Effective Gene Silencing by Multilayered siRNA-Coated Gold Nanoparticles

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Small interfering RNA (siRNA) has been widely proposed to treat various diseases by silencing genes, but its delivery remains a challenge. A well controlled assembly approach is applied to prepare a protease-assisted nanodelivery system. Protease-degradable poly-L-lysine (PLL) and siRNA are fabricated onto gold nanoparticles (AuNPs), by alternating the charged polyelectrolytes. In this study, up to 4 layers of PLL and 3 layers of siRNA (sR3P) are coated. Due to the slow degradation of PLL, the incorporated siRNA is released gradually and shows extended gene-silencing effects. Importantly, the inhibition effect in cells is found to correlate with the number of siRNA layers.

1. Introduction

The discovery of RNA interference (RNAi) in *Caenorhabditis elegans* and the birth of the concept of short interfering RNA (siRNA) is only a bit longer than a decade.[1,2] Its gene-silencing effect has been demonstrated in numerous biological models and thus siRNA has been widely proposed as a future therapy to treat many kinds of diseases, including cancers. Although systemically delivered siRNA has been advanced into phase-I clinical trials, more effective delivery systems are required to transport therapeutic siRNA to specific cells and tissues.[3,4] There are two major strategies for nucleic acid delivery: viral and nonviral vector systems. Recombinant viral systems have been commonly used in clinical trials but the associated severe side reactions, including immune response, have led to death and mutagenesis and has onco-genic potential.[4–6] Because of such serious concerns on the recombinant viral vectors, various nonviral delivery systems, prepared by complexing with cationic lipids and polymers, have been validated.[7–13] Of many cationic polymers, poly-ethyleneimine (PEI) is one of the most widely used carriers

to transport genes, oligonucleotides, and siRNAs.[14–17] Despite considerable transfection efficiencies, the properties of PEIs need to be further improved because of its associated toxicity and nonspecific interactions with nontargeted cells.[15,18,19]

Layer-by-layer (LbL) fabrication is a gentle assembly procedure, which is based on charge–charge interactions between positively and negatively charged polymers, to add multiple layers of thin films onto a surface or a particle.[20,21] The LbL method is simple and versatile and therefore a large variety of charged molecules can be used, including natural and synthetic polyelectrolytes.[22,23] It has been applied to many different areas, including biological, materials, and electronic science.[24] We thought that this simple LbL assembly approach would be suitable for preparing a new type of enzyme-assisted siRNA delivery system. High loading of siRNA can be achieved by coating multiple layers of siRNA onto a nanoparticle and the enzyme-assisted release of siRNA can be controlled by the number of layers and the degradability of the positively charged polymers. In addition, the shielding layers could protect the siRNA from degradation.

In this Full Paper, in order to fabricate the proposed assembly, we selected gold nanoparticles (AuNPs) as the core for their unique properties, including uniform size, shape-dependent optical and electronic features, biocompatibility, and feasibility for surface modification.[25,26] A polypeptide, poly-L-lysine (PLL), which has previously been used as a gene-delivery vector and drug carrier, was selected as the positively charged polyelectrolyte for its protease degradability.[27] Recently, we have used PLL as a template to

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prepare self-quenched protease-sensitive fluorescent probes for in vivo imaging of cancer and other diseases.[28–30] In vivo, the PLL was cleaved by lysosomal cathepsin B, which is often up-regulated in cancer cells and inflamed cells, resulting in a bright fluorescent signal in the area with high enzymatic activity.[31–34] Similarly, the progressive degradation of PLL inside of the cell is expected to trigger slow release of siRNA, resulting in a prolonged gene-silencing effect.

