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ARTICLE TYPE

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

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In this study, we report a synthesis of surface modified heparin-based nanogel by using D, L-alpha-lipoicacid. Heparin-PEI-LA nanogel could exist stably in aqueous solution with uniform size distribution. Transmission electron microscope (TEM) observation showed that the developed Heparin-PEI-LA nanogel could adsorb gold nanorod to form AuNRs-nanogel complexs with the aid of a reducing agent DTT. Then, the photothermal conversion efficiency and photothermal conversion to killing tumor cell *in vitro* of these nanogel complexes were detected. The improved cellular killing efficiency of AuNRs-nanogel might induce by an increased cellular uptake of gold nanorod *in vitro*. Moreover, Cy-7-labled heparin-PEI-LA nanogel could be enriched in the lungs, suggesting the potential use of the nanogel system for a targeting delivery of gold nanorods and a great potential application in the tumor photothermal therapy.

Polymeric drug vectors of nanoscale size range and noble metal used for biomedical treatment have both attracted increasing attention in recent years[1, 2]. More than ever, metal nanoparticles, such as gold and silver nanoparticles, could be delivered by polymeric carriers to improve their diagnosis or treatment effect[3-7].

As a class of intelligent biomaterials, hydrogel is defined as hydrophilic three-dimensional polymer networks containing large amounts of water or physiological fluid[8, 9]. The internal network structure of hydrogels can payload therapeutic agents and release them in a targeting site. Nanogel is the hydrogel with nanoscopic dimensions and easily accessed in the lesion areas. Therapeutic drugs, such as peptide, siRNA, nucleoside analogs and water soluble chemotherapeutic agent