Enhancement of photothermal toxicity and lung targeting delivery of Au nanorods via Heparin-based nanogel

Materials and methods

Materials

Heparin (molecular weight was between 19-20 kDa) was purchased from Shenzhen Hepalink Pharmacetical Co. (China). Polyethyleneimine (Mw=800 Da), polyethyleneimine (Mw=2000Da, PEI2K), 2-morpholino-ethanesulfonic acid (MES), N,N-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), anhydrous dimethyl sulfoxide (DMSO), D,L-lipoic acid, D,L-Dithiothreitol and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co.(St.Louis, MO). Silver nitrate ($AgNO_3$), sodium borohydride, antiscorbic acid, chlorauric acid and citric acid were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Synthesis of Heparin-PEI-LA nanogel

The disulfide bond contained Heparin-PEI-LA nanogels were prepared via amide bond formation between amine and carboxyl groups, which contained in heparin, polyethyleneimine and D,L-lipoic acid. Firstly, heparin-polyethyleneimine nanogels were constructed according to previous report[21]. Heparin (150 mg) was dissolved in 100 mL of MES buffer solution after adjusting the pH to 5.5. Then, NHS and EDC were added into the heparin solution above a weight ratio 1.5. After activating these carboxyl groups of heparin for 4 h, the heparin solution was added into polyethyleneimine (700 mg diluted in 50 mL water solution) drop by drop. The Heparin-PEI nanogel solution was collected after reacting at 25 ^{o}C for 24 h and then dialyzed in distilled water for 2 days. Secondly, the carboxylic acid group of D,L-lipoic acid were activated. 200 mg of D,L- lipoic acid was dissoved in DMSO (30 mL) followed by DCC (100 mg) and NHS (120 mg). After stirring at room temperature for

4 hours and a filtration of insolubles, a dimethyl sulfoxide solution of lipoic acid was obtained. At the end, lipoic acid solution after activated was added into heparin-PEI nanogel solution dropwise. The reaction was stirred for 24 hours, and then its solution was filtered and dialyzed for the purification of the Heparin-PEI-LA nanogel construct. The scheme was shown in Fig. S1.

Characterization of Heparin-PEI-LA nanogels

The particle size, polydispersity index and surface charge of these obtained nanogel were measured by using a Nano-ZS ZEN3600 (Malvern Instruments) instrument at 25 ^{o}C. All experimental groups were test three times.

The morphology of Heparin-PEI and Heparin-PEI-LA nanogels were observed by TEM (JEM-1230, Japan) at an acceleration voltage of 200 kV. After centrifugation and resuspending, nanogel samples of Heparin-PEI and Heparin-PEI-LA suspension (5mg/mL) were administed onto a 400-mesh copper grid. The Heparin-PEI nanogels were followed stained with 2% (w/V) phosphotungstic acid for 5 min at room temperature, while the sample of Heparin-PEI-LA nanogels were observed directly without negatively stained.

Cytotoxicity Assays

To examine the biocompatibility of the obtained nanogels, the cytotoxicity *in vitro* was evaluated by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using L929 cell line. Cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 0.1 mL medium and incubated for 24 h. Next, these medium in each well were replaced by 0.2 mL of fresh Dulbecco's modified Eagle's medium (DMEM) containing different concentrations of heparin-PEI-LA nanogel. After incubated for 24 h, the cell viability was tested by MTT assay.

Preparation of nanogel-Au nanorod complexes

First of all, the gold nanorod was prepared through seed-mediated growth method as reported previously. A 5 mL of o.5 mmol/L $HAuCl_4$ was mixed with 5 mL

of 0.2 M CTAB solution. A 0.6 mL of fresh 0.01 M $NaBH_4$, with a temperature 0 ^oC, was dropped into the Au(lll)-CTAB solution under stirring. After vigorous stirred for 5 min, the mixture was aged at room temperature for 2 h to prepare seed solution.