2. Results and Discussion

Figure 1 shows the preparation flow of multilayered siRNA gold nanoparticles (sRAuNPs). It is known that the solubility of the coated AuNPs is largely affected by size and surface charges. The concentration and molecular weight of polycations and siRNA all have been optimized to prevent aggregation.[17,35] Using an optimized procedure, densely packed sRAuNPs were obtained. To assemble the multilayered sRAuNPs, the negatively charged gold particles in water were dropped into the positively charged PLL solution (average M_w = 22.5 KDa) for the first layer of coating. The reaction solution was incubated for 30 min and then the coated particles were spun down using a centrifuge. After several washes with sterilized water, the PLL-coated AuNPs were added to the negatively charged siRNA (21 bp against luciferase) solution. After incubation, free unbound siRNAs were removed by centrifugation and the particles were resuspended in sterilized water. As shown in Figure 1, by repeating these procedures, multiple layers – a total of 4 layers of PLL and 3 layers of siRNA – were successfully deposited on Au surface by electrostatic interactions.

Transmission electron microscopy (TEM) images of bare AuNPs (40 nm) and polyelectrolyte-coated AuNPs were collected (**Figure 2**a). The visualization of the polyelectrolyte layers was achieved after negative staining with methylamine tungstate. Under TEM, all coated particles (sR1P, sR2P, and sR3P) were found to be ≈50 nm in diameter. For comparison,

the hydrodynamic diameter of the formulated particles was also measured by dynamic light scattering (DLS) after each layer of coating. The size of initial bare AuNPs was 40 nm, while the particle size increased steadily with the number of layers (sR1: 104 nm; sR1P: 151 nm; sR2P: 159 nm; sR3P: 183 nm). The differences between DLS and TEM might be caused by the hydrodynamic structure of sRAuNPs. The initial zeta potential of bare AuNPs was –42 mV. The PLL loading brought the surface charge up to about +46 mV, while the subsequent siRNA layer dragged it down to about –30 mV again. This characteristic zigzag pattern of zeta potential indicates the successful layering of the alternately charged molecules (Figure 2b).

