

Dual target gene therapy to EML4-ALK NSCLC by a gold nanoshell-based system

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Method:

Characterization of DOX@siRNA@HAuNs drug delivery system

The UV absorption spectra of the microcapsules was acquired by a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, USA). Morphology and size of the prepared nanoshells were characterized using a JEOL JEM-2100 high-resolution transmission electron microscope (HR-TEM). The hydrodynamic size and zeta-potential of the

DOX@siRNA@HAuNs were measured by Zetasizer (Brookhaven Instruments Corporation, USA) at a temperature of 25 °C. The relatively stability of the HAuNs was further investigated. We incubated the HAuNs before and after PEG coupling at different pH values and different concentrations of salt solution. Meanwhile, we also investigate the release of siRNA and DOX at different pH values and different concentrations of salt solution.

Cell transfection (RNA interference)

H2228 cells or A549 cells were plated at 50% to 60% confluence in 6-well plates and then incubated for 24 hours before transient transfection for the indicated times with siRNAs mixed with the Lipofectamine RNAiMAX reagent (Invitrogen). The siRNAs specific for ALK mRNA (ALK-1, 50-ACACCCAAAUUAAUACCAA-30; ALK-2, 50-UCAGCAAUUCAACCACCA-30), ERK mRNA (ERK-1, 50-CAAGAGGAUUGAAGUAGAA-30; ERK-2, 50-UCAGCCCCUUGAGCACCA-30), or BIM mRNA (BIM-1, 50-GGAGGGUAUUUUUGAAUAA-30; BIM-2, 50-AGGAGGGUAUUUUUGAAUA-30) as well as a nonspecific siRNA (50-GUUGAGAGAUUUAGAGUU-30) were obtained from Sangon. The cells were then subjected to qRT-PCR, western blot or the annexin V-binding assay.

Real-Time Polymerase Chain Reaction

Cells were seeded in 24-well plates at 5×10^4 cells per well. Cells of three groups were respectively treated with siALK@HAuNs and siALK@lip2000, PBS only was used as negative control. The amount of siRNAs and nonsense RNAs delivered by gold nanoshells and Lipofectamine2000 was 5×10^{-9} mol for 5000 cells. Total RNA

was extracted by using Trizol reagent (Invitrogen), and the reverse-transcription reactions were performed using an M-MLV Reverse Transcriptase kit (Invitrogen). The gene-specific stem-loop RT primers for miRNAs, which were released by the Sanger Institute, were designed according to Chen et al. Real-time PCR was performed using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) and a Rotor-Gene RG-3000A (Corbett Life Science, Sydney, New South Wales, Australia) according to the instructions from the respective manufacturer. U6 and GAPDH were used as references for miRNAs and RNAs, respectively. Each sample was analyzed in triplicate. The 2DDCt method was used to quantify the relative levels of gene expression.

Western blot

Western blot analysis was carried out on 10% SDS-PAGE. The siALK and a non-specific siRNA sequence were transfected into the H2228 cells and A549 cells. NC counterparts concurrently, investigate the effect of miRNA 301a inhibitor towards the apoptotic Bim protein expression was investigated. Consequently, miRNA 301a inhibitor and miRNA 301 mimics were added to H2228 cells, respectively. Briefly, equal amounts of protein were separated by 10% SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Thermo Scientific). The membranes were blocked for 1 h with 5% defatted milk and then incubated at room temperature with specific primary antibodies. The primary antibodies used included Bim (Cell Signaling Technology) and GAPDH (Bioworld Technology). Detection was carried out by the use of second antibody (Bioworld Technology) and developing agent (Thermo).

MTT assay

H2228 cells and A549 cells were seeded and cultured in 96-well plates to the density of 1×10^4 cells per well, respectively. Then the Cells was incubated with a range of concentration of HAuNs solution. Furthermore, in order to discover the most effective laser time, different illumination time gradient was tested with RGD @ DOX @ siRNA @ HAuNs, and the HAuNs was used as control group. At last, single treatment and combined treatment on cells were investigated. There are five groups of cells treated with saline, laser, HAuNs and laser, DOX, RGD @ DOX @ siRNA @ HAuNs. Cells were washed with PBS and incubated with fresh medium containing MTT solution (20 μ L, 5 mg mL⁻¹). After 4h incubation, 150 μ L dimethyl sulfoxide (DMSO) was added after removal of the medium. Absorbance was measured at wavelength of 570 nm using a microplate reader and cell viability was then calculated.

