<Supplementary Information>

Gold-Installed Biostable Nanocomplexes for Tumor-Targeted siRNA Delivery *In Vivo*

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Experimental methods

1. Materials

Sodium hyaluronate (MW = 4.7 kDa, Lifecore Biomedical, Chaska, MN, USA) was used following dialysis against distilled water and lyophilization. Monomethoxy PEG-amine (PEG, MW = 5 kDa), branched PEI (MW = 25 kDa), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), Gold(Ⅲ) chloride trihydrate, thiazolyl blue tetrazolium bromide (MTT), ethidium bromide (EtBr), and ammonium peroxodisulfate (APS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). DAPI Fluoromount-G was purchased from SouthernBiotech Co. (Birmingham, AL, USA). The RFP siRNA (sense strand: 5'-UGU AGA UGG ACU UGA ACU Cdtdt-3', anti-sense strand: 5'-GAG UUC AAG UCC AUC UAC AdTdT-3') and a scrambled negative control siRNA (AccuTarget^{TM}) were obtained from Bioneer Corporation (Daejeon, Korea). The YOYO-1 fluorescence dye was purchased from Life Technologies Korea LLC (Seoul, Korea). The LysoTracker Red was purchased from Invitrogen (Carlsbad, USA). The NIR dye, Cy5.5 (λ_{ex} = 675 nm, λ_{em} = 695 nm), was purchased from Amersham Biosciences (Piscataway, NJ, USA). The murine melanoma cell line (B16F10) and mouse embryo fibroblast cell line (NIH3T3) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and RFP-expressing melanoma cells (RFP-B16F10) were kindly donated by Kyungpook National University (Daegu, Korea). RPMI 1640, DMEM and OPTI-MEM media were obtained from Welgene Inc. (Daegu, Korea). All other chemicals were reagent grades and used without further purification.

2. Preparation and characterization of GICs

PEGylated HA, bearing five PEG molecules per 100 sugar residues of HA, was prepared by chemical modification of HA with PEG in the presence of EDC and HOBt as descried previously.[1, 2] The PEG content was determined by integration ratio between the characteristic peaks of HA at 2.0 ppm(a) and PEG at 3.6 ppm(b) using ^{1}H NMR (UnityPlus500, Varian, CA, USA). The GICs were prepared using the layer-by-layer method. PEI (0.5 mg/ml in RNase-free distilled water) was mixed with siRNA (1 mg/ml in RNase-free distilled water) at different weight ratios of PEI to siRNA, followed by incubation for 30 min at room temperature. For growth of AuNPs on PEI/siRNA complexes, gold chloride solution (0.5 mole ratio of PEI amine, 1 mg/ml in RNase-free distilled water) was slowly added to the complex solution. Afterwards, the solution was incubated at 60 ^{o}C until the solution color changed from yellow to pink. The PEGylated HA solution (0.5 mg/ml in RNase-free distilled water) was added to the complex solution, which was incubated for 30 min at room temperature to obtain GICs. The GICs with a scrambled negative control siRNA (scrambled GICs) were also prepared to evaluate gene silencing effect *in vivo*.

The formation of complexes was confirmed by a gel retardation assay, and siRNA was visualized using a GelDoc-it 310 imaging system (UVP LLC, CA, USA) after being stained with ethidium bromide. The formation of gold nanoparticles was analyzed with an UV-visible spectrophotometer (Optizen3320, Mecasys Inc., Korea). The hydrodynamic diameter and zeta-potentials of the complexes were determined at room temperature using the dynamic light scattering device (Zetasizer Nano ZS, Malvern Instrument Ltd., Worcestershire, UK) at 632 nm. To visualize the morphology of the complexes, the complex solution was dropped onto a carbon-coated copper grid and dried at room temperature. The complexes were then

observed using transmission electron microscopy (TEM, JEM-2100F, JEOL, USA).

3. *In vitro* stability of the complexes

The *in vitro* stability of complexes was evaluated in a 50% serum solution.[3] Briefly, the free siRNA, GDCs and GICs were incubated with rat serum solution at 37 °C. An aliquot was removed from the samples at predetermined time points. The heparin solution (1 μl, 40 mg/ml) was added to the GDC solution to evaluate the dissociation behavior of the complexes. For the GIC solution, the glutathione solution (1 μl, 10 mM) was also added to observe dissociation of AuNPs from the complexes. The siRNAs released were observed by electrophoresis in 8% polyacrylamide gel containing ethidium bromide. For the quantitative analysis, the relative bands of siRNA at different time points were detected by measuring the intensity at the region of interest (ROI) using Image J software (NIH, USA).