The next critical step was to confirm that the fabricated siRNA could be released from sRAuNPs by proteases. PLL is made of a natural amino acid, lysine, so that it could be degraded by many different types of proteases, including lysosomal cathepsin B and trypsin. Enzyme-assisted release of siRNA was determined by incubating various sRAuNPs with trypsin in buffer and fractions of the solution were collected at different time points to determine the concentration of released siRNA. As expected, the release
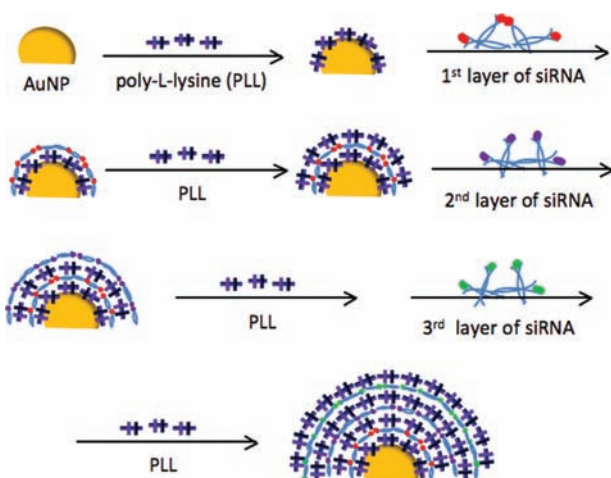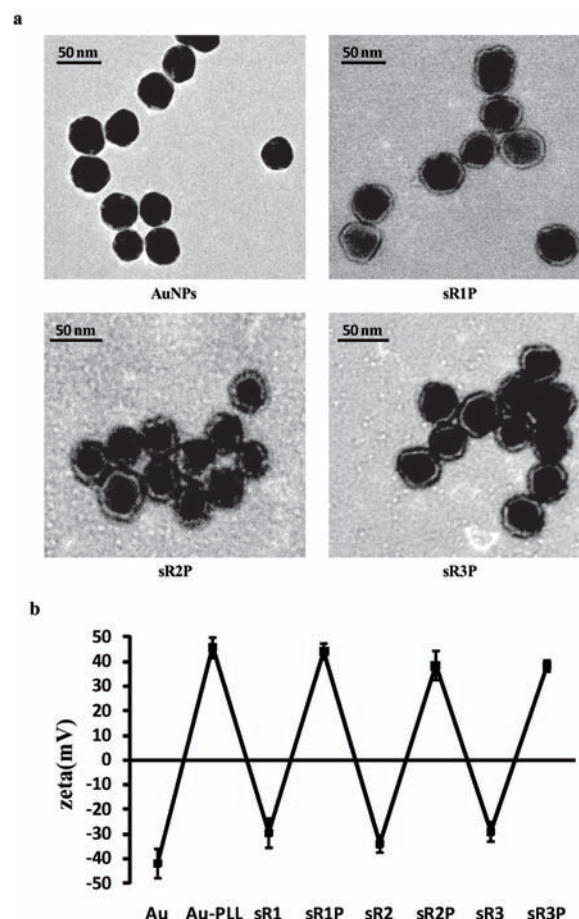
Figure 2. Characterization of sRAuNPs. a) TEM images of bare AuNPs and polyelectrolyte-coated AuNPs. Negative staining by methylamine tungstate was used for all images. b) Zeta potential after each coating of polyelectrolytes. The values represent the standard deviation of three independent experiments.
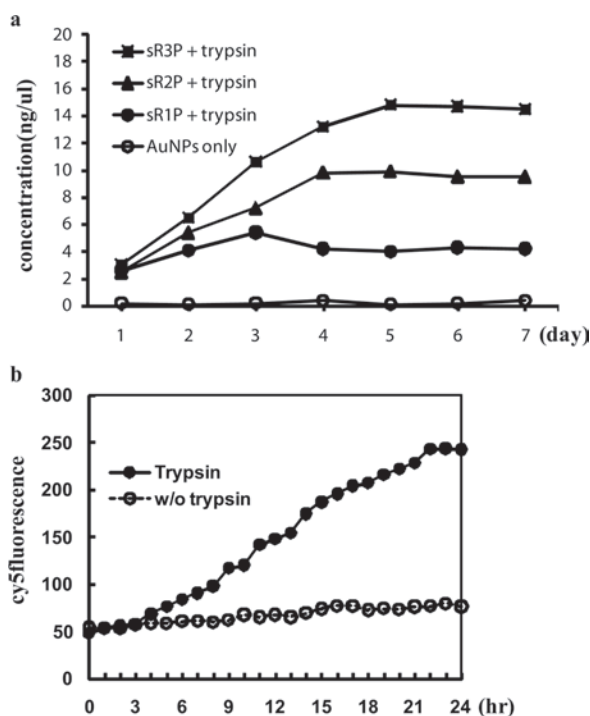
Figure 1. Preparation of multilayer siRNA-coated AuNPs (sRAuNPs) using siRNA and PLL as the charged polyelectrolytes.

Figure 3. Release of siRNA after proteolytic cleavage of PLL. a) Multilayer sRAuNPs were incubated with or without trypsin in phosphate-buffered saline (PBS) and the concentration of released siRNA in supernatant was measured. b) sR1(cy5)P AuNPs were incubated in RPMI 1640 medium containing serum with or without trypsin for 24 h and the fluorescence was examined every 1 h.

kinetics of siRNA from sRAuNPs depended on the number of layers (sR1P > sR2P > sR3P) (**Figure 3**a). It took about 3 days for siRNA to be fully released from the sR1P particles, which had one layer of siRNA and two layers of PLL under the testing condition, whereas it required 4 and 5 days for sR2P and sR3P, which had 2 siRNA/3 PLL and 3 siRNA/4 PLL, respectively. The data also validates the hypothesis that more siRNA could be carried on a single particle by multiple layering. The final siRNA concentrations released from sRAuNPs for sR1P, sR2P, and sR3P were 0.3, 0.7 and 1.1 μM, respectively. Similar results were observed in serum condition using sR1(cy5)P, which coated siRNA tagged with cyanine dye, cy5. As shown in Figure 3b, the fluorescence intensity of cy5 increased persistently because of the trypsin-assisted release and, importantly, the particle remained stable without trypsin, indicating that no siRNA was released from the particle during the experimental period.