To obtain Au nanorods, a growth solution mixture containing 5 mL CTAB solution (0.2 M), 0.3 mL $AgNO_3$ solution (4 mM), 5 mL Au(lll) solution (1 mM), 70 μL antiscorbic acid solution (0.0788 M) and 12 μL amount of seed solution was prepared by adding these components successively. Then the mixture was allowed to stand at 28 ^oC for 2 h before use.

Characterization of Heparin-PEI-LA/Au nanorod complexes

The morphology of Heparin-PEI-LA/Au nanorod complexes was observed by TEM (JEM-1230, Japan) with an accelerating voltage of 120 KV.

The temperature versus time curve of both Au nanorod and nanorod complexes were measured using an electronic contact thermometer (IKA ® ETS-D5). The equal volume of 1 mL suspension of Au nanorod and nanorod compound were, respectively, added into eppendorf tubes (1.5 mL) and irradiated with a 808 nm laser at 1 W/cm^2 for 5 min. Temperatures were recorded every 10 seconds. The concentration of Au NRs was 0.2 mM.

Cellular uptake and photothermal effect

As a photothermal agent, the photothermal effect of free Au nanorods compared with these delivered by nanogels was tested *in vitro*. A549 cells were seeded in 6-well plates at a density of 1×10^5 cells/well in 2 mL medium and incubated for 24 h. Followed by adding Au nanorod or nanorod-nanogels respectively for 3 h. The concentration of gold nanorod used in cell co-culture was 1 nM. Each group was exposed to an 808 nm laser at $1W/cm^2$ for 3 min. The death or survival cells were stained using cell death detection kit, containing both calcein AM and propidium iodide (PI).

Distribution of the Nanogel *In Vivo*

The nanogel was labeled with Cy7-NHS and the animal model used was 6 week old nude Balb/c. These unused amino groups on the surface of Heparin-PEI–LA nanogel were chemically modified by using Cy7-NHS, and thus Cy7-labled nanogel could be traced via fluorescence signal. The fluorescence-labeled nanogel was administered by intravenous injection. The near infrared images were taken at predetermined time points, including 1 h, 3 h, 6 h and 24 h.

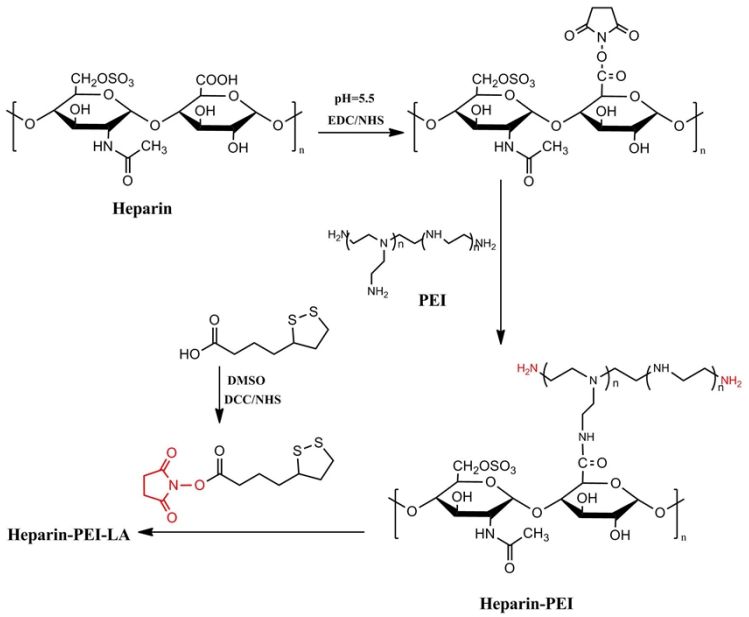

Fig.S1 The synthesis Heparin-PEI-LA copolymer for the nanogel preparation.

