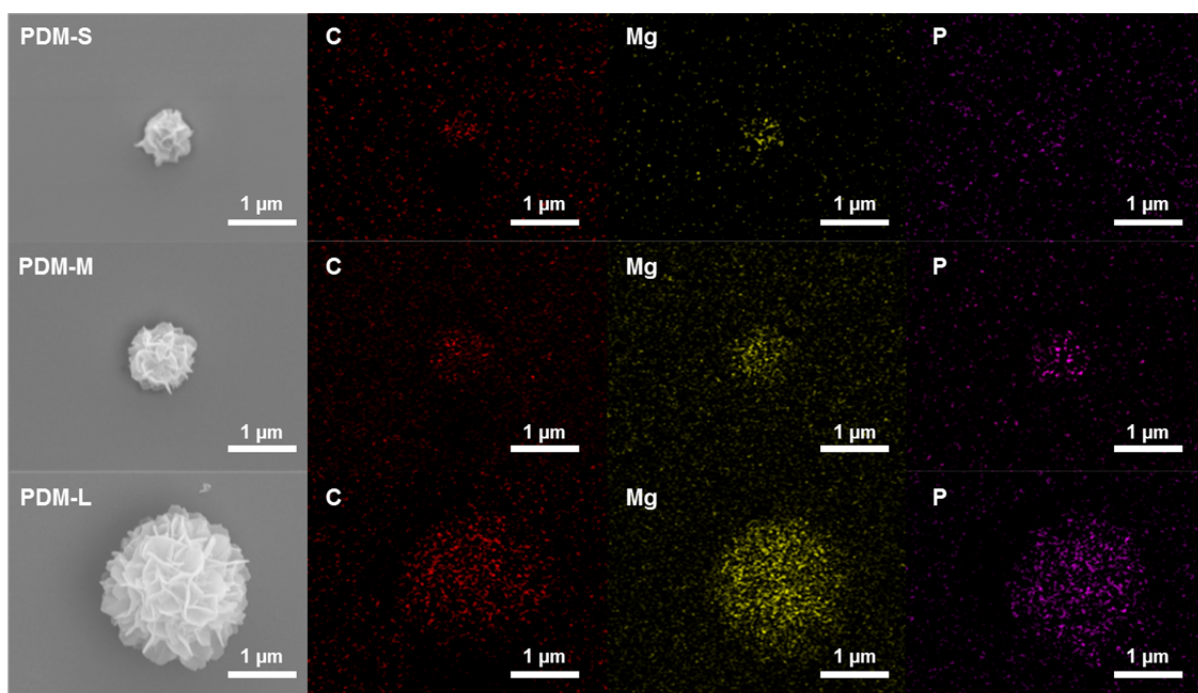


Fig. S3 SEM-based energy dispersive spectrometer. Mapping and line scan results of size-tuned PDMs.

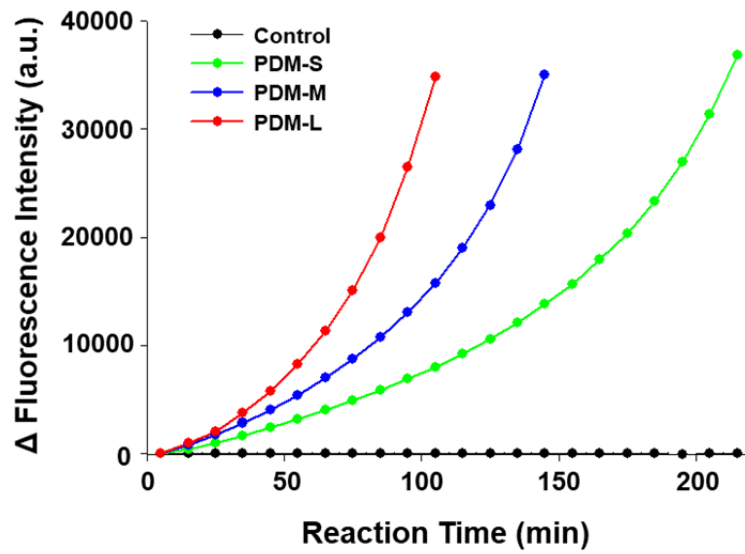


Fig. S4 Real-time analysis of polymerized DNA synthesis during the RCA reaction of PDMs. Fluorescence intensities resulting from intercalation of SYBR Green II to the polymerized DNA were measured at an interval of 10 min until saturation of the fluorescence intensity.