The ability of sRAuNPs to enter cells was investigated by real-time fluorescence microscopy and flow cytometry. MDA-MB231-luc2 (**Figure 4**a) and LNCaP-luc2 (Figure 4c) cell lines were incubated with sR1(cy5)P AuNPs (1.58×10^9 particles) for 8 h. Since a cy5 label was anchored to the siRNA, the fluorescence images reveal the location of the siRNA. Spotty fluorescence signal was seen in the early time points, while the signal diffused into cytoplasm as time went on (Figure 4a and c). Cellular uptake of sRAuNPs by both cell lines was also confirmed
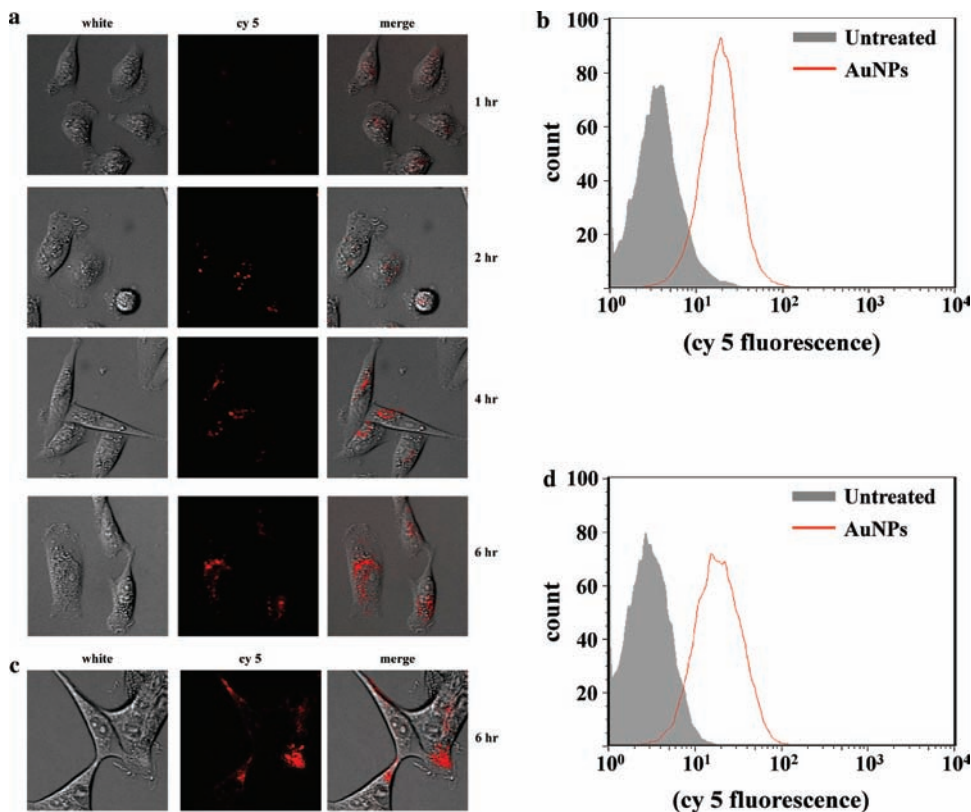

Figure 4. Cellular uptake of sRAuNPs. Real-time image of the uptake of siRNA was visualized by fluorescence microscopy after 8-h incubation with sR(cy5)P AuNPs in a) MDA-MB231-luc2 and c) LNCaP-luc2. b,d) After 24-h incubation with sR(cy5)P AuNPs, cellular uptake of siRNA was confirmed by flow-cytometry analysis in both cell lines.

by flow-cytometry analysis. A strong cy5 fluorescence signal was obtained 24 h after incubating with sR1(cy5)P AuNPs (Figure 4b and d). These cellular-uptake data indicate that sRAuNPs require no transfection agent to enter cells, and, once it is internalized, the siRNA could be freed from particles slowly, as proposed.

