Published in final edited form as:

Adv Healthc Mater. 2014 October ; 3(10): 1629–1637. doi:10.1002/adhm.201400103.

Multifunctional Gold Nanorods for siRNA Gene Silencing and Photothermal Therapy

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Abstract

Cancer is a complex disease that usually requires several treatment modalities. Here, we have designed a multifunctional nanotherapeutic system incorporating small interfering RNA (siRNA) and gold nanorods for photothermal therapy. Surface engineered gold nanorods with polyethylenimine were synthesized using a layer-by-layer assembly and siRNA was absorbed on the surface. The siRNA was efficiently delivered into breast cancer cells, resulting in subsequent gene silencing. Cells were then irradiated with near-infrared (NIR) light, causing heat-induced anticancer activity. The combination of gene silencing and photothermal therapy resulted in effective inhibition of cell proliferation.

Keywords

gold nanorods; layer-by-layer assembly; siRNA; photothermal therapy

1. Introduction

In the past few years there have been several breakthroughs in the field of nanomedicine.^[1] The emergence of various classes of nanoparticles has enabled the development of diverse nanotherapeutics.^[2] Metallic nanoparticles have proven especially useful, due to their optical and electronic properties. Gold nanoparticles are among the most promising, owing to their unique characteristics, such as stability, biocompatibility, photothermal responsiveness and ease of preparation and modification.^[3, 4] In particular, gold nanorods (Au NRs) have been extensively exploited as agents for biomedical applications including optical imaging (OA), homogeneous assays (HA), plasmonic photothermal therapy (PPT), drug carriers (DC), biosensing (BS), surface enhanced Raman spectroscopy (SERS) and other functions.^[5-7] Moreover, RNA interference (RNAi) using small interfering RNA (siRNA) is also an emerging technology that holds promise for cancer therapy. siRNAs can be used to silence gene activity by preventing the translation of messenger RNAs (mRNAs) into proteins.^[8, 9] Indeed, siRNAs are viable tools for suppressing a multitude of oncogenes that contribute to malignancy. However, the delivery of naked siRNA *in vitro* and *in vivo* remains challenging, due to rapid degradation by nucleases, poor intracellular uptake and inefficient endosomal escape.^[10-13] Therefore, the successful implementation of siRNA requires the use of delivery vehicles that can efficiently overcome such biological barriers.

In this study we have used Au NRs as a delivery platform for siRNA gene silencing agents. Gold particles were selected as the carrier of choice, in hopes of creating a safe platform that could improve the cytosolic delivery of siRNA, thus increasing therapeutic efficacy. In addition, the Au NRs could simultaneously be exploited for photothermal therapy, which could further enhance the anticancer activity of the carrier. The surface of Au NRs was modified with polyethylenimine (PEI) through layer-by-layer assembly and siRNA was absorbed on the surface. PEI served as a cationic coating that attracted the anionic nucleic acids, resulting in the formation polyplexes. Functionalized delivery vehicle was evaluated in MDA-MB-231 and SUM-159 breast cancer cell lines. The intracellular uptake, release kinetics, endosomal escape and transfection efficacy of siRNA against pyruvate kinase isoenzyme type M2 (PKM2) was measured. In addition, following transfection, the Au NRs

were irradiated with near infrared light. Upon NIR irradiation, the excited conduction band electrons decay into the surrounding medium, resulting in the production of heat. Thus, gold nanorods have been referred to as “nanoheaters”.[14]

Here, the combined anticancer activity of thermal therapy and PKM2 gene silencing was assessed *in vitro*. Moreover, the potential cytotoxicity and immunotoxicity of the delivery vehicle was evaluated in cancer and macrophage cell lines. Previously, gold nanoparticles have been used for successful delivery of siRNA and subsequent suppression of gene expression.[15,16] However, to our knowledge, this is the first report using Au NRs for combined gene silencing and thermal therapy. This nanodelivery system may constitute a promising non-viral platform for future medical applications of RNAi.