Cell apoptosis evaluation

Cells of four groups were, respectively, treated with PBS, siALK, 301inhibitor, both ALK siRNA and miRNA 301a inhibitors, the amount of ALK siRNA and miRNA 301a inhibitor were delivered by Lipofectamine2000. Meanwhile, there are also another four groups, and the amount of ALK siRNA and miRNA 301a inhibitor were delivered by gold nanoshells. Group 1 with PBS alone delivered by Lipofectamine2000 or gold nanoshells were used as negative and positive controls, respectively. Then the Cells were cultured in 6-well plates overnight and treated with inhibitors at indicated doses for 48 hours. Consequently, cells were collected by centrifugation and stained with annexin V and PI following the manufacturer's procedure of the Apoptosis Detection

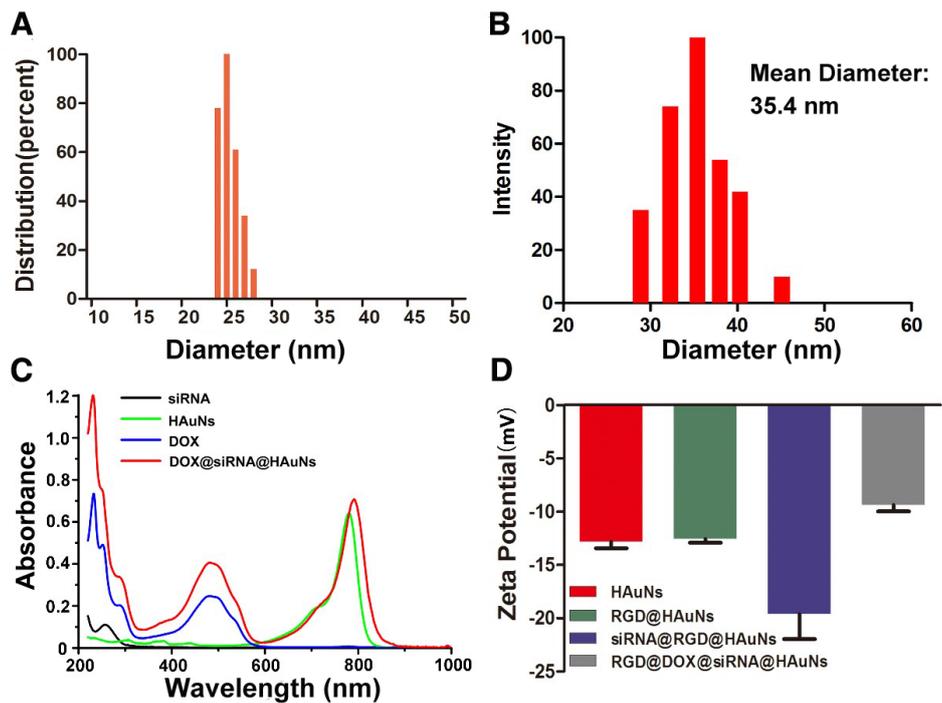
Kit (Beyotime Biotechnology). Binding of annexin V and PI to cells was measured by flow cytometry or laser confocal scanning microscopy (LCSM).