It has been reported that the toxicity of the formulated AuNPs depends on the chemical composition of the surface molecules and high-molecular-weight poly-cationic carriers in nonviral vector-delivery systems could be toxic.[18,36,37] Therefore, the cytotoxicity of the prepared sRAuNPs was evaluated in MDA-MB231-luc2 and LNCaP-luc2 cell lines by comparing it with Lipofectamine 2000, which is a widely used transfection agent. As shown in **Figure 5**, no significant toxicity was detected for all sRAuNPs, while some toxicity was observed with Lipofectamine 2000 in both cell lines (cell viability: less than 80%).

Finally the siRNA gene-silencing effect was investigated by measuring the luciferase activity. MDA-MB231-luc2 cells stably expressing firefly luciferase were incubated with sR1P, sR2P, sR3P, siRNA/Lipofectamine, or free siRNA (**Figure 6**). In addition, a control sR3P was prepared with a nonsense siRNA. After incubation with different sRAuNPs for 5 d, the luminescence of MDA-MB231-luc2 cells was measured immediately after addition of luciferin. It was found that the luminescence was reduced to about 43% by sR1P (siRNA 0.3 μM; 1.26×10^8 particles). Furthermore, the luciferase silencing effect was dependent on the number of siRNA layers. With the same amount of particles (1.26×10^8), the luminescence intensity was reduced to 28% by sR2P (siRNA 0.7 μM) and to 18% by sR3P (siRNA 1.1 μM). No significant silencing effect was observed when the luciferase siRNA in sR3P was replaced by a nonsense control siRNA. In comparison, Lipofectamine formulation maintained 62% of luminescence, even though the siRNA concentration had been doubled (2.2 μM). Free unformulated siRNA (2.2 μM) did not show any appreciable effect under the same conditions, either. Similar results were observed with LNCaP-luc2 cell lines (data not shown). All results suggested that trilayer siRNA-coated (sR3P) AuNPs was the best formulation in gene silencing
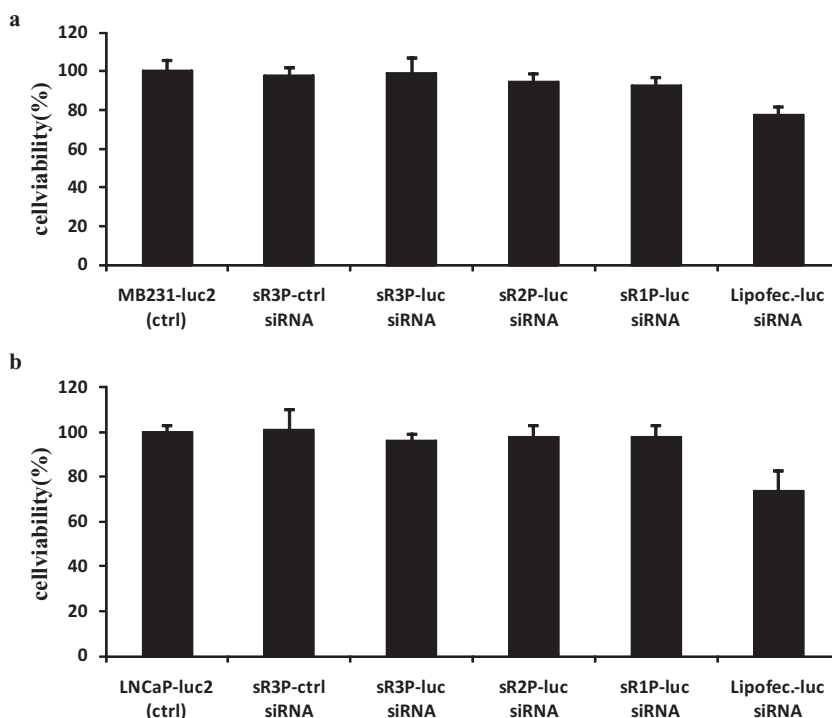