2. Results and discussion

2.1. Synthesis and Characterization of PEI-Au NRs System

A three-step procedure for the fabrication of the PEI-Au NR delivery vehicle is illustrated in **Scheme 1**. The synthesis of cetyltrimethylammonium bromide (CTAB)-Au NRs was based on the seed-mediated growth method.[17] Functional surface modification was obtained via a well-developed layer-by-layer assembly approach. Firstly, a polystyrene sulfate (PSS) layer was assembled on the original CTAB-Au NR in order to obtain a negatively charged nanorod. PEI was then coated on the NR surface, enabling the electrostatic bonding with siRNA. The purified nanorod complexes were dissolved in water and stored at 4 °C.

Next, transmission electron microscope (TEM) was used to analyze the morphology of the final product (**Figure 1a**). Dynamic light scattering (DLS) was also employed to confirm the size of PEI-Au NRs (**Figure 1b**). The results indicate that the PEI-Au NRs are 90 nm in length and 15 nm in width, with an average aspect ratio of 6. The zeta potential values of the CTAB-Au NRs, PSS-Au NRs, and PEI-Au NRs were +32.3 mV, -23.1 mV, and +66.9 mV, respectively (**Figure 1c**). PEI-Au NRs had two surface plasmon (SP) bands that are shown in **Figure 1d**. The first SP band, corresponding to the transverse oscillation mode, was located in the visible region at 515 nm, while the second band, corresponding to the longitudinal oscillation mode, was between the far-red and NIR region at 752 nm. A water medium containing different concentrations of nanorods was subjected to NIR irradiation and the temperature was measured. **Figure 1e** and **Figure S1** show that at a various concentration of nanorods, the maximum increase in temperature is 25.5 °C. To visualize the delivery system, Alexa555 fluorescently labeled RNA oligos were used. Confocal microscopy images show the presence of distinct fluorescent spots (**Figure 1f**). This observation suggests that the siRNA molecules had successfully been incorporated into the delivery system.

2.2. Optimization and Release Kinetics of PEI-Au NRs/siRNA System

The most important factors to consider when developing a siRNA delivery platform are the binding affinity, stability, and release of siRNA. In order to study the binding capability of siRNAs to PEI-Au NRs, agarose gel electrophoresis was performed using various vectors to siRNA weight ratios (5:1 to 30:1). The migration of PEI-Au NR/siRNA was prevented at weight ratios higher than 15 (**Figure 2a**, left panel), indicating that the binding between the

carrier and the siRNA was strong enough to withstand dissociation during electrophoresis. The stability and release of PEI-Au NR/siRNA complexes were then examined by RNase digestion followed by sodium dodecyl sulfate (SDS) replacement. The SDS treatment results in the release of siRNA from the delivery vehicle (**Figure S2**). In **Figure 2a** (right panel) the band for the free siRNA sample is missing, indicating that the siRNA was degraded by the RNase treatment. On the contrary, the PEI-Au NR/siRNA complexes display visible bands, demonstrating that the siRNA was protected from degradation due to association with the vehicle. In addition, the zeta potential of the delivery vehicle was measured using different weight ratios between the vehicle and the siRNA. PEI-Au NR/siRNA complexes showed a negative charge at the lower weight ratios (5:1 and 10:1), a moderate positive charge at a weight ratio of 15:1 and a high positive charge at the higher weight ratios (20:1, 25:1 and 30:1) (**Figure 2b**). A negative charge can lower the intracellular uptake of particles, while a high positive charge can have cytotoxic effects. By taking into consideration the zeta potential and binding affinity, a weight ratio of 15:1 was selected for further experiments. To evaluate the stability of the delivery system naked siRNA and siRNA bound to PEI-Au NR particles were incubated in 50% FBS at 37 °C for different time periods, and then separated in a 2% agarose gel (**Figure S3**). After 12 h of incubation, the naked siRNA had degraded while the particle-bound siRNA remained intact. The result indicates that the delivery system protects siRNA from degradation by serum enzymes. Next, the release of siRNA from the delivery vehicle was evaluated at pH 5.5 and 7.4. The amount of released siRNA was measured by determining the absorbance of the supernatant at 260 nm. After 24 h of incubation the cumulative siRNA release from the PEI-Au NR/siRNA complexes reached 41% at pH 5.5 and 37% at pH 7.4 (**Figure 2c**). The release of siRNA from the delivery vehicle is crucial, since it enables the binding of siRNA to the RNA-induced silencing complex (RISC), which is essential for translational repression.