In vivo imaging of mice

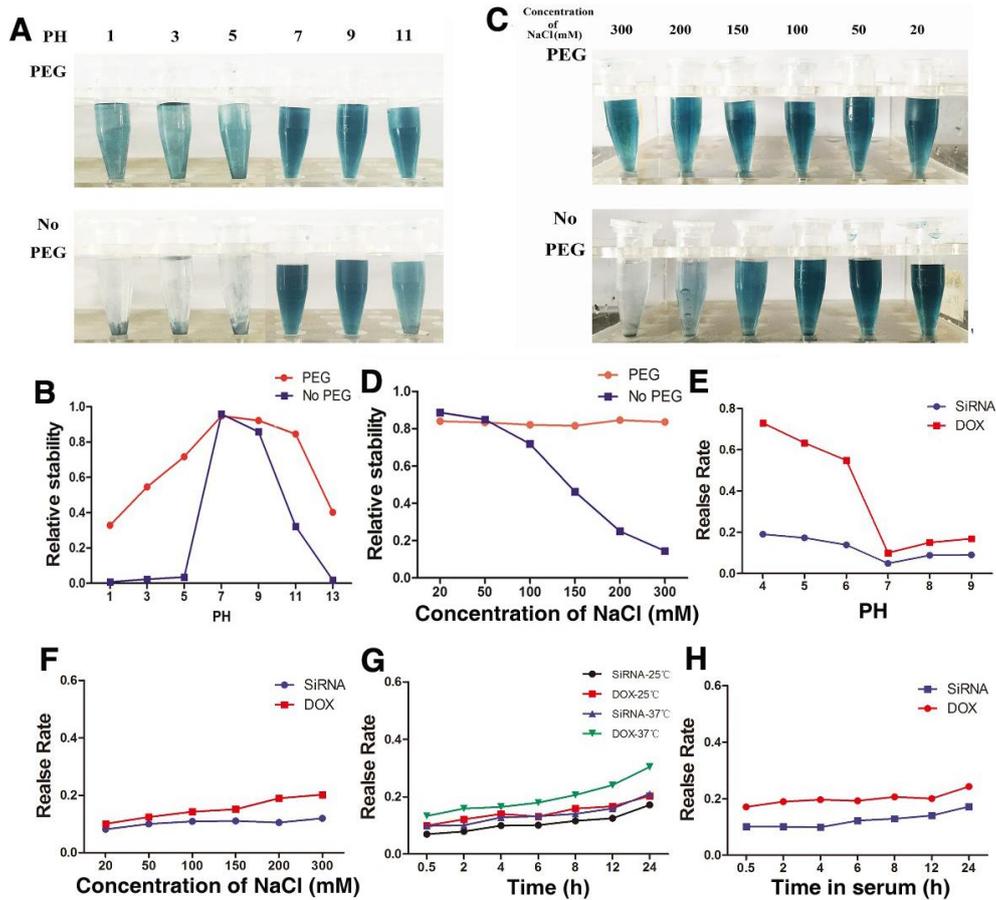
All animal experiments were conducted according to the Animal Management Rules of the Ministry of Health of the People's Republic of China (document No. 55, 2001) and the guidelines for the Care and Use of Laboratory Animals of the China Pharmaceutical University. The mice model was established by injecting a suspension of about H2228 cells (5×10^6), subcutaneously injected into the upper left axillary fossa of each mouse. In vivo study began when the tumors reached 0.5 cm in diameter. The NIR dye MPA (Indocyanine green) adhered to the DOX@siRNA@HAuNs drug delivery system by electrostatic adsorption. The mice were randomly assigned into two groups. Tumor bearing mice were intravenously injected with ICG@DOX@siRNA@HAuNs and MPA respectively at a dye dose of 10 mg/kg ($5 \mu\text{l/g}$ of mouse body weight) and then imaged at various time (0.5h, 2 h, 4h, 6 h, 8 h, and 12 h) post-injection using an NIR imaging system. Athymic nude mice were purchased from Yangzhou University.

ICP-MS assay

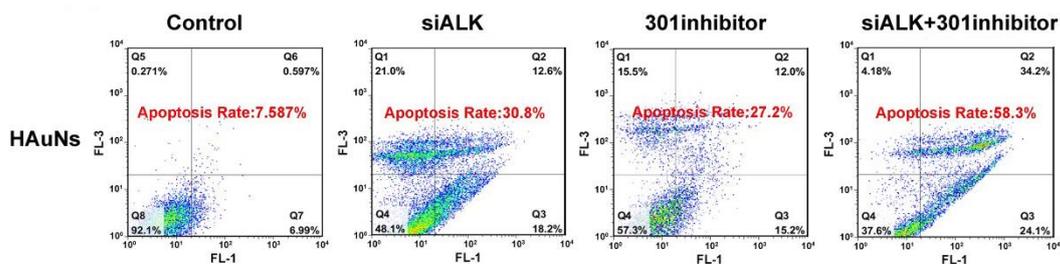
For ICP-MS analysis, H2228 cells were seeded in six-plate well and incubated with different constructs (5×10^{-9} M), respectively. After 2 h incubation, cells were washed with cold PBS and enzymatically detached from the plates. Cells were then dissolved by aqua regia for ICP-MS analysis.