4. *In vitro* cytotoxicity and cellular uptake behavior of complexes

B16F10 and NIH3T3 cells were cultured in RPMI 1640 and DMEM medium, respectively, containing 10% FBS and 1% penicillin-streptomycin at 37 °C in a CO_2 incubator. The cells were seeded onto 96-well plates at a density of 5×10^3 cells/well and incubated for 24 h at 37 °C. After cell attachment, the medium was replaced with serum-free medium containing GDCs or GICs, and incubated for 12 h at 37 °C. Cell viability was assessed using the MTT assay.

For the cellular uptake tests, RFP-B16F10 and NIH3T3 cells were dispersed into a 6-well plate at 5×10^4 cells/well and incubated for 24 h at 37 °C. siRNA was labeled with YOYO-1 dye (λ_{ex} = 491 nm, λ_{em} = 509 nm) to obtain intracellular fluorescence images. Free siRNA,

GICs, or GDCs (at a concentration of 50 nM siRNA) in serum-free OPTI-MEM medium were incubated with the cells for 3 h in a 37 ^{o}C incubator. The media was then removed and the cells were washed with Dulbecco's PBS (DPBS). After fixation with a 4% paraformaldehyde solution, cells were stained for 10 min using DAPI Fluoromount-G solution, followed by visualization of cellular morphology using a confocal LSM 700 microscope (Carl Zeiss, Göttingen, Germany).

For the flow cytometry analysis, B16F10 cells were plated in 6-well plates and treated with YoYo-1 dye-labeled free siRNA, GDCs, and GICs. After the 3 h incubation at 37 ^{o}C, the cells were washed with DPBS, trypsinized and diluted with 10 ml of an OPTI-MEM medium. Following centrifugation (1500 rpm, 3 min), the cell pellet was suspended in flow buffer (PBS supplemented with 2% FBS). The cells were analyzed using a flow cytometry (Guava Easycyte, Millipore, USA). The data were analyzed using a Guava EasyCyte software.

5. *In vitro* cellular distribution of complexes

For the investigation of intracellular distribution of the siRNA, the B16F10 cells seeded on the glass coverslips in a 12-well plate were treated for 3 h with free siRNA, GDCs and GICs. Thereafter, LysoTracker Red with a concentration of 100 nM was used to stain the endosomes and lysosomes. After fixation with a 4% paraformaldehyde solution, cells were stained for 10 min using DAPI Fluoromount-G solution, followed by visualization of fluorescences from YOYO-1-labeled siRNA and LysoTracker Red-labeled lysosomes using a confocal LSM 700 microscope (Carl Zeiss, Göttingen, Germany).

6. *In vitro* gene silencing behavior

RFP-B16F10 cells were seeded at a density of 1×10^5 cells/well. Following a 24 h incubation, the media was replaced with the free-siRNA, GDCs or GICs (at a concentration of 200 nM siRNA) in serum-free OPTI-MEM medium for 3 h. Afterwards, the cells were washed with DPBS, incubated for 48 h, and fixed using 4% paraformaldehyde solution. The cells were stained for 10 min using DAPI Fluoromount-G solution and observed using a confocal LSM 700 microscope.

7. *In vivo* biodistribution

All animal experiments were conducted under the relevant laws and institutional guidelines of Sungkyunkwan University and the institutional committees approved the experiments.

For the *in vivo* biodistribution tests, GICs and GDCs were prepared using Cy5.5-labeled PEG-HA which was synthesized according to the procedure reported.[1-3] Briefly, Cy5.5-amine was conjugated to PEG-HA in the presence of EDC and HOBt. To prepare animal tumor models, 1×10^6 SCC7 cells were subcutaneously injected into the left flank of mice. When the tumors volume reached approximately 80 – 150 mm^3, 200 μl of the Cy5.5-labeled complexes (10 μg of siRNA/mouse) was intravenously injected into the tumor-bearing mice ($n = 3$ for each group). Tumor volumes were calculated as $a \times b^2/2$, where a and b were the largest and smallest diameters, respectively. The time-dependent *in vivo* biodistribution of the complexes was observed with a whole-body animal imaging system (Optix MS3, ART Advanced Research Technologies Inc., Montreal, Canada). The excitation and emission points were raster-scanned in 1-mm steps over the selected polygon region of interest (ROI) to obtain whole body NIRF images. By measuring the NIRF intensity at the tumor site, the

tumor accumulation profiles of the complexes were evaluated. To observe the organ distribution of complexes, each group of mice was sacrificed after 24 h. The major organs and tumors were then excised and observed using the above described imaging system. All the data were calculated using ROI function of the Analysis Workstation software (n = 3, ART Advanced Research Technologies Inc., Montreal, Canada).