2.3. Cellular Uptake of PEI-Au NRs/siRNA System

The cellular uptake of PEI-Au NR/AF 555-labeled siRNA complexes (15:1 weight ratio) was evaluated in MDA-MB-231 and SUM-159 breast cancer cell lines. Confocal microscopy was used to monitor the intracellular accumulation of the complexes at different time points. As shown in Figure 3a, AF 555 siRNA was observed in the cells after an incubation period of 1, 3 and 7 days. 3D and Z slice images were obtained to confirm the presence of siRNA inside the cell (**Figure S4 and S5**). Furthermore, TEM images were taken to visualize the nanorods in the cells (**Figure 3b**). Flow cytometry was also used to measure the cellular uptake of AF 555-labeled siRNA. Breast cancer cell lines were transfected with the delivery vehicle and incubated for 1 day. The results indicate that the PEI-Au NR/AF 555-labeled siRNA complexes were internalized into 96.5% and 90.4% of the MDA-MB-231 and SUM-159 cells, respectively (**Figure 3c**). The various techniques used to evaluate the presence of the delivery vehicle inside the cells, are evident of efficient intracellular uptake.

2.4. Intra-cellular Trafficking of PEI-Au NRs/siRNA Nanoparticle

The majority of nanodelivery systems are taken up through endocytosis and travel from the early endosomes to the lysosome, which contains degradative enzymes. Since siRNAs are sensitive to degradation and exert their biological activity in the cytoplasm, endosomal

escape is important for achieving post-transcriptional gene silencing.^[18, 19] To determine whether the siRNAs in the PEI-Au NR complex could be released from the endosome/lysosome, a stain for acidic organelles was used (LysoTrack Red). FAM-siRNA (green) was absorbed onto PEI-Au NRs and MDA-MB-231 cells were treated with the delivery system. After 2 and 4 h incubation periods the siRNAs had colocalized with the LysoTrack Red dye (**Figure 4**). However, at 6 and 8 h of incubation, the siRNA could be visualized in multiple areas that did not overlap with the red dye (Figure 4), suggesting successful endosomal escape. The potential mechanism behind this phenomenon is the PEI-induced proton sponge effect, which causes disruption of endosomal/lysosomal compartments.^[20]

2.5. Cell Viability

For the clinical implementation of a technology, the biocompatibility and safety should be carefully evaluated. The cytotoxicity of PEI-Au NR complexes was determined in MDAMB-231 and SUM-159 cells using an MTS assay. After 24 h of incubation the cell viability was over 90 % at a concentration of 0-80 $\mu\text{g/mL}$ and 80 % at a concentration of 100 $\mu\text{g/mL}$ (**Figure S6a**). In addition, the cytotoxicity of 80 $\mu\text{g/mL}$ PEI-Au NR complexes after 48 and 72 h was above 85 % (**Figure S6b**). Free PEI displayed higher cytotoxicity than the PEI-Au NR complex (Figure S6), suggesting that the layer-by-layer assembly approach mitigates PEI-induced toxicity. PEI is known to interact with the glycocalyx on the cell surface, resulting in the formation of large clusters that can cause cytotoxicity.^[21] It is likely that the conjugation of PEI to the PSS-Au NRs decreases non-favorable interactions with the cell surface. Moreover, the assembly of PEI with PSS-Au NRs reduces the number of PEI primary amine groups, which are more toxic than secondary or tertiary amines. In addition, the cytotoxicity of PEI-Au NR/siRNA (scrambled) complexes in MDA-MB-231 and SUM-159 cells was evaluated (**Figure 5a**). The results indicate that the incorporation of control siRNA does not compromise the biocompatibility of the delivery vehicle.