Supplementary Material 1. A, The dynamic light scattering (DLS) characterization of HAuNs. B, the dynamic light scattering (DLS) characterization of drug carrier system RGD@siRNA@DOX@HAuNs. C, UV-vis spectrum of siRNA, HAuNs, DOX, RGD@siRNA@DOX@HAuNs. D, Zeta potential of HAuNs, RGD@HAuNs, siRNA@RGD@HAuNs, and RGD@DOX@siRNA@HAuNs.

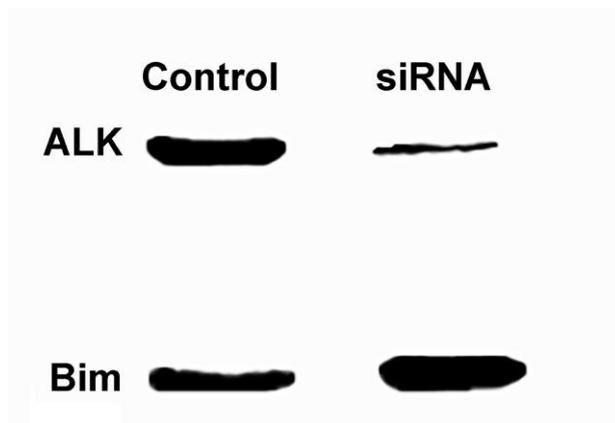


Supplementary Material 2. A, B, image of HAuNs stability in different pH and ion strength solution with or without PEG modification, C,D, quantification of image A and B. E, siRNA and DOX release from drug carrier system in different pH situation. F, siRNA and DOX release from drug carrier system in different NaCl concentration. G, siRNA and DOX release from drug carrier system in different temperature. H, siRNA and DOX release from drug carrier system in serum with different time.

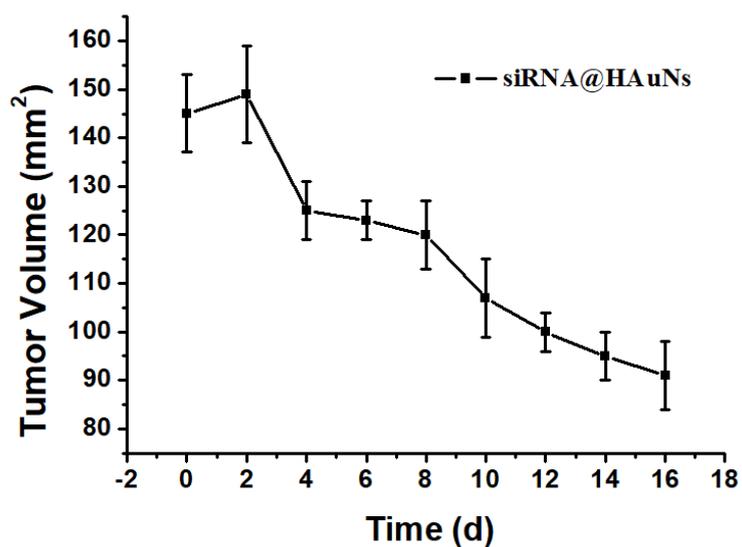


Supplementary Material 3. The apoptosis rate of cells treated under the above

conditions assayed by AV-PI kit.



Supplementary Material 4. Western blot assay of tumor tissue after RGD@DOX@siRNA@HAuNs injected into H2228 tumor bearing mice.



Supplementary Material 5. Tumor volume of mice-bearing H2228 tumors under siRNA@HAuNs injected.