Figure 5. Cytotoxicity of multilayer sRAuNPs. Cell viability was evaluated by MTT assay after transfection with multilayer sRAuNPs or Lipofectamine 2000 in a) MDA-MB231-luc2 and b) LNCaP-luc2 cell lines. Results are representative of three independent experiments.
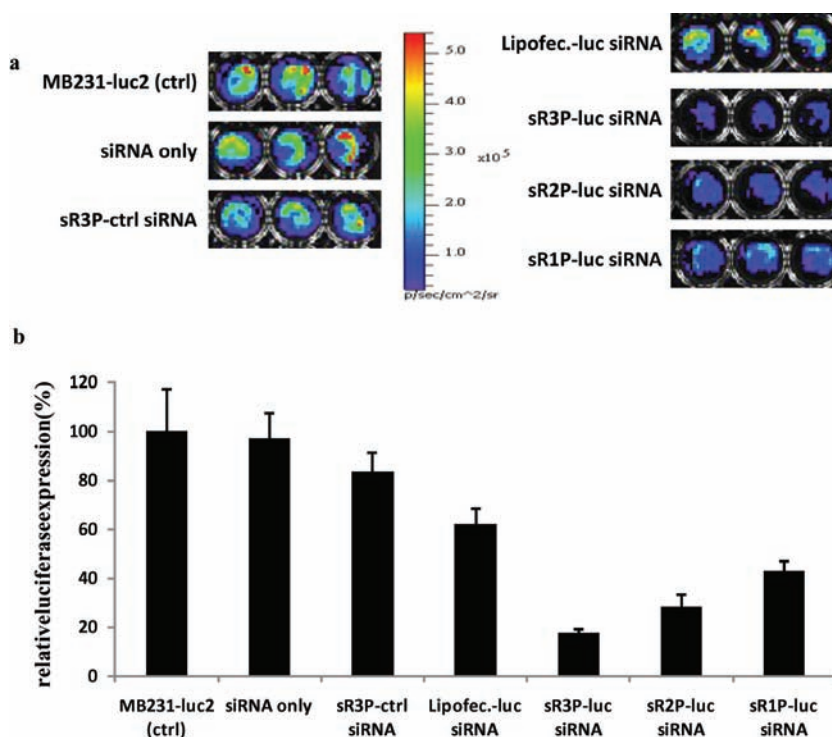

Figure 6. Gene-silencing effect of multilayer sRAuNPs in the MDA-MB231-luc2 cell line. a) The luminescence signal in MDA-MB231-luc2 cells after incubation with different sRAuNPs or Lipofectamine 2000 was evaluated by IVIS 200. b) The value of the luminescence intensity (photons s^{-1}) in MDA-MB231-luc2 cells without treatment was set as 100%. The results are representative of three independent experiments.

(>80%) among all the different kinds of siRNA-delivery formulations.

The positive charge made the assembly straightforward and also assisted the cellular delivery. It is known that positively charged NPs are most effective in crossing negatively charged cell membranes and localizing in the cytosol or nucleus.[38–40] Due to slow release of siRNA, an excellent silencing effect was achieved even 5 or 6 days after single-sRAuNPs treatment. Previously, the importance of biodegradable polymers for the improvement of siRNA release has been recognized.[35] PLL degraded slowly inside of the cells, resulting in a persistent siRNA effect. In addition, the multilayered sRAuNPs carried more siRNA into the cells and silenced target genes more effectively than the monolayer sRAuNPs did.