2.6. Evaluation of Immunotoxicity *In Vitro*

Certain nanoparticles may evoke an immunological response, which is an undesirable effect of a delivery system.^[22] The release of cytokines from cells treated with nanomaterials is usually used as an indicator of possible immunotoxicity.^[23, 24] Interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF- α) are key inflammatory cytokines that mediate an immunological response. The immunotoxicity of PEI-Au NRs and PEI-Au NR/siRNA complexes were studied by measuring the levels of IL-6, and TNF- α in Raw-264.7 mouse macrophage cells. The results demonstrate that the cells exposed to the delivery vehicle had similar cytokine levels as the control group, suggesting the lack of immunotoxicity *in vitro* (**Figure S7** and **Figure 5b**). Although not as dramatically as the positive control lipopolysaccharide (LPS), free PEI displayed an immunological response (Figure S7, Figure 5b). Thus, the Au NRs were able to eliminate PEI-induced activation of the immune system

2.7. Gene Silencing

Two of the most important features of an siRNA delivery vector is safety and efficient gene silencing. The transfection efficacy of PEI-Au NR/siRNA complexes (weight ratio 15:1) was evaluated in MDA-MB-231 and SUM-159 cells. The siRNA used in the experiments

was against PKM2, which is overexpressed in breast cancer cells. The inhibition of PKM2 can result in the suppression of cancer cell proliferation and migration.^[25] In this study, the siRNA delivery system effectively reduced the protein levels of PKM2 (**Figure 6a**). Moreover, in both cell lines the messenger RNA (mRNA) expression decreased with approximately 77 % (**Figure S8**). As anticipated, free PKM2 siRNA did not have an effect on protein or mRNA levels. The PEI-Au NR/siRNA complexes had similar transfection efficacy as the commercial reagent oligofectamine and PEI. Nevertheless, the PEI-Au NR vehicle has a distinct therapeutic advantage, due to decreased cytotoxicity and immunotoxicity.

2.8. Combination of Gene Silencing and Thermal Therapy

The major advantage of nanoparticles in comparison to conventional therapeutics is multifunctionality.^[24, 26] Indeed, nanodelivery systems can incorporate several materials, each of which has distinct properties that contribute to therapeutic success. Gold nanoparticles display unique optical properties that have been exploited for photothermal applications. Recently, gold nanoparticles have served as energy transducers that convert light into heat upon laser irradiation.^[27-29] MDA-MB-231 and SUM-159 cells were transfected with the delivery vehicle, and 3 days later exposed to NIR light (wavelength 808 nm) for 7 min. When gene silencing (PKM2) or thermal therapy was applied separately the cell viability reduced to approximately 60% (**Figure 6b**). When these therapeutic applications were combined the cell viability dropped to almost 20%. In contrast, the oligofectamine/siRNA (PKM2) group did not display any further reduction in cell viability upon laser irradiation, confirming that the gold nanoparticles are responsible for heat-induced cytotoxicity. In addition, following the combination treatment, live and dead cells were stained with calcein AM and ethidium homodimer-1 (EthD-1), respectively. The results indicate the presence of multiple dead cells in the irradiated region (**Figure 6c**). In essence, the suppression of an oncogene in combination with heat induction is more effective than either treatment alone.

3. Conclusion

In conclusion, by using a layer-by-layer assembly method we have developed a multifunctional nanocarrier with the capability of gene silencing and thermal therapy. Gene silencing is achieved through the incorporation of siRNA with the cationic polymer PEI, while heat is generated by the irradiation of gold nanorods. We show that the PEI-Au NRs protect siRNA from degradation and facilitate endosomal escape, both of which are requisites for successful gene silencing. By using a siRNA against PKM2 we demonstrate the effective suppression of corresponding protein and mRNA levels. Furthermore, the combined anticancer activity of PKM2 inhibition and heating dramatically reduced the viability of breast cancer cells. Moreover, the PEI-Au NR/siRNA (scrambled) complexes did not display any signs of cytotoxicity or immunotoxicity *in vitro*. PEI-Au NRs represent a promising platform for siRNA delivery in combination with photothermal therapy.

4. Experimental Section

Materials

Hydrogen tetrachloroaurate (III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), PSS (molecular weight: 70,000), and sulphuric acid (H_2SO_4) were bought from Sigma-Aldrich. Branched PEI (MW 25 kDa), sodium borohydride (NaBH_4), CTAB, silver nitrate (AgNO_3) and L-ascorbic acid (AA) were purchased from Alfa Aesar. All chemicals were of analytical grade and used as received. Dulbecco's Modified Eagle Medium high glucose (DMEM) and fetal bovine serum (FBS) were from Fisher Scientific. PKM2 and β -actin antibodies were purchased from Cell Signaling. siRNA-Alexa Fluor 555 was purchased from Qiagen. Scrambled siRNA and PKM2 siRNA were ordered from Sigma. PKM2 sense and anti-sense sequences are CCAUAAUCGUCCUCACCAA[dT] and UUGGUGAGGACGAUUAUGG[dT], respectively. Milli-Q water (18M Ω .cm) was used for the preparation of all aqueous solutions. Nuclease free water was used for siRNA experiments.