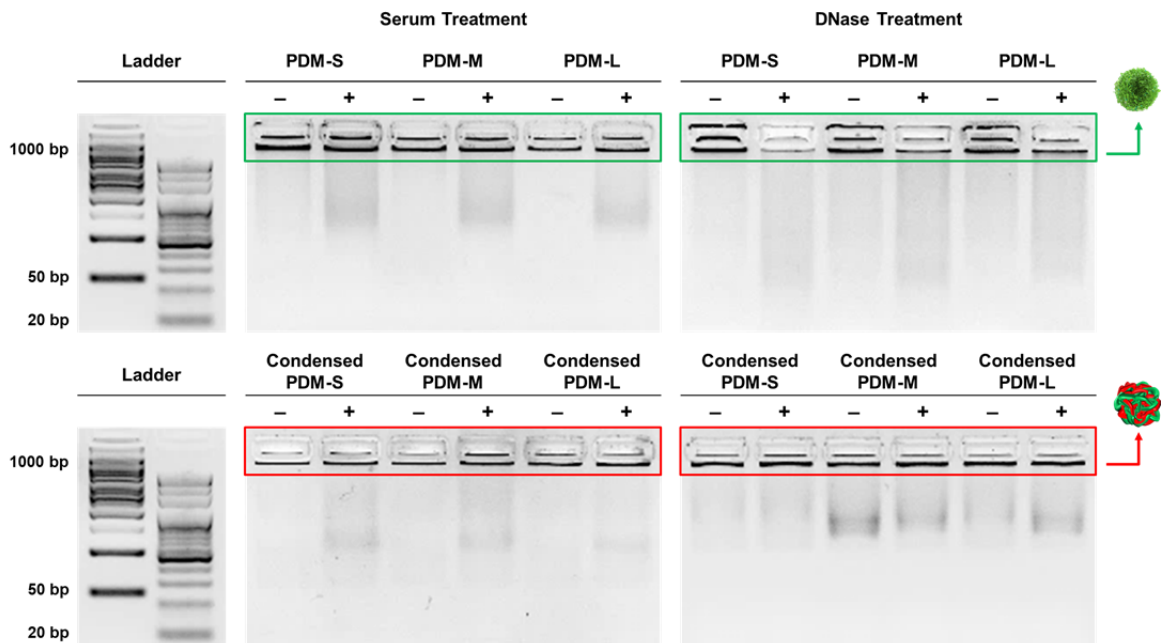


Fig. S5 Gel electrophoresis images of polymerized DNA for biostability characterization. Polymerized DNAs in PDMs and condensed PDMs of all size were treated with serum (50%) and DNase I (5 units/mL). Green and red boxes indicate polymerized DNAs in PDMs and condensed PDMs, respectively. The polymerized DNAs of PDMs remained in the well due to their high molecular weight, whereas degraded and depolymerized DNAs of PDMs created smear bands owing to their relatively lower molecular weight.

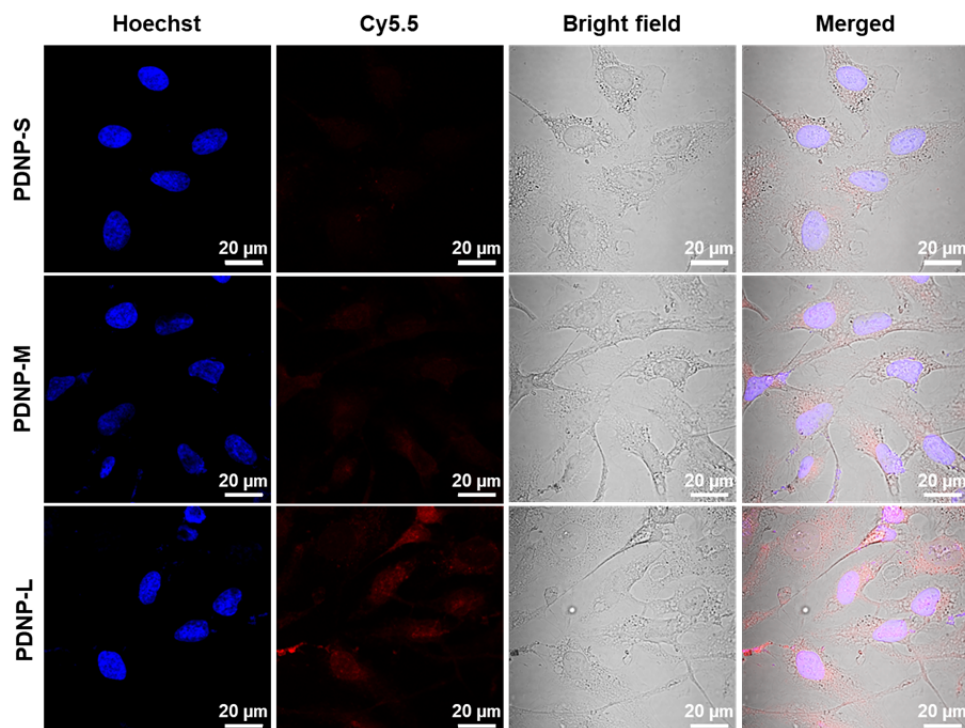


Fig. S6 Intracellular delivery of PDNs in SKOV3 cells. After 2 h incubation of Cy5.5 labeled PDNs with SKOV3 cells, fluorescence images were obtained with CLSM. Nuclei were stained with Hoechst-33342 (blue) and PDNs were labeled with Cy5.5 (red).