LbL technology has been reported for some time. However, it was not popular in siRNA delivery. A monolayer siRNA particle formulated with PEI has been prepared for siRNA delivery and a thin-film-based multilayered siRNA was prepared with PEI for electroporation purposes.[35,41] Most recently, PLL layered with siRNA has been applied to a thin film and albumin NPs.[42,43] In contrast, our design is a particle-based multilayered sRAuNPs design, which could have great impact in siRNA therapy. Our results suggested that a multilayer sRAuNPs system is much more effective than the monolayer system. Furthermore, two or three different siRNAs that target different genes could be conveniently formulated on a single multilayer sRAuNP, achieving a synergistic gene-silencing effect. Dual silencing of target siRNA has been reported to be more effectively than single siRNA silencing.[44,45]

In conclusion, we successfully fabricated densely packed multilayered sRAuNPs by layering oppositely charged PLL and siRNA on the surface of AuNPs. The prepared multilayered sRAuNPs, whose outer surface layer is PLL, could deliver siRNA into tumor cells and silence its target gene effectively and without side toxicity. A persistent siRNA inhibition effect was achieved as a result of incorporated protease-assisted slow-release design.

3. Experimental Section

Chemicals and Materials: All siRNA and poly-L-lysine (M_w = 15 000–30 000 g mol^{-1}) were obtained from Sigma-Aldrich (St. Louis, MO). Bare AuNPs (40 nm) were purchased from BB International (Cardiff, UK), Lipofectamine 2000 from Invitrogen (Carlsbad, CA), D-Luciferin from Regis Technologies (Morton Grove, IL), and MTT solution from Promega (Madison, WI).

Preparation and Characterization of Multilayer sRAuNPs: Using the LbL method, multilayers of siRNA and PLL were successfully deposited on the Au surface. The sequences of siRNA against luciferase are: sense strand 5′-CGUACGCGGAAUACUUCGAdTdT-3′ and antisense strand 5′-UCGAAGUAUUCCGCGUACGdTdT-3′. The sequences of control nonsense siRNA are: sense strand: 5′-AGCUUCAUAAGGCGCAUGCdTdT-3′ and antisense strand: 5′-GCAUGCGCCUUAUGAAGCUdTdT-3′.[46] For cellular-uptake experiments, a fluorochrome cyanine dye, cy5, was tagged on

the 5′ end of the sense siRNA. Au solution (3.15×10^9 particles in 0.7 mL) was added dropwise into a PLL solution (0.5 mL of 5 mg mL^{-1}) in pure water. After incubating for 30 min in the dark with gentle shaking, the solution was centrifuged for 30 min at 16 100 g using a microcentrifuge (Eppendorf, Hauppauge, NY). The supernatant was removed and the gel-like deep-red pellet was resuspended with pure water and centrifuged for 30 min at 16 100 g. After one more wash, PLL-coated AuNPs were stored in pure water. Next, a polyelectrolyte layer was deposited by adding PLL-coated AuNPs (in 0.5 mL pure water) to siRNA solution (4.0 μM, 0.5 mL). The reaction solution was incubated in the dark for 30 min with gentle shaking, followed by three washes. The deposition procedures were repeated to have a total of 7 layers of polyelectrolytes (4 layers of PLL and 3 layers of siRNA). Sizes and zeta potentials of AuNPs in water were measured using a Zetasizer Nano-ZS (Malvern, Worcestershire, UK) according to the manufacturer's instructions.

TEM Images: The sizes of the AuNPs were measured by TEM using a JEOL 2010 FasTEM (JEOL Ltd., Tokyo, Japan). In brief, all samples were prepared by placing a drop of the NP solution onto a carbon-coated copper TEM grid (Ted Pella Inc., Redding, CA) of mesh-size 300. After 5 min, the excess amount of the solution was removed using blotting paper. Negative staining of the sample was performed with one drop of Nano-W (methylamine tungstate, Nanoprobes, Yaphank, NY) for 45 s. The excess reagent was blotted away and the grids were allowed to dry overnight before the microscopy was performed. TEM measurements were operated at an accelerating voltage of 200 KV with a LaB6 filament.