Cell lines

The MDA-MB-231, SUM-159, and RAW 264.7 cell lines were obtained from ATCC (Rockville, MD). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin under conditions of 5% CO_2 atmosphere and 95% humidity at 37 °C.

Synthesis of CTAB-Au nanorods

CTAB-Au NRs were synthesized by a seed-mediated growth method.^[17] Briefly, 15 mL 0.1 M CTAB was mixed with HAuCl_4 (200 μL , 24 mM) and diluted with water to 18.8 mL. Then, 1.2 mL ice-cold NaBH_4 (0.01 M) was added while stirring magnetically. After another 2 min of vigorous stirring, the seed solution was kept at room temperature and used within 2-5 h. The growth solution for the Au NRs was obtained by mixing CTAB (200 mL, 0.1 M), HAuCl_4 (4 mL 0.024 M), H_2SO_4 (4 mL, 0.5 M), AgNO_3 (1 mL, 10 mM), and ascorbic acid (1.6 mL, 0.1 M). Finally, 480 μL of seed solution was added to initiate the growth of the Au NRs. After 12 h, the reaction was stopped by centrifugation (8000 rpm for 10 min). The precipitate was re-dispersed in deionized water.

Synthesis of PEI-Au nanorods

PEI-Au NRs were obtained through a layer-by-layer assembly approach.^[17] A PSS layer was first assembled on the original CTAB-Au NRs in order to obtain negatively charged nanorods. Briefly, 12 mL of CTAB-Au NRs were centrifuged at 15000 rpm for 30 min and the precipitate was dispersed in 12 mL of PSS aqueous solution (2 mg/mL with 6 mM NaCl). The solution was stirred for 3 h, followed by centrifugation at 15,000 rpm for 30 min. The precipitate that formed during centrifugation was then re-dispersed in deionized water. PEI-Au NRs were obtained following the same procedure as outlined above.

Assembly of PEI-Au NR/siRNA complexes

The PEI-Au NRs were precipitated through three rounds of centrifugation at 12000 g/min for 30 min, followed by resuspension in nuclease free water. To load siRNA onto the

prepared vehicles, 1 $\mu\text{g}/\mu\text{L}$ of siRNA solution was combined with PEI-Au NRs at various w/w ratios of Au-PEI NRs to siRNA. The resulting solutions were mixed by pipetting and incubated at 4 °C for 30 min prior to use.

Characterization of PEI-Au nanorods

Electron micrographs were obtained using a transmission electron microscope (TEM) (Tecnal G2 20 S-TWIN, FEI company, Hillsboro, OR). Samples were prepared by dipping 5 μL of PEI-Au NR solution onto a carbon-coated copper grid, followed by air-drying. Visible-near infrared (Vis-NIR) spectra were recorded in the wavelength range of 400-900 nm using a DU® 730 UV/Vis spectrophotometer. Zeta potential and size were determined on a Nano-ZS Zetasizer (Malvern, Worcestershire, UK) using the DTS (nano) program.

The photothermal effect of different concentrations of PEI-Au NRs in aqueous solution (2.5, 5.0, 7.5, 10, 20, 30, 40, and 50 $\mu\text{g}/\text{mL}$) was measured and analyzed with a GCSLX-05-1600m-1 fiber coupled diode laser (DHC, China Daheng Group, Beijing, P. R. China). The center wavelength of 808 ± 10 nm was powered by a DH 1715A-5 dual-regulated power supply. The samples were placed in a quartz cuvette containing 200 mL of PEI-Au NRs. The thermo coupler was inserted into the solution perpendicular to the path of the laser light. The temperature was measured over a period of 10 min.