8. *In vivo* gene silencing

To assess the *in vivo* gene silencing efficacy of complexes, RFP-B16F10 tumor-bearing mice were prepared by injecting a suspension of 1×10^6 RFP-B16F10 cells in saline (100 μl) into the left flank of nude mice. Mice were divided into four groups: (i) saline, (ii) free siRNA, (iii) GDCs, (iv) GICs, and (v) scrambled GICs. When the tumors grew to approximately 60 – 80 mm^3 in volume, 200 μl of the complexes (10 μg of siRNA/mouse) was injected daily for 3 days into the tail veins of the mice. The red fluorescence signal, expressed in the tumor tissue, was observed for 3 days using a whole-body animal imaging system. A 532-nm pulsed laser diode was used to excite the RFP cells. All of the data were calculated using the ROI function of the Analysis Workstation software (n = 3).

9. Statistical analysis

The statistical significance among the groups was analyzed using one-way ANOVA. The figure, marked with an asterisk, was considered significant if p-value was less than 0.05.

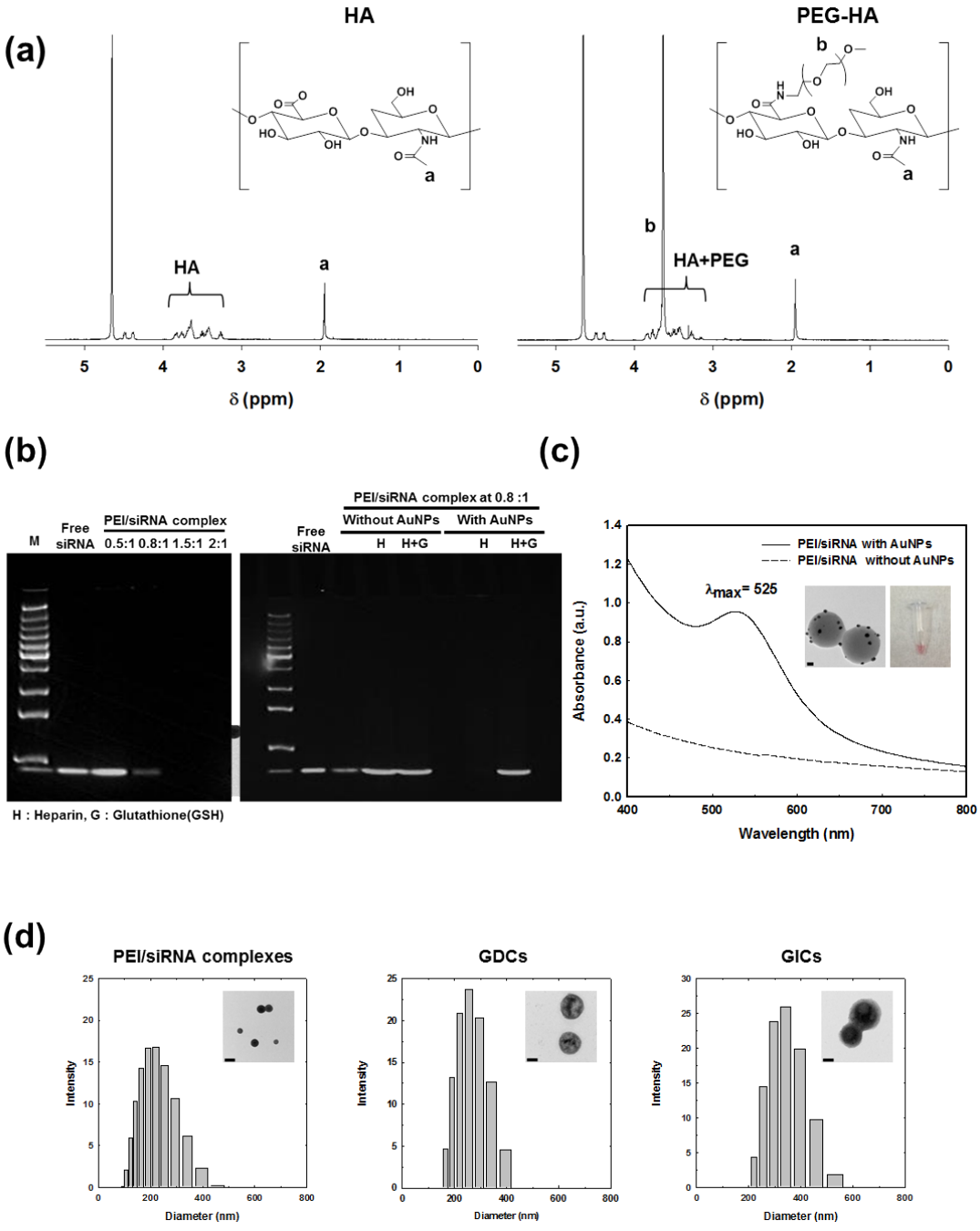

Fig. S1. (a) ^{1}H NMR spectra of HA and PEG-HA. (b) Gel retardation assay of PEI/siRNA complexes with and without AuNPs. (c) UV-vis spectroscopy of PEI/siRNA complexes before and after gold installation. The inset is the TEM image (scale bar: 20 nm). (d) Size distribution of PEI/siRNA, GDCs and GICs. The insets are TEM images (scale bars are 200 nm for PEI/siRNA complexes and 100 nm for GDCs and GICs).

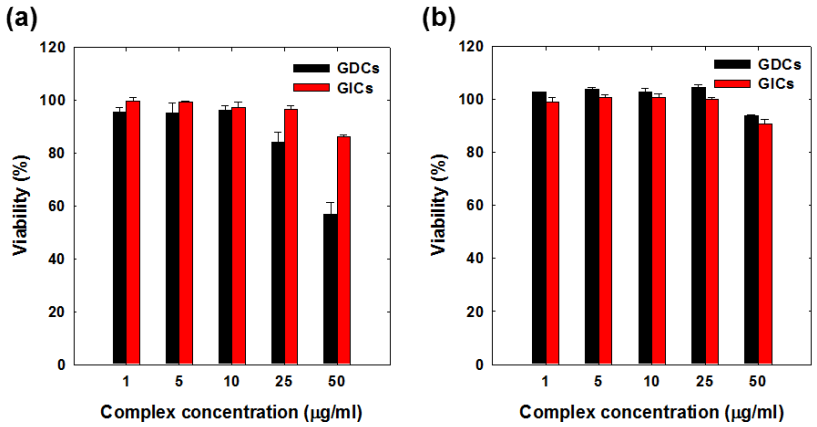

Fig. S2. Cellular cytotoxicity of the GDCs and GICs in (a) B16F10 (b) NIH3T3 cells.