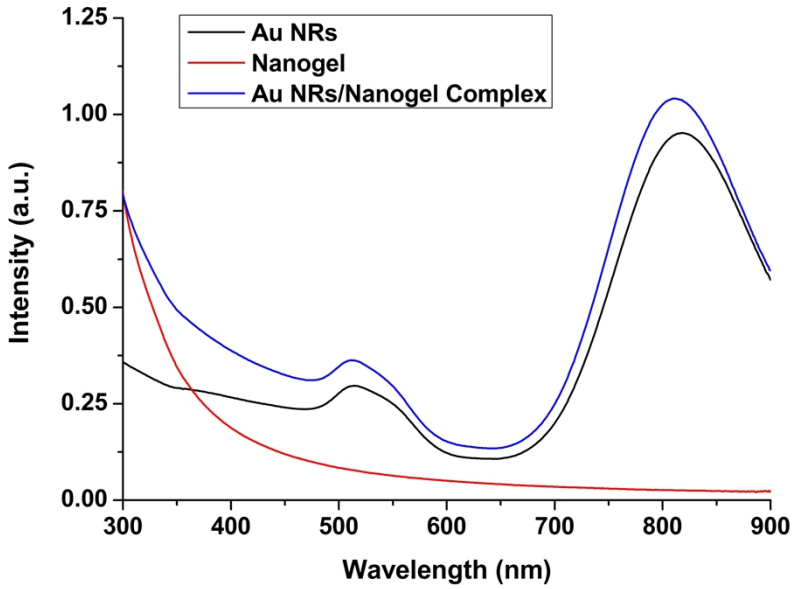

Fig. S2 UV-Vis spectra of Au nanorods and heparin-PEI-LA/Au NRs nanogels complexes at room temperature. The concentration of Au nanorod in each group was all 0.5 nM.

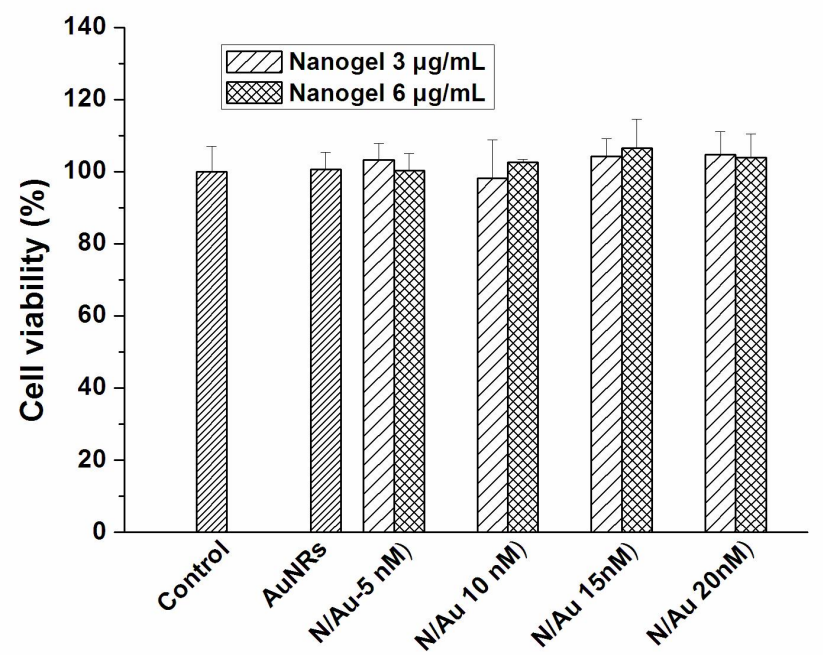

Fig. S3 A biocompatibility evaluation of Nanogel/Au NRs complexes by using L929 cell line *in vitro*. N is an abbreviation of Heparin-PEI-LA nanogel.

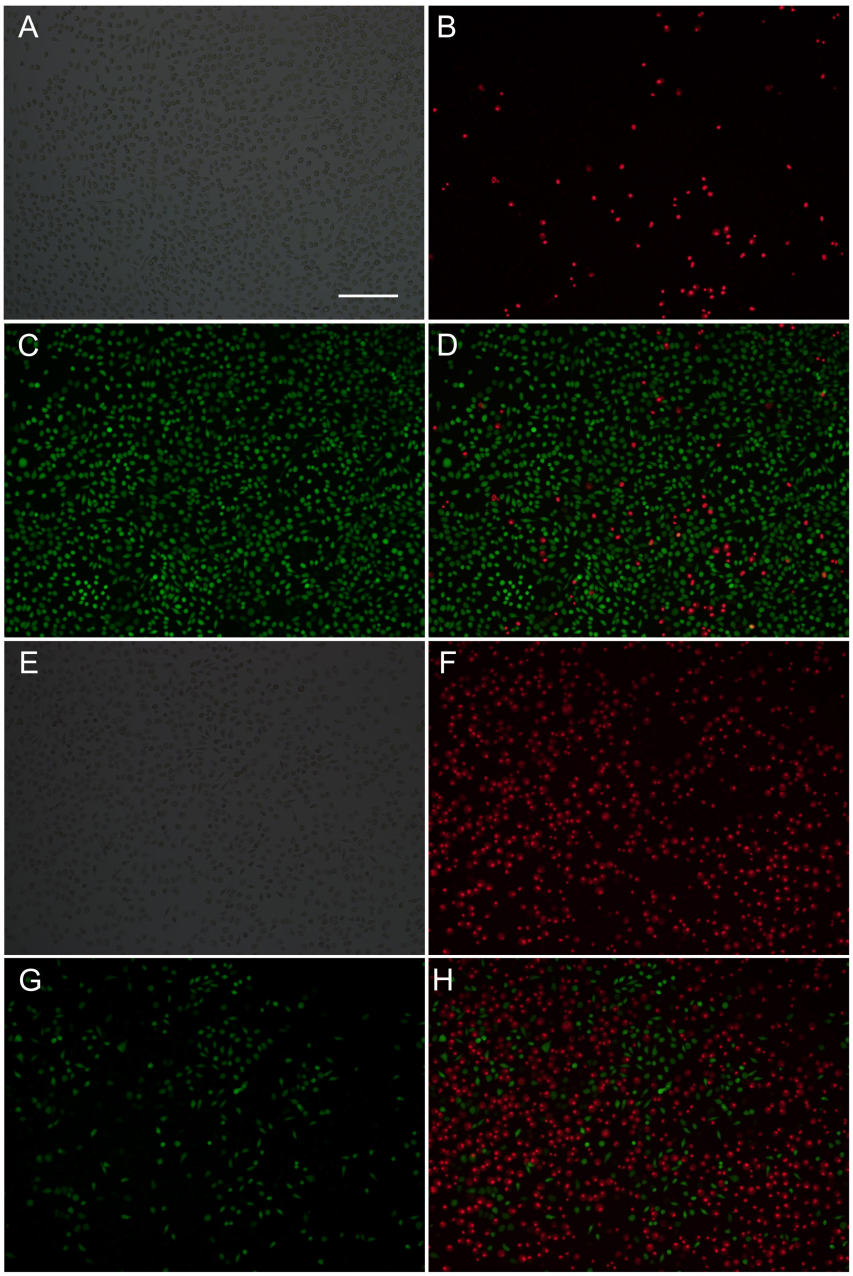

Fig. S4 Fluorescence microscopic images (10×) of calcein AM (green, live cells) and propidium iodide (red, dead cells) indicated cell survival of MCF-7 cells after photothermal treatment. All these cells were incubated with AuNRs/Heparin-PEI-LA nanogels complexes or free single AuNRs with a nanogel concentration of 10 μg/mL at 37 °C. The scale bar was 200 μm.