Optimization of PEI-Au NR/siRNA complexes

PEI-Au NRs were mixed with scrambled siRNA in nuclease free water at various weight ratios (PEI-Au/siRNA = 5:1, 10:1, 15:1, 20:1, 25:1, 30:1), and incubated for 30 min at 4°C. Nanoparticle stability and siRNA integrity was investigated using a gel retardation assay. The binding of siRNA to PEI-Au NRs was determined by 1% agarose (Promega, low melting point) gel electrophoresis containing ethidium bromide. Electrophoresis was carried out at a constant voltage of 120 V for 20 min in TAE running buffer (2 M Tris, 250 mM sodium acetate, 50 mM EDTA, pH 7.8).

To evaluate the protection of siRNA by PEI-Au NRs, 1 μL RNaseA (1 $\mu\text{g}/\mu\text{L}$) was used to digest 0.2 mg of siRNA formulated with PEI-Au NRs at 37°C for 1 hour. The RNaseA was then inactivated with 4 mL of EDTA (0.25 M) and incubated for 10 min at room temperature. After digestion, 5 μL of 1 % SDS NaOH solution (1M) was added to dissociate siRNA from the PEI-Au NR/siRNA complexes. siRNA bands were visualized in a UV transilluminator.

PEI-Au NRs were mixed with Alexa 555 siRNA at a w/w ratio of 20 in nuclear free water and dropped on the surface of a microscope slide. Premium Superslip was used to cover the samples and images were obtained using a Fluo View TM 1000 confocal microscope (Olympus Inc., USA).

siRNA stability in serum

Naked scrambled siRNA and PEI-Au NRs/scrambled siRNA were incubated in 50% FBS at 37°C. Samples were taken at 30 min, 1 h, 3 h, 6 h, 12 h, 24 h and 48 h, and immediately mixed with gel loading buffer containing 2% SDS. After the final aliquot was collected, the

samples were subjected to electrophoresis in a 2% agarose gel and the presence of siRNA oligos was visualized by ethidium bromide staining.

siRNA release from PEI-Au NR/siRNA complexes

To measure the release profile of siRNA, PEI-Au NRs were complexed with Alexa 555 siRNA (w/w ratio: 20) in 10 mM HEPES buffer at pH 5.5 or 7.4 and incubated in a 96 well culture plate at 37 °C. Samples were removed from the plate at scheduled time points and centrifuged at 12000 rpm for 30 min. The supernatant was then collected and the fluorescence intensity was measured with a plate-reader.

Cellular uptake of PEI-Au NR/siRNA complexes

The cellular uptake of siRNA delivered by PEI-Au NRs was examined by confocal microscopy and flow cytometry. MDAMB-231 and SUM 159 cells were plated in 4-well chamber slides at a seeding density of 5×10^4 cells/well and incubated for 24 hours. The complexes containing 100 nM Alexa 555 siRNA were prepared according to the procedure described in the previous paragraph. The distribution of siRNA in the cells was examined at different time points using a confocal microscopy. Cell lines were stained with AF 488 Phalloidin (Invitrogen) to visualize the cell membrane and with DAPI to label the nuclei. TEM images of the distribution of PEI-Au NR/siRNA complexes were obtained via transmission electron microscopy. Cells were incubated with PEI-Au/siRNA for 24 hours, washed with phosphate buffered saline (PBS) and then fixed with EM Fixative (2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M sodium cacodylate, pH 7.4). The cells were transferred onto a carbon-coated copper grid and air-dried prior to imaging.

For flow cytometry analysis cells were incubated with PEI-Au NR/siRNA complexes (100 nM siRNA) for 24 h, collected and washed in 2 % FBS solution three times. The fluorescence intensity of Alexa 555 siRNA was then determined using an LSRII Flow Cytometer (BD Bioscience, USA).

Intracellular trafficking of PEI-Au NR/siRNA complexes

To monitor intracellular trafficking, PEI-Au NR/FAM-labeled siRNA nanorods were incubated with MDA-MB-231 cells at various time points. Cells were harvested at schedule time points, and fixed with 4 % paraformaldehyde. Samples were then blocked with 1 % bovine serum albumin (BSA) in PBS with 0.1 % Tween-20 solution (PBST) and stained with LysoTracker (Invitrogen) and DAPI. Confocal microscopic images were obtained using a Fluo View TM 1000 confocal microscope.