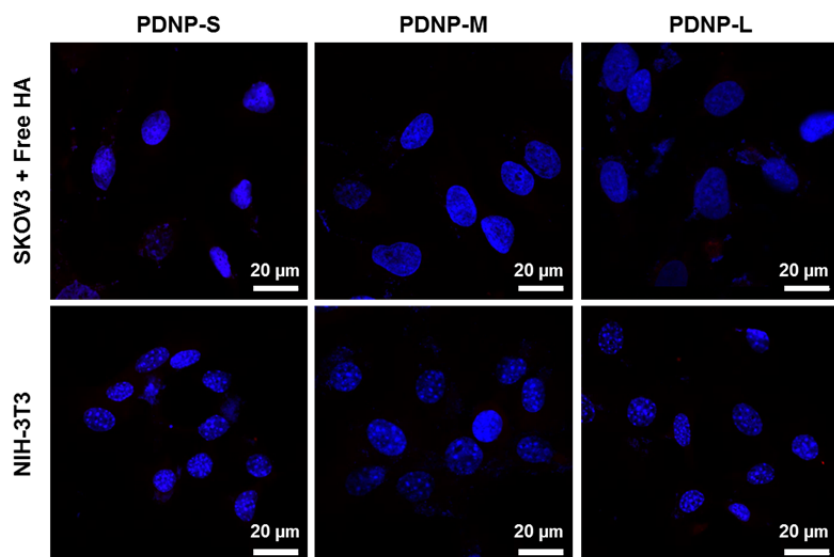


Fig. S7 Intracellular delivery of PDNs to HA pretreated SKOV3 and NIH-3T3 Cells was examined by CLSM after 2 h incubation of Cy5.5 labeled PDN-L (red). SKOV3 cells were pretreated with 40 mg/mL of HA for 1 h and stained with Hoechst-33342 (blue). Red fluorescence is indicative of the intracellular delivery of PDNs.

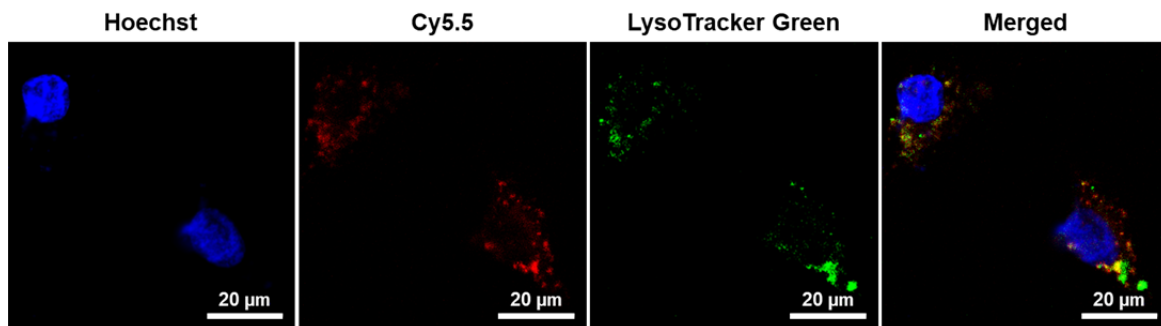


Fig. S8 Intracellular trafficking of PDN-L was examined by CLSM. Fluorescence images were obtained after 4 h incubation with Cy5.5 labeled PDN-L (red). Lysosomes were stained with LysoTracker Green (green) and nuclei were stained with Hoechst-33342 (blue). Red fluorescence of the merged image is indicative of the successful endosomal escape of PDN-L.

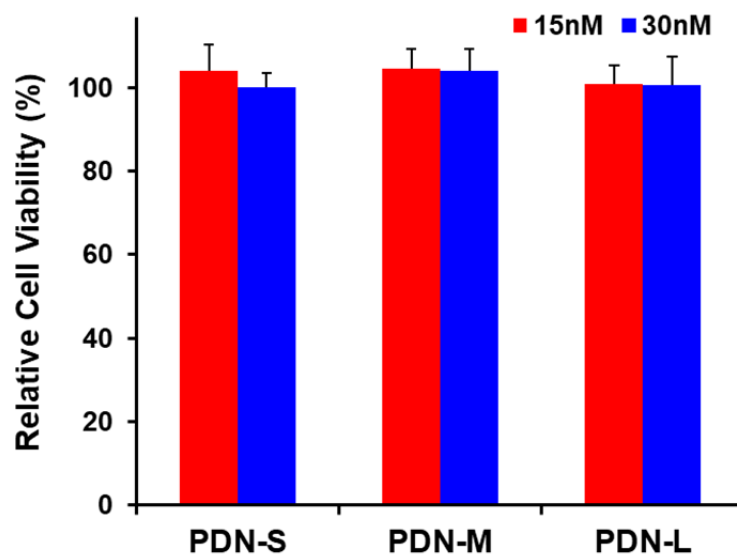


Fig. S9 Cytotoxicity of SKOV3 cells treated with PDNs at viable ODN concentrations (15 and 30 nM).