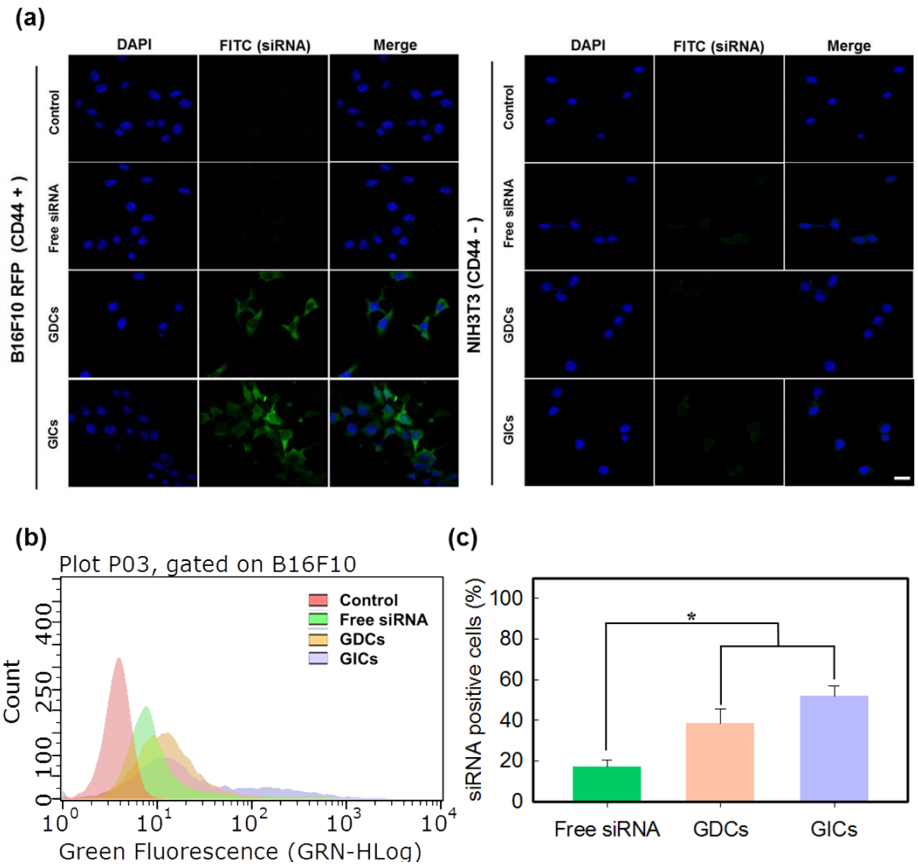

Fig. S3. *In vitro* cellular uptake of complexes (siRNA 50 nM). (a) Confocal LSM images of cellular uptake with free siRNA, GDCs and GICs. Each sample was treated for 3 h. The scale bar represents 20 μm. (b) Flow cytometric histogram profiles of B16F10 cells. (c) Quantification of siRNA positive cells after treatment with free siRNA, GDCs, and GICs.

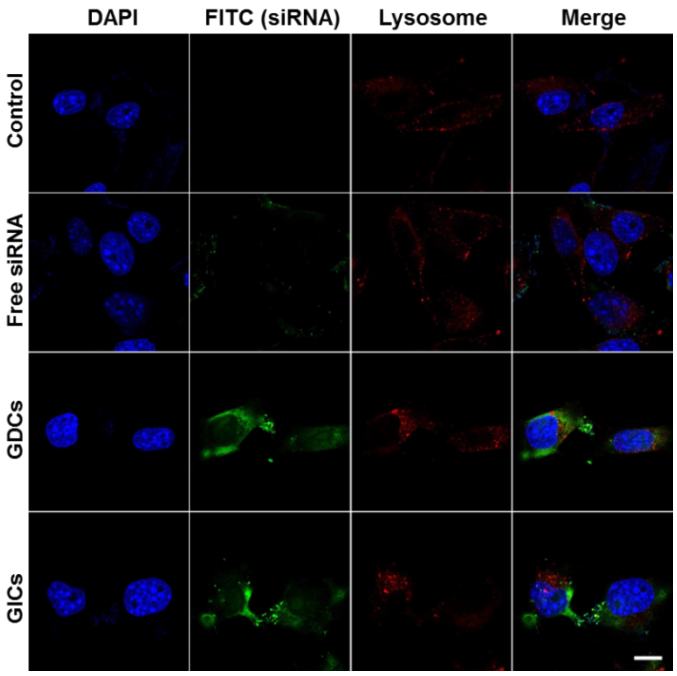

Fig. S4. Confocal images of the intracellular distribution of free siRNA, GDCs and GICs. The scale bar represents 10 μm.

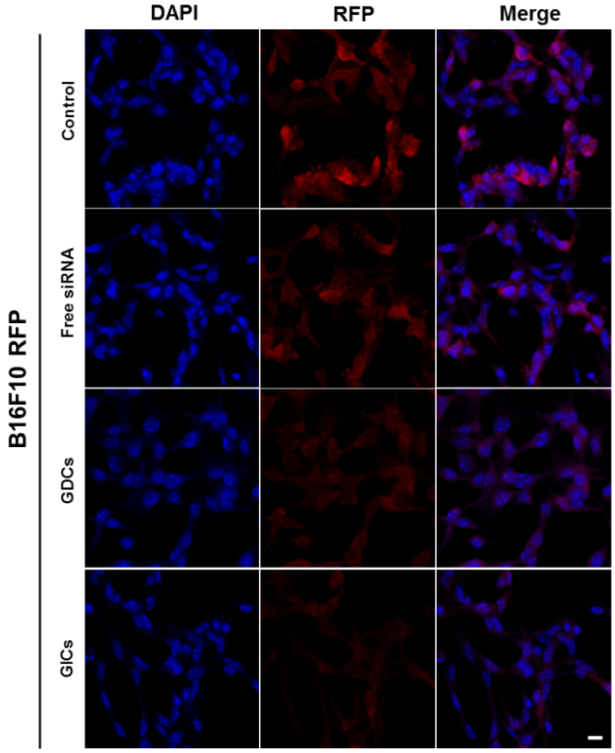

Fig. S5. *In vitro* gene silencing efficacy of (siRNA 200 nM). Each sample was treated for 3 h then incubated for an additional 48 h. The scale bar represents 20 μm.

Table S1. Characteristics of the PEI/siRNA complexes, GDCs and GICs.

Sample	Size (nm)[a]	ζ (mV)[b]
PEI/siRNA complexes	213.0 ± 5.56	44.4 ± 2.46
GDCs	247.6 ± 1.63	- 5.58 ± 0.18
GICs	327.6 ± 10.24	- 7.58 ± 0.81

[a]Mean diameters were measured using dynamic light scattering
[b]Zeta potential of the complexes.

References

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