Evaluation of the toxicity and immunotoxicity in vitro

The proliferation of MDA-MB-231 and SUM-159 cells in the presence of PEI-Au NRs and PEI-Au NR/siRNA complexes was determined using an MTS-based colorimetric assay (CellTiter 96 cell proliferation assay kit; Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were seeded in 96-well culture plates (5×10^3 cells/well) and left to adhere overnight at 37 °C in medium containing 10 % serum. Cells were then treated for 72 h with different concentrations of gold nanorods with or without siRNA. At

the end of the treatment period, the cell viability was measured. The experiments were performed in triplicates on three separate occasion.

RAW 264.7 cells were seeded in a 96-well tissue culture plate at a density of 2×10^4 cells/well in 100 μ L DMEM containing 10 % FBS. Lipopolysaccharide served as a positive control, and untreated cells as a negative control. Cells were treated with PEI-Au NRs (with or without siRNA) for 24 hours and the levels of murine TNF- α and IL-6 were measured using an ELISA assay, according to the manufacturer's instructions.

Gene silencing efficiency in vitro

MDA-MB-231 and SUM-159 cells were seeded in 6-well tissue culture dishes at a density of 2×10^5 cells/well in 2 mL DMEM containing 10 % FBS. Cells were incubated with PEI-Au NR/siRNA complexes for 72 hours and then harvested for Western blot analysis. Proteins were extracted using a mammalian protein extraction reagent (M-PER, Pierce Inc., USA), separated on a 12 % gel by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Inc., USA).

For qPCR analysis, the cells were seeded in 6-well plates (2×10^5 cells/well) and incubated with PEI-Au NR/PKM2 siRNA complexes for 48 hours. Total RNA was collected from transfected cells and isolated using a TRIzol reagent according to the manufacture's protocol (Qiagen, Vllencia, CA). Oligofectamine and PEI were used as positive controls, while untreated cells served as a negative control. 2 μ g of total RNA was transcribed into cDNA using the PrimeScript First Strand cDNA Synthesis Kit (Takare, Dalian, China). Thereafter, 2 mL of cDNA was subjected to qPCR analysis using the SYBR Premix Ex Taq. The primer sequences for PKM2 and β -actin mRNA amplification were as follows: PKM2 forward 5'-ATAACGCCTACATGGAAAAGTGT-3', and reverse 5'-TAAGCCCATCATCCACGTAGA-3', with a product size of 108 bp. β -actin forward 5'-AAATCGTGCGTGACATTAA-3', and reverse 5'-CTCGTCATACTCCTGCTTG-3', with a product size of 375 bp. Analysis was performed with the Applied Biosystems StepOne Real-Time PCR Systems. Standard curves were generated and the relative amount of target mRNA was normalized to mRNA levels of β -actin.

Photothermal efficiency in vitro

MDA-MB-231 and SUM-159 cells were seeded in 96-well tissue culture dishes at a density of 5×10^3 cells/well in 100 mL DMEM containing 10 % FBS. Cells were treated with PEI-Au NRs and PEI-Au NRs/siRNA for 72 h. The cells were then washed three times with serum-free media and irradiated with NIR light (wavelength: 808 nm) at an output power of 0.5 W for 7 min. Finally, cell viability was measured using an MTS assay. Moreover, cells were then exposed to NIR irradiation for 7 min, live cells were stained green with calcein AM and dead cells were stained red with ethidium homodimer-1 (EthD-1). We used the DMSO solution of 20 μ L EthD-1 and 10 μ L AM to stain the cells, and incubated 30 min at 37 $^{\circ}$ C. Finally, the confocal images were obtained.

Statistical analysis

All quantitative data are presented as the mean $\pm$ the standard deviation. Statistical analysis was performed with the Student's t-test. Differences were considered statistically significant with $p < 0.05$ (*) and $p < 0.01$ (**).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge financial support from the following sources: Department of Defense grants W81XWH-09-1-0212 and W81XWH-12-1-0414, National Institute of Health grants U54CA143837 and U54CA151668, the CPRIT grant RP121071 from the state of Texas, the Ernest Cockrell Jr. Distinguished Endowed Chair, the National Natural Science Foundation of China (Nos., 21231007, 21172274 and 21121061), Program for Innovative Research Team in University of China (No. IRT1298), Key Project of Chinese Ministry of Education (No. 313058), the National Basic Research Program of China (973 Program No. 2014CB845604), and the Fundamental Research Funds for the Central Universities.