Protease-Assisted siRNA Release: To measure the release of siRNA from sRAuNPs, formulated particles (1.26×10^8 particles) were incubated in a 96-well culture plate at 37 °C with or without 50 μL trypsin–ethylenediaminetetraacetic acid (EDTA) (0.25%, Sigma-Aldrich) in PBS. After incubation, the concentration of siRNA in supernatant (1.5 μL) was determined using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) at different time points. The protease-induced fluorescence change of siRNA was determined by incubating the sR1(cy5)P AuNPs (1.26×10^8 particles) in a 96-well culture plate with or without trypsin (50 μL) in RPMI 1640 medium (Thermo Scientific, Rockford, IL) containing serum (Sigma-Aldrich) at 37 °C and the increase of the cy5 fluorescence signal was analyzed by a spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) with 649 nm excitation and 670 nm emission for 24 h.

Cell Lines: The human breast cancer cell line stably expressing firefly luciferase (MDA-MB231-luc2) and the human prostate cancer cell line stably expressing firefly luciferase (LNCaP-luc2) were purchased from Caliper (Alameda, CA). MDA-MB231-luc2 cell lines were cultured in minimum essential medium (Invitrogen, Carlsbad, CA), while the LNCaP-luc2 cell line were cultured in RPMI 1640 medium (Thermo Scientific) and both cell lines were supplemented with 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, and 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) in a humidified atmosphere of 5% CO$_2$ at 37 °C.

Cellular Uptake of sRAuNPs: MDA-MB231-luc2 cells (5.0×10^4) and LNCaP-luc2 (2.5×10^5) cells were seeded on a 35-mm culture dish with a glass-bottomed microwell (Met-Tek Inc, Clackamas, OR). After 24 or 48 h, the culture medium was replaced with fresh sR1(cy5)P AuNPs (1.58×10^9 particles) containing medium and

further cultured for 8 h. Cells were then washed twice with PBS and cultured in the phenol-red-free medium. Real-time fluorescence images of cells were acquired using a LCV-110 incubator fluorescence microscopy system (Olympus Corporation, Tokyo, Japan). For flow-cytometry analysis, MDA-MB231-luc2 cells (5.0×10^4) and LNCaP-luc2 cells (2.5×10^5) were seeded on a 6-well culture plate (BD Falcon, San Jose, CA) and cultured for 24 or 48 h. sR1(cy5)P AuNPs $(1.58 \times 10^9$ particles) were added and further cultured for 24 h. After removal of the cultured medium, the cells were washed with PBS and then detached from the wells by trypsin−EDTA. After three more washes with PBS in a tube, the cy5 fluorescence signal inside of cells indicating the uptake of siRNA were measured by BD FACSAria III flow cytometry (BD Biosciences, San Jose, CA).

Cytotoxicity Measurement of sRAuNPs: MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed to determine the cytotoxicity of Lipofectamine 2000 (Invitrogen) and the preparations of sRAuNPs. Briefly, MDA-MB231-luc2 cells were collected by trypsinization, counted, and plated in a 96-well culture plate at a density of 5×10^3 (or 2.5×10^4 of LNCaP-luc2) cells well^{-1}. After 1 d, sRAuNPs $(1.26 \times 10^8$ particles) and Lipofectamine 2000 (0.2 μL) were added and the cells were further cultured for 24 h. MTT solutions (20 μL, Promega) were then added to each well. After incubation for an additional 3 h, absorbance was measured at 570 nm using a SpectraMax plate reader (Molecular Devices).

Gene Silencing in MDA-MB231-luc2 and LNCaP-luc2 Cell Lines: For the examination of the gene-silencing effect, bioluminescence measurements were performed after incubating with various multilayered AuNPs. Cells were seeded in a 96-well black clear-bottom culture plate at a density of 2.5×10^3 (or 1.25×10^4 of LNCaP-luc2) cells well^{-1}. After 1 d, different sRAuNP $(1.26 \times 10^8$ particles) were added to each well and cultured for an additional 24 h. The cells were further cultured in phenol-red-free medium for another 5 d. The manufacturer's direction was followed for the transfection with Lipofectamine. Bioluminescence measurement was performed using an IVIS 200 (Caliper) immediately after addition of 125 μg mL^{-1} of D-Luciferin (Regis).

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