Supporting information

Atom transfer radical polymerization of multi-shelled cationic corona for the systemic delivery of siRNA

*Hye Sung Kim[†], Young Ju Son[†], Wei Mao[†], Kam W. Leong[‡], and Hyuk Sang Yoo[†,§,]**

[†]Department of Biomedical Materials Engineering, Kangwon National University, Chuncheon, 24341, Republic of Korea, [‡]Department of Biomedical Engineering, Columbia University, New York, NY, 10027, USA, [§]Institute of Bioscience and Bioengineering, Kangwon National University, Chuncheon, 24341, Republic of Korea

*Corresponding Author: Hyuk Sang Yoo (hsyoo@kangwon.ac.kr)

Experimental section

Materials

Gold(III) chloride hydrate ($HAuCl_4$), Bis[2-(2-bromo isobutyryloxy) undecyl] disulfide (disulfide initiator), 2-(dimethylamino)ethyl methacrylate (DAMA), 2-hydroxyethyl methacrylate (HEMA), copper bromide (CuBr), 2,2'-bipyridyl (Bpy), DL-dithiothreitol, and plasma from mouse were purchased from Sigma-Aldrich (St. Louis, MO). RPMI1640 medium, fetal bovine serum (FBS), TRIzol® reagent, and APO-BrdU™ TUNEL Assay Kits were obtained from ThermoFisher Scientific (Grand Island, NY). The primers for the real-time PCR, c-Myc siRNA, and FITC-labeled siRNA were purchased from Bioneer (Daejeon, South Korea). The sequences of c-Myc siRNA suppressing the expression of human MYC proteins were 5'-GACAGUGUCAGAGUCCUGA-3' (sense) and 5'-UCAGGACUCUGACACUGUC-3' (antisense). The human lung carcinoma cell line (A549) was obtained from Korea Cell Line Bank (Seoul, South Korea). The 5x Reverse Transcription Premix and HiFi Real-Time PCR 2x Master Mix (with SYBR Green) were purchased from Elpis Biotech (Daejeon, South Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Trevigen Inc. (Gaithersburg, MD). An *in situ* cell death detection kit (POD) was purchased from Roche (Basel, Switzerland).

Preparation of polymer-multilayered AuNP

Gold nanoparticles (~18 nm in diameter) (AuNP) were prepared via reducing chloroauric acid according to the literature and were subsequently decorated with methacrylate chains via surface-initiated atom transfer radical polymerization (SI-ATRP)[11-15]. Briefly, 1 mM $HAuCl_4$ solution (100 ml) was boiled, and 38.8 mM trisodium citrate solution (5 ml) was subsequently

added under vigorous stirring. After a 10-min reaction, the mixture was cooled to room temperature for further use. The SI-ATRP was performed on the AuNPs and repeated three times to prepare the multilayers on the AuNPs. For immobilization of the initiator on the AuNPs, a disulfide initiator in N,N-dimethylformamide (DMF) (1ml) was slowly added to the gold nanoparticle suspension in DMF (2mg/ml, 1ml) and incubated at room temperature for 24 h with stirring (Au on the surface of the AuNPs and disulfide initiator = 1:5 molar ratio). The initiator-immobilized AuNPs (initiator@AuNP) were collected via centrifugation at 12,000 rpm for 10 min and then washed with DMF (1ml) 3 times via centrifugation. For the growth of the polymer chains on the surface of the AuNPs, DAMA, HEMA (molar ratio of DAMA and HEMA = 4:1, the monomer concentration is to be 20% (v/v) in the mixture), CuBr, and bpy were added to the initiator@AuNP in a DMF/DW/isopropanol(IPA) mixture (1/4/2 (v/v/v)) (molar ratio of initiator@AuNP, monomers, CuBr, and bpy = 1:100:1:2), and the reaction was conducted at room temperature under nitrogen for 3 h. The reaction mixtures were thoroughly washed with DMF/methanol (1/1 (v/v), 1ml) 5times by centrifugation to completely remove the unreacted chemicals. After the first SI-ATRP on the AuNPs (L1@AuNP), the second and third initiation/SI-ATRP cycles were repeated using the same procedure as for the first SI-ATRP (L2@AuNP and L3@AuNP). For 2^{nd} initiation, a disulfide initiator in DMF was added to the L1@AuNP dispersed in DMF and incubated at room temperature for 24h (HEMA of the L1@AuNP and disulfide initiator = 1:5 molar ratio). The initiator-immobilized L1@AuNPs (initiator@L1@AuNP) were washed with DMF 3 times via centrifugation at 12,000 rpm for 10min. For the 2^{nd} ATRP, monomers (molar ratio of DAMA and HEMA=4:1) and catalyst were added to initiator@L1@AuNP in a DMF/DW/IPA mixture (molar ratio of initiator@L1@AuNP, monomers, CuBr, and bpy= 1: 100:1:2) and reacted at room temperature under nitrogen for 3h. The reaction mixtures were thoroughly washed with DMF/methanol mixture 5times by centrifugation to completely remove the unreacted chemicals.

Characterization of the polymer-multilayered AuNPs

The particle size and ζ-potential of the polymer-multilayered AuNPs were measured using dynamic light scattering (DLS; 90Plus, Brookhaven Instrument, Holtsville, NY). Samples were measured three times for 30 s each, and the average diameters and standard errors were recorded. The morphologies of the polymer-layered AuNPs were observed via energy filtering-transmission electron microscopy (EF-TEM; LEO 912 AB, Carl Zeiss, Germany) at the Korea Basic Science Institute. The UV-Vis spectra of the polymer-layered AuNPs were measured on a UV-Vis spectrophotometer (NanoVue Plus, GE Healthcare Life Sciences, Pittsburgh, PA) to analyze the transmittance change of the surface plasmon resonance (SPR) of the gold nanoparticles. The poly(DAMA-HEMA) decorated on the surface of the AuNPs was examined using Fourier transform infrared spectroscopy (FT-IR; Nicolet 6700, ThermoFisher Scientific Inc., Waltham, MA). The amount of the polymers decorated on the AuNPs was determined through quantifying the amount of atomic gold in the polymer-layered AuNPs via inductively coupled plasma optical emission spectroscopy (ICP-OES; OPTIMA 7300 DV, PerkinElmer, Waltham, MA). The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses of the AuNPs were performed on a Voyager DE (JMS-700, JEOL, Japan) at an accelerating potential of 20 kV and a laser power of 581. A positive reflectance mode was used, and α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix. The molecular weight was determined by gel permeation chromatography (GPC) (UltiMate 3000, Dionex) with a LC column (Shodex OH pak® SB-803 HQ, 6 μm, 100 Å, Shodex). A mobile phase of DMF was employed at 0.5ml/h and signals were detected by a reflective index detector.

Incorporation of siRNA into the polymer-multilayered AuNPs

To test the feasibility of the polymer-multilayered AuNPs as gene carriers, siRNA was electrostatically incorporated into L1, L2, and L3@AuNP, and the siRNA released from the siRNA-incorporated L#@AuNP was subsequently determined. For the siRNA incorporation, the polymer-multilayered AuNPs (equivalent amount of AuNPs = 22.2 μg) in PBS (pH 7.4, 500 μl) was slowly added to siRNA (15 μg) in a silanized tube for 1 min and further incubated at 37 °C with gentle shaking for 12 h. The unincorporated siRNA was removed via centrifugation at 12,000 rpm for 10 min, and then, it was quantified at 260 nm to determine the amount of incorporated siRNA in the polymer-layered AuNPs. In addition, the number of siRNA incorporated to the polymer-multilayered AuNP was calculated by the weight ratio between siRNA and gold nanoparticle (mass of a gold nanoparticle (18-20nm in diameter) is 8.08×10^{-17}g). The particle size and ζ-potential after the siRNA incorporation were measured using DLS. To monitor the association and dissociation behaviors of the siRNA with the AuNPs, the siRNA-incorporated AuNPs were incubated in 10 mM DTT solution at 37 °C for 24 h and subjected to agarose gel electrophoresis analysis (1.2%, w/v). The released fractions were also separated via centrifugation, and the released siRNA was quantified. The morphological changes and elemental mapping of a single siRNA/polymer-layered AuNP structure were observed using a TEM equipped with electron spectroscopy imaging (ESI-TEM; JEM-2100F, JEOL, Japan) at the Central Laboratory of Kangwon National University. Elemental mapping was performed for the gold and phosphate to confirm the configuration of the AuNPs and siRNA, respectively, in the siRNA-incorporated AuNP.

Particle and siRNA stability assay

To confirm the stability of the siRNA-incorporated AuNPs, the size changes of the AuNPs were monitored, and the RNase-resistance of the AuNPs was determined[76,77]. Particles with or

without siRNA incorporation were incubated in PBS (pH 7.4) that contained 10% (v/v) fetal bovine serum, and their size changes were monitored using DLS. Naked siRNA and siRNA incorporated into the polymer-layered AuNPs (siRNA-equivalent weight: 11 μg) were incubated either in PBS (pH 7.4) containing RNase (200 μg/ml) or plasma from mouse at 37 °C for 72 h. The cationic poly(DAMA-HEMA) complexed with siRNA was removed through treatment with a heparin sodium salt solution (10 mg/ml) at 37 °C for 30 min; then, the mixture was analyzed via gel electrophoresis.

***In vitro* cellular uptake of polymer-multilayered AuNPs**

To assess the effects of the polymeric multilayers on the cellular uptakes of the AuNPs, A549 cells were seeded on a 12-well plate ($1x10^5$ cells/well) and allowed to attach in RPMI 1640 that contained 10% FBS medium for 24 h. The cells were fed with fresh culture medium and treated with FITC-siRNA-incorporated bare AuNPs or the polymer-layered AuNPs (siRNA-equivalent concentration=150nM). After 3 h of transfection, the cells were fixed with 1% formaldehyde and observed with confocal laser scanning microscopy (CLSM; FV 1000 SPD, Olympus, USA). The excitation and emission wavelength were 488 nm and 505–525 nm, respectively. The nucleus was counter-stained with DAPI. To quantify the intracellular uptake of the AuNPs, the cells were dissolved in aqua regia (500 μl) and were subsequently diluted with 3% aqua regia for the ICP-OES analyses. Real-time PCR analyses were performed to determine the gene silencing of c-Myc. A549 cells were treated with free c-Myc siRNA, c-Myc siRNA/L#@AuNP, scrambled siRNA/L#@AuNP and c-Myc siRNA/lipofectamine 3000 (siRNA-equivalent concentration=30nM or 150nM). After 3 h of transfection, the cells were fed again with fresh culture medium and incubated for an additional 24 h. The total RNA was harvested with TRIzol® reagent, synthesized into cDNA, and amplified with a HiFi Real-Time

PCR 2x Master Mix (with SYBR Green). The reaction was performed as follows: cDNA was synthesized at 42 °C for 60 min and at 94 °C for 5 min. The initial denaturation was performed at 94 °C for 3 min, followed by 40 cycles of 94 °C for 10 s, 52 °C for 25 s, and 72 °C for 30 s. The following primers were used: GAPDH, 5'-TCC CTG AGC TGA ACG GGA AG-3' (forward primer) and 5'-GGA GGA GTG GGT GTC GCT GT-3' (reverse primer); c-Myc, 5'-TCA AGA GGC GAA CAC ACA AC-3' (forward primer) and 5'-GGC CTT TTC ATT GTT TTC CA-3' (reverse primer).

Cytotoxicity assay

To evaluate the cytotoxicity of the polymer-layered AuNPs and siRNA/polymer-layered AuNPs, an MTT-based cytotoxicity assay and a TUNEL assay were performed against the A549 cells, respectively. For the MTT assay, the cells were seeded on a 12-well plate at a density of $1x10^5$ cells per well in RPMI 1640 with 10% FBS. After incubation for 24 h, the cells were treated with bare AuNP or polymer-layered AuNPs (AuNP concentration =0–10 μg/ml) or with c-Myc siRNA/L#@AuNP or scrambled siRNA/L#@AuNP (siRNA concentration = 150nM). After 3h of incubation, the cells were washed with PBS 3 times to remove non-internalized gold nanoparticles and fed again with fresh culture medium. After an additional 24 h of incubation, the MTT solution (10 μl, 5 mg/ml) was added to each well and incubated for 3 h. Formazan crystals were dissolved with DMSO, and the absorbance at 570 nm was measured (Mutiskan GO, ThermoFisher Inc., USA) to determine the cell viabilities compared with the cells in the untreated group.

For the TUNEL assay, A549 cells were seeded on a 6-well plate at a density of $5x10^5$ cells per well. After 24 h of incubation, polymer-layered AuNPs with or without siRNA

incorporation (siRNA-equivalent concentration=150nM) were treated in each well and further incubated for 3 h. After an additional 24 h of incubation, the cells were fixed in 1% (w/v) formaldehyde in PBS (5 ml) and permeabilized in 70% (v/v) ice-cold ethanol (5 ml). A DNA-labeling solution (50 μl) that contained terminal deoxynucleotidyl transferase (TdT enzyme) and 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) was treated in the cells and incubated at 37 °C for 60 min. After washing the cells with a rinse buffer, Alexa Fluor 488-labeled anti-BrdU antibody solution (100 μl) was added to stain the cells at room temperature for 30 min. The stained cells were analyzed using flow cytometry (FACS Calibur, BD Biosciences, USA) and CLSM with an excitation wavelength of 488 nm and an emission wavelength of 505–525 nm.

Animal studies

Tumor model and siRNA treatment

For solid tumor formation, human lung carcinoma cells (A549, $1x10^7$ cells/animal) were subcutaneously injected into the right flank of female athymic nude mice at 4 weeks (n = 10) (Daihan Biolink, South Korea). When the volume of the A549 tumor xenograft reached approximately 40–50 mm^3/tumor, naked c-Myc siRNA, c-Myc siRNA/L3@AuNP, scrambled siRNA/L3@AuNP, and L3@AuNP alone (equivalent amount of siRNA = 1.6 mg/kg) suspended in PBS (100 μl) were intravenously injected through the tail vein on days 1, 7, 13, and 19. The control group was injected with PBS. The body weight of the mice and the tumor sizes were recorded every 2 days; the tumor growth was determined through measuring the perpendicular diameters using calipers and was calculated by (major axis) × $(\text{minor axis})^2/2$. All of the animal experiment procedures were conducted in accordance with the institutional guidelines for the care and use of experimental animals (KW-150331-1).

Quantification of c-Myc expression in the tumor tissue

To determine the intratumoral level of c-Myc mRNA, the tumors were harvested from the tumor-bearing mice at 20 and 28 days, respectively. The tumors were weighed and homogenized, and the total RNA was isolated from the tissue homogenates using TRIzol® reagent. Real-time PCR was performed using the same method as was used for the *in vitro* real-time PCR.

***In vivo* fluorescence imaging and bio-distribution**

The A549 tumor-bearing mice were injected via the tail vein with L3@AuNP that incorporated Alexa Fluor 647-labeled siRNA (100 μg of siRNA). At 6 h and 24 h post-injection, the mice were anesthetized and fluorescently imaged using an In Vivo Imaging System (IVIS 200, Caliper Life Sciences Inc., USA) with an excitation at 640 nm and an emission at 650–670 nm in Korea Basic Science Institute (KBSI). In addition, the mice were euthanized at 24 h post-injection, and the major organs (liver, lungs, spleen, kidneys, and heart) and tumor tissue were excised, and their fluorescence images were obtained. The amount of Au that accumulated in the major organs and tumor tissues was quantified using ICP-OES. The tissues were homogenized and dissolved in aqua regia/hydrogen peroxide (7/3, v/v). After incubation at 80 °C for 12 h, the homogenates were diluted and then filtrated through a 0.45-μm filter membrane.

Immunohistochemical staining

The immunohistochemical staining was performed in accordance with the manufacturer's

instructions (In situ cell death detection kit, POD, Roche). The tissue samples were fixed with a 4% formaldehyde solution for 1 h, dehydrated in a graded ethanol series, and then embedded in paraffin. The tissue sections were prepared using a microtome with a 4 μm thickness and deparaffinized with xylene and a series of graded ethanol (100%, 70%, and 30%). The rehydrated tissue sections were subjected to antigen retrieval in a 0.1 M sodium citrate buffer (pH 6.0) and microwave irradiation for 5 min. After immersing the sections in 0.1 M Tris-HCl (pH 7.5) that contained 3% BSA and 20% normal bovine serum at room temperature for 30 min, the TUNEL reaction mixture (50 μl) was treated and incubated at 37 °C for 60 min. After washing the sections with PBS three times, the converter-POD (50 μl) was added and subsequently incubated at 37 °C for 30 min. Then, the DAB substrate (100 μl) was treated and incubated for 10 min. The tissue sections were counter-stained with Harris hematoxylin. For c-Myc staining, the tissue sections were treated with 0.3% hydrogen peroxide for 30min to block endogenous peroxidase activity and saturated in diluted normal horse serum for 20min. The tissue sections were incubated with an anti-c-Myc antibody (rabbit polyclonal IgG) (1/50) at 4°C overnight and a secondary antibody (biotinylated ‘‘universal’’ anti-mouse IgG/anti-rabbit IgG) was subsequently incubated for 30min at room temperature. A streptavidin horseradish peroxidase reagent (VECTASTAIN® R.T.U ABS reagent) was treated for 30min and developed in freshly prepared 3,3’-diaminobenzidine substrate for 10min. The tissue sections were counter-stained with Harris hematoxylin.

Table S1. Polymer shell growth according to HEMA contents. Polymer shell thickness was calculated based on particle size changes measured by DLS.

HEMA content	1st layer thickness (nm)	Polymer shell thickness after 2nd ATRP (nm)	Thickness increase (nm)	Thickness increase (%)
10%	28.3±2.1	37.4±2.7	9.1±0.6	32.2
20%		40.3±2.4	12.0±0.3	42.4
50%		31.1±2.3	2.7±0.2	9.5

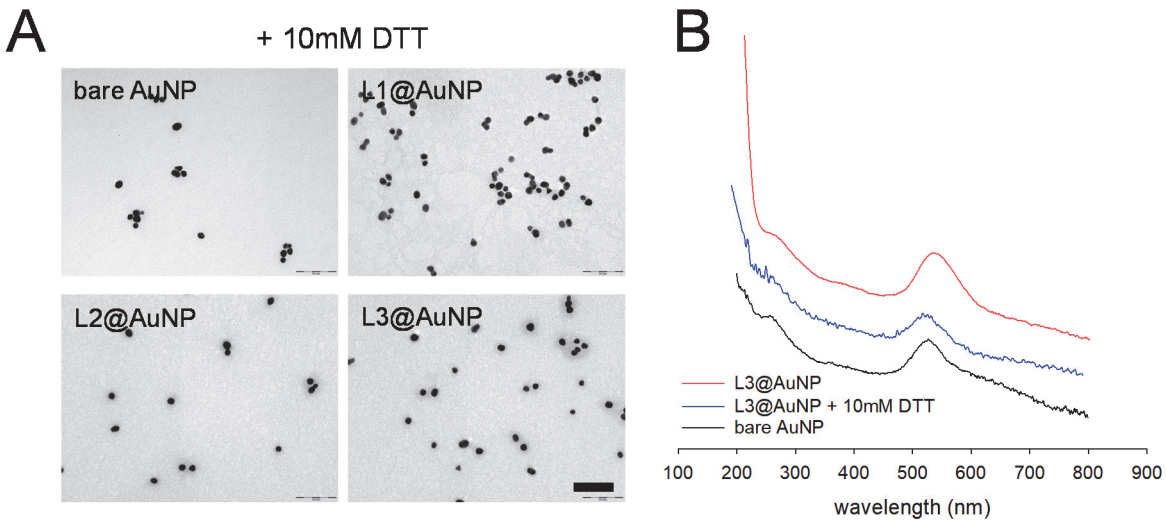

Figure S1. (A) TEM images of polymer-multilayered AuNP treated with 10mM DTT (scale bar =100nm). (B) UV-Vis spectra of bare AuNP and polymer-multilayered AuNP with or without 10mM DTT.

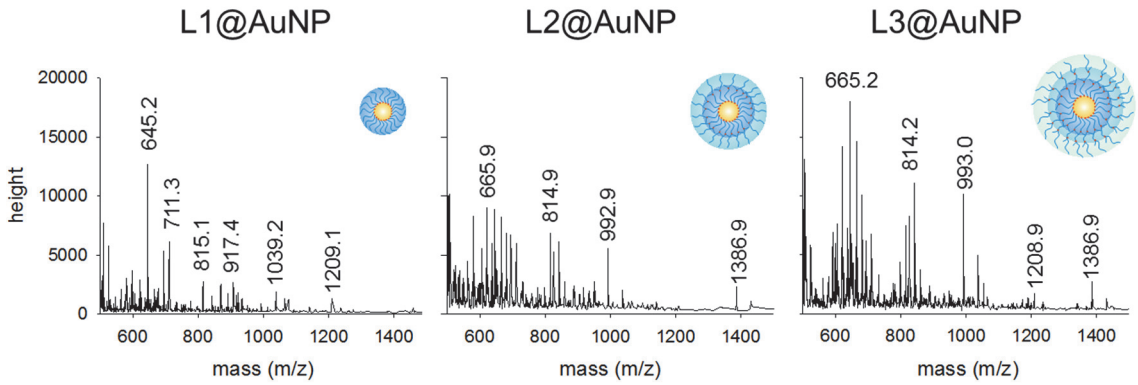

Figure S2. MALDI-TOF mass spectra in a reflector mode of polymer-multilayered AuNP in the low range from 500-1500Da.

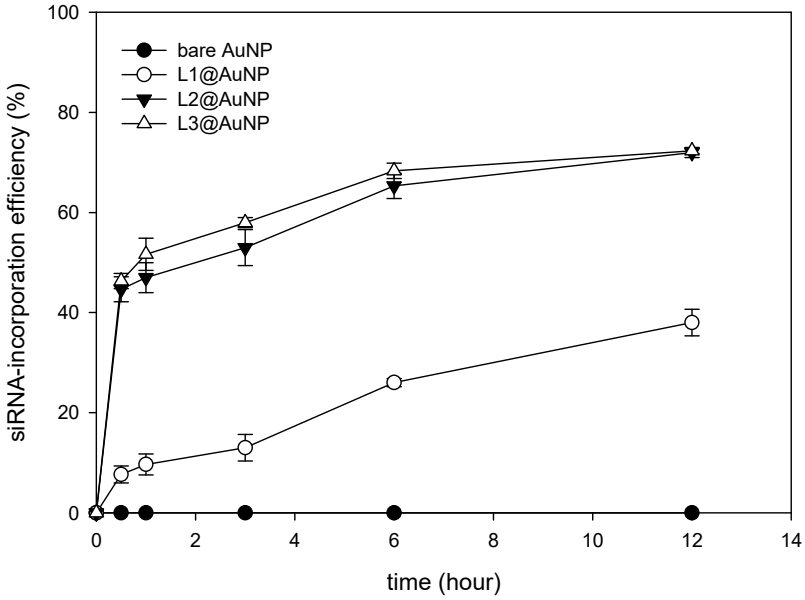

Figure S3. SiRNA incorporation profile of polymer-multilayered AuNP.

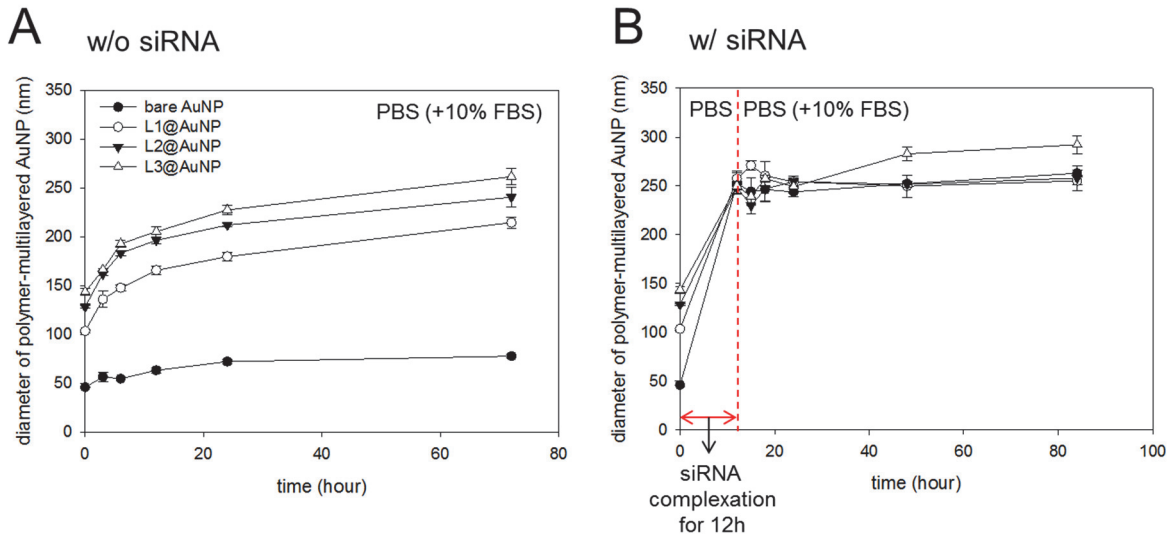

Figure S4. Particle stability test in the presence of high concentration of serum (10% FBS). Size change of the polymer-multilayered AuNP with or without siRNA was monitored by DLS.

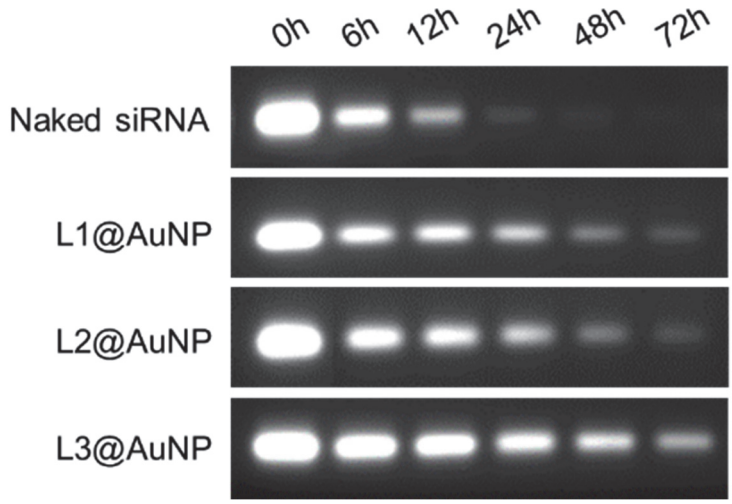

Figure S5. Stability test of siRNA-incorporated AuNPs in the presence of mouse plasma for 72h.

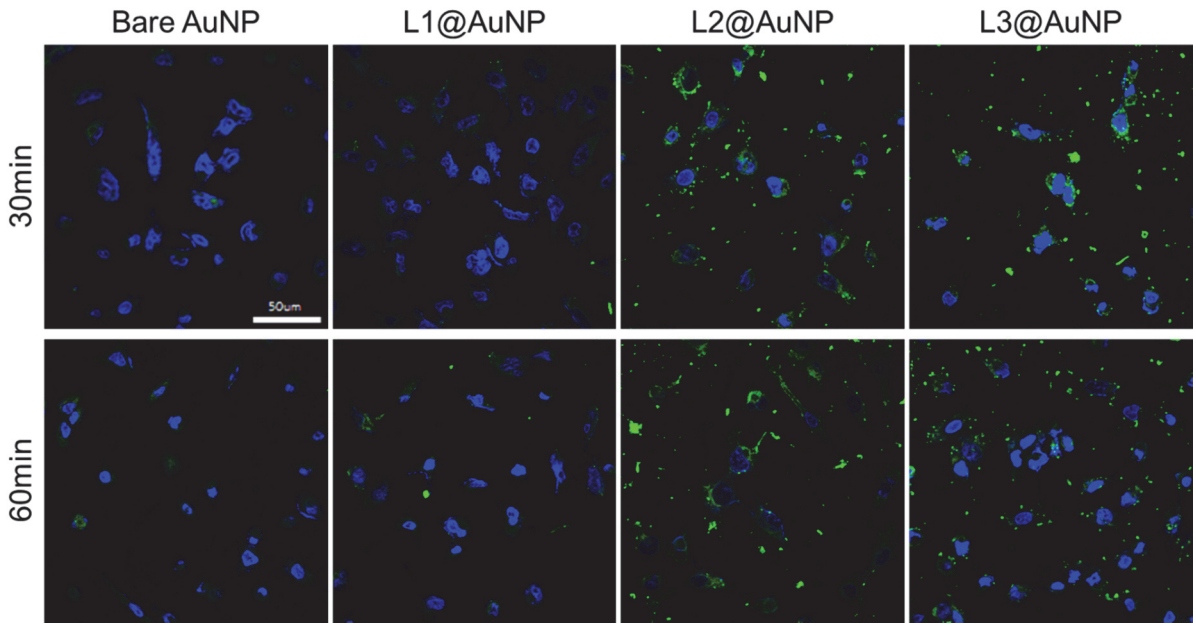

Figure S6. Fast adsorption of FTIC-siRNA/L2 or L3@AuNP in 30min.

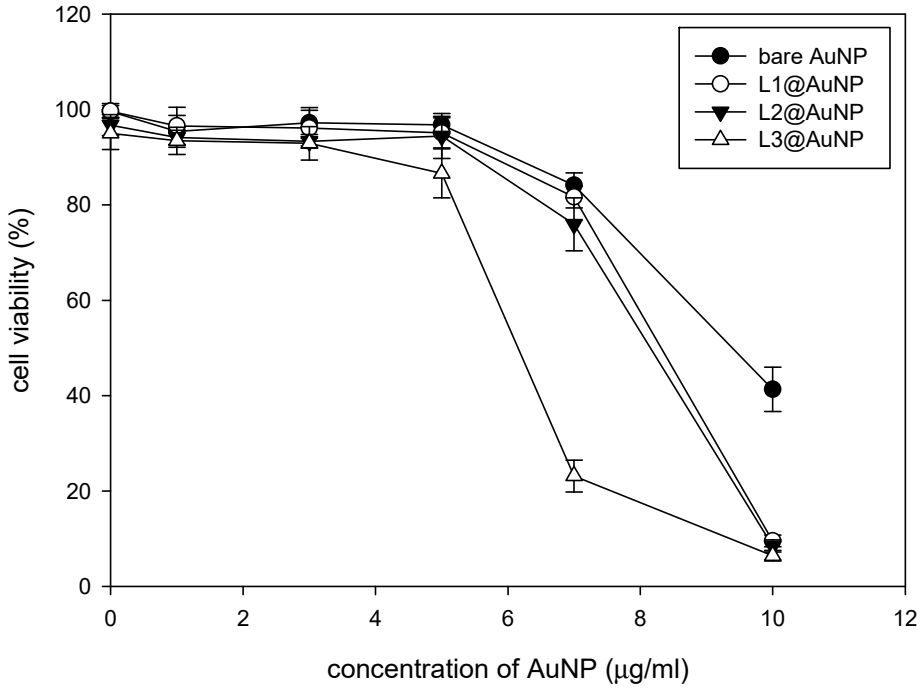

Figure S7. MTT-based cytotoxicity assay against A549 cells cultivated with various concentration of bare AuNP and polymer-multilayered AuNP.

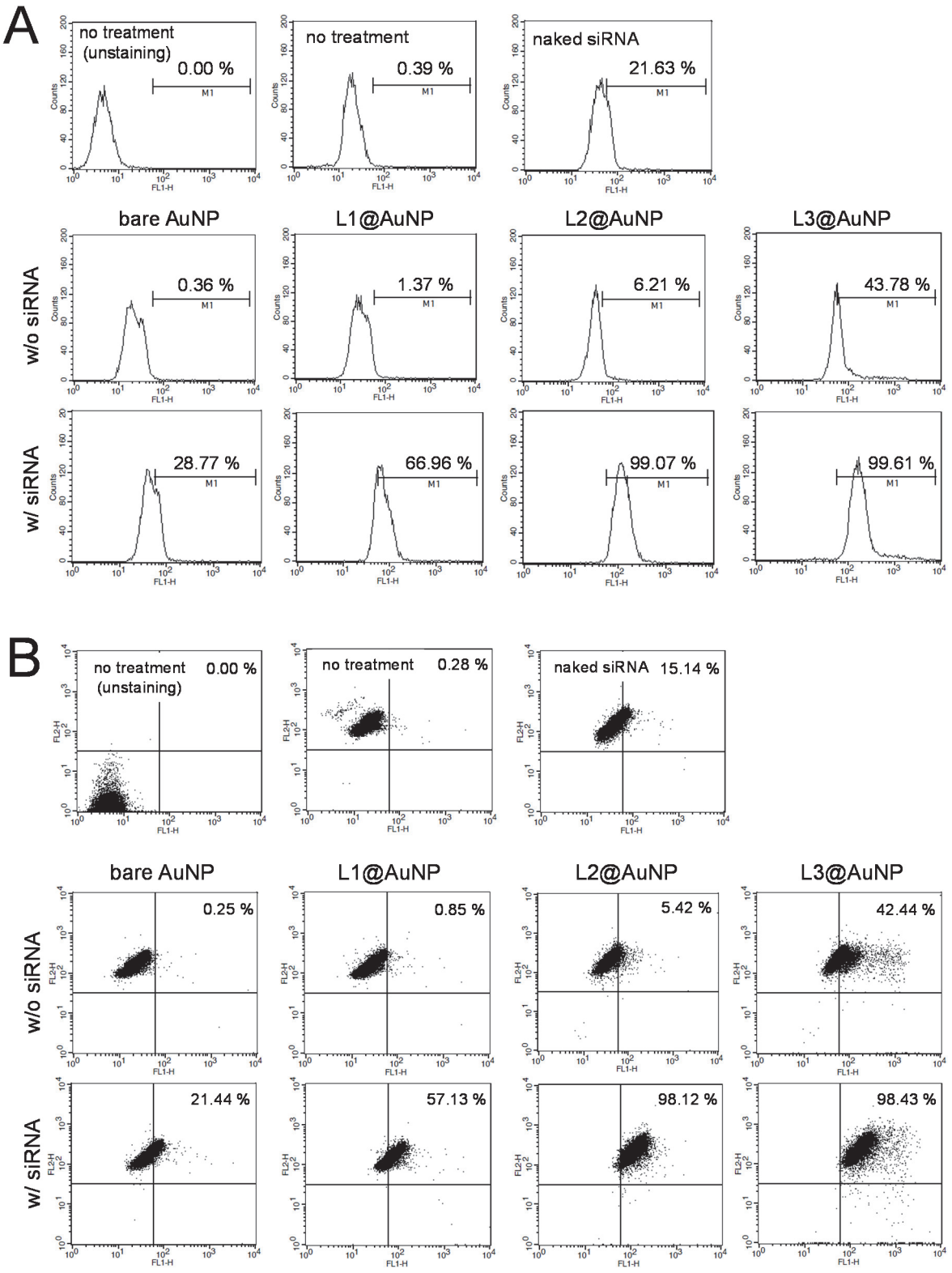

Figure S8. Flow cytometry analysis of a TUNEL-positive population for apoptosis induced by siRNA-incorporated AuNP displayed as histograms (A) and dot plots (B), respectively. The percentage of apoptotic cells are marked in the respective graph.

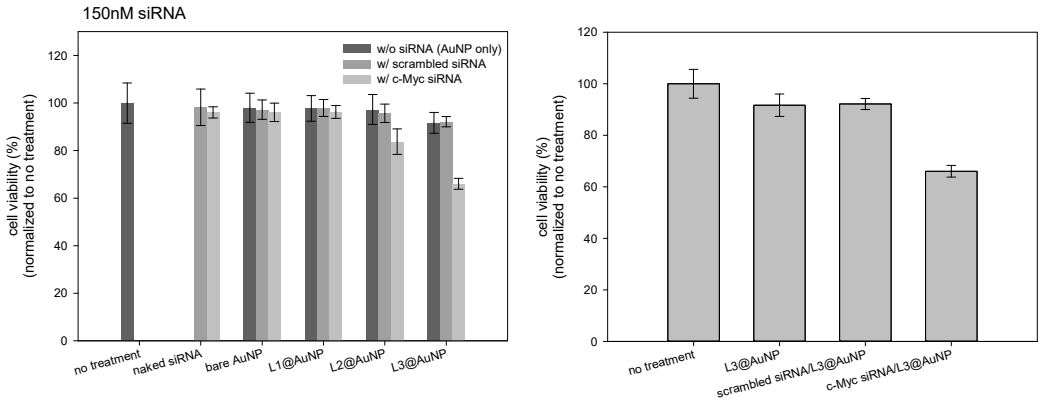

Figure S9. Cell proliferation rate of A549 treated with naked c-Myc siRNA, c-Myc siRNA/L#@AuNP, and scrambled siRNA/L#@AuNP, respectively.

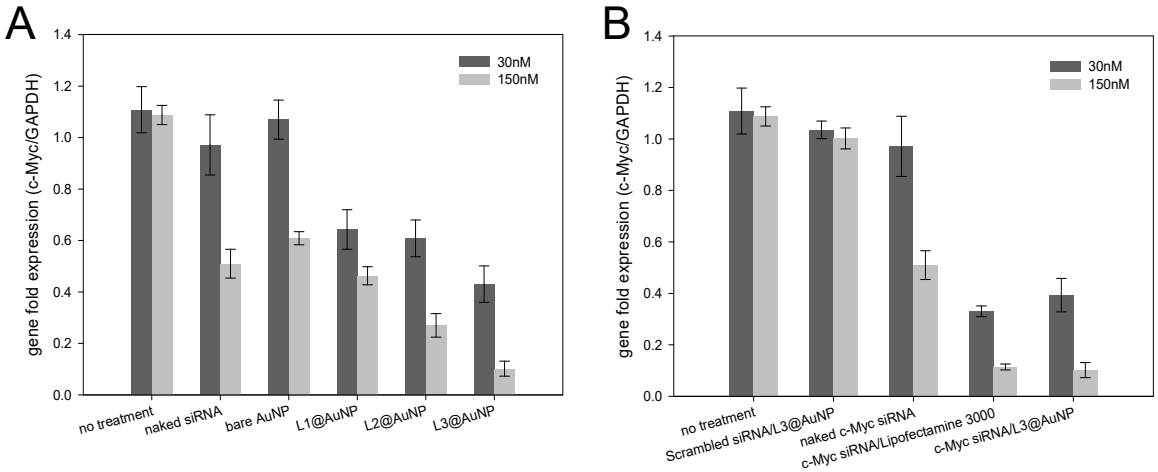

Figure S10. *In vitro* expression levels of c-Myc in A549 cells exposed to L#@AuNP with (A) low (30nM) or high (150nM) c-Myc siRNA dose and (B) scrambled siRNA/L3@AuNP or c-Myc siRNA lipoplex.

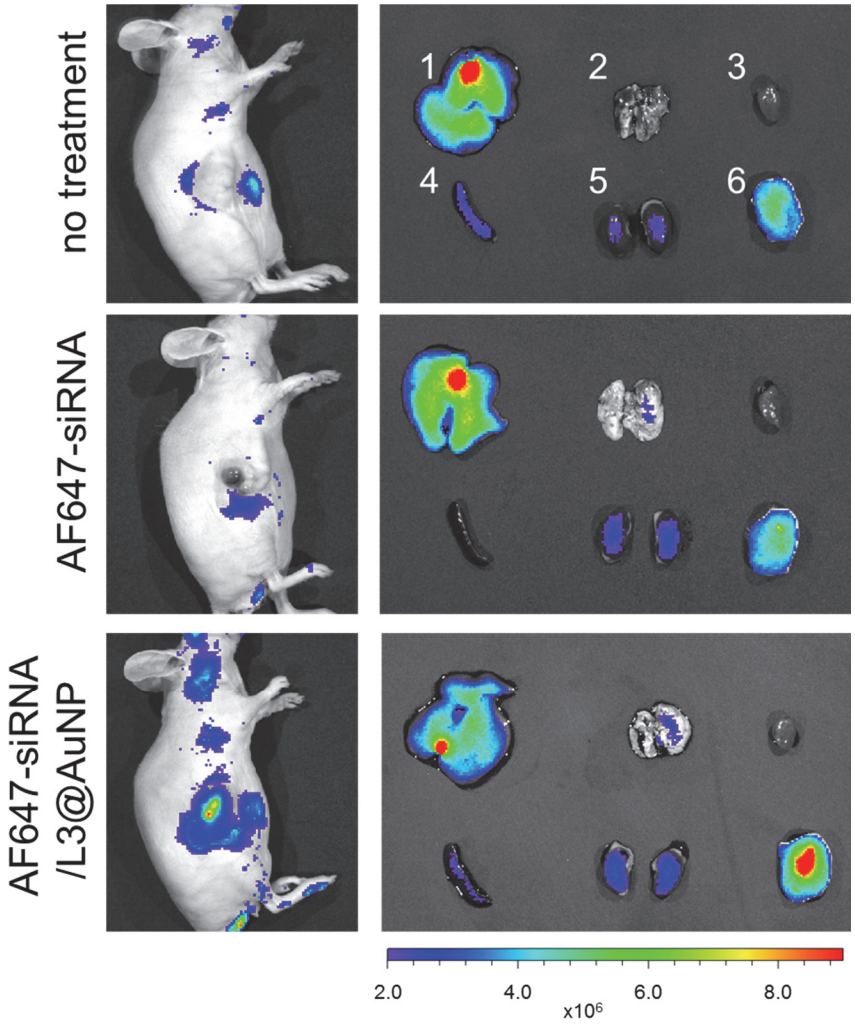

Figure S11. *Ex vivo* fluorescence images of tissues including (1) liver, (2) lung, (3) heart, (4) spleen, (5) kidney, and (6) tumor collected at 24h post-injection. A group of mice injected with PBS (no treatment) was used as a control.

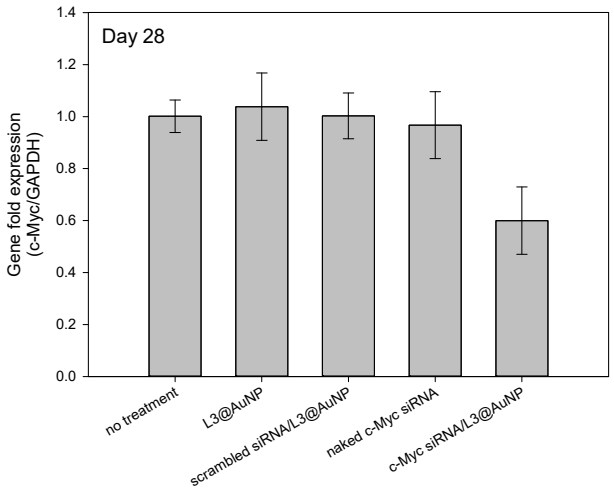

Figure S12. *In vivo* expression level of c-Myc in A549 tumor xenografts at day 28.

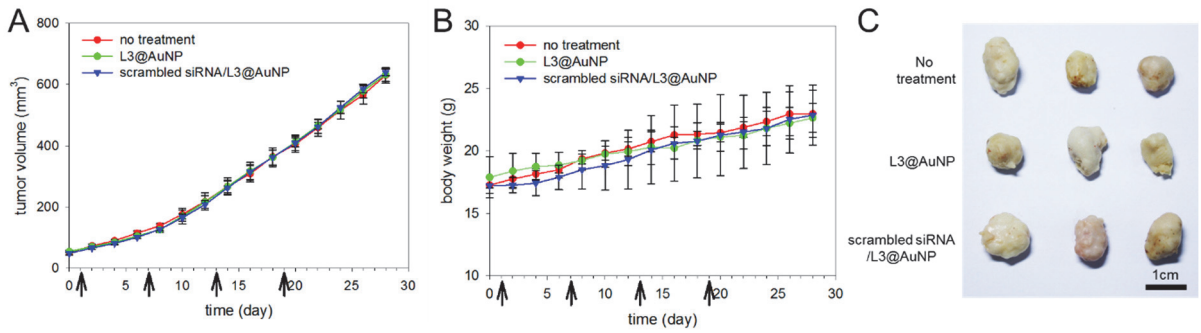

Figure S13. (A) Tumor volumes and (B) the average body of the tumor-bearing mice treated with L3@AuNP alone or L3@AuNP with scrambled siRNA. (C) Representative images of A549 tumor xenografts at day 28. I.V. injection of each sample was performed every week (arrow indicates the injection time).