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Scheme 1.

Schematic illustration of the fabrication and function of PEI-Au NRs for the delivery of siRNA.

Figure 1. Characterization of PEI-Au NRs delivery system. (a) Transmission electron microscopy (TEM) images of PEI-Au NRs. (b) Size distribution of PEI-Au NRs measured by dynamic light scattering. (c) Zeta potential of particles at various stages of fabrication. Results are presented as the mean of five measurements $\pm$ standard deviation. (d) Ultraviolet visible (UV-Vis) spectra of PEI Au NRs in water solution. (e) Heat-generation kinetics of free PEI-Au NRs with serial concentrations in response to near infrared light. (f) Confocal microscope images of PEI-Au NR/siRNA complexes. Red fluorescence originates from Alexa555-conjugated siRNA.

Figure 2.

Optimization and siRNA release from the PEI-Au NR/siRNA. (a) Left panel: agarose gel retardation assay of PEI-Au NRs/siRNA complexes under various weight ratios (5:1, 10:1, 15:1, 20:1, 25:1, and 30:1). Right panel: protection of siRNA against RNase digestion and siRNA release by competitive binding sodium dodecyl sulfate (SDS) with carriers after degradation. Naked siRNA was used as a control. (b) Zeta potential changes with different weight ratios. Results are presented as the mean of five measurements $\pm$ standard deviation. (c) Profile of siRNA release from PEI-Au NR/siRNA complexes prepared at a weight ratio of 15:1.

Figure 3.

Cellular uptake and transfection efficiency of PEI-Au NRs/siRNA complexes. (a) Time-dependent release of Alexa555-siRNA (red) inside MDA-MB-231 and SUM-159 breast cancer cells. The cell membrane and nuclei were stained with AF 488 Phalloidin (green) and DAPI (blue), respectively. (b) Transmission electron microscopy (TEM) images of the cellular distribution of PEI-Au NR/siRNA complexes. (c) Quantitative flow cytometry analysis of fluorescence intensity after transfection with PEI-Au NR/Alexa555-siRNA complex. The cell only was used as a control.

Figure 4. Intracellular trafficking of polyethylenimine (PEI)-gold (Au)-nanorod (NR)/small interfering RNA (siRNA) complexes. Cells were harvested at the indicated time points and stained with Lysotracker for imaging the late endosome/lysosome (in red) and DAPI for imaging the nuclei (in blue). Fluorescent images were captured with a confocal microscope.

Figure 5.

Evaluation of the cytotoxicity and immunotoxicity of polyethylenimine (PEI)-gold (Au)-nanorod (NR)/small interfering RNA (siRNA) complexes. (a) Cell viability of MDA MB-231 and SUM-159 breast cancer cells was evaluated using an MTS assay. (b) Immunotoxicity was evaluated in Raw-264.7 mouse macrophage cells by measuring the levels of tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6) in the supernatant by ELISA. Lipopolysaccharide (LPS) was used as a positive control. Experiments were performed in triplicates and data is presented as mean + standard deviation. ** $p < 0.01$.

Figure 6.

Pyruvate kinase isoenzyme type M2 (PKM2) protein expression and cell viability in response to gene silencing and thermal therapy using polyethylenimine (PEI)-gold (Au)-nanorod (NR)/small interfering RNA (siRNA) complexes. (a) Western blot analyses of PKM2 protein expression in MDA-MB-231 and SUM-159 breast cancer cells. β -actin was used as a loading control. Oligofectamine and PEI represented positive controls. (b) Cell viability (MTS assay) in response to PEI-Au NR/siRNA and near infrared light. (c) Cell viability staining after NIR treatment of MDA-MB-231 and SUM-159 cells incubated with PEI-Au NRs/siRNA and treated with NIR. Live cells were stained green with calcein AM, and dead cells were stained red with EthD-1. The boundary of NIR laser beam was marked with a white line in each well. Experiments were performed in triplicates and data is presented as mean + standard deviation. ** $p < 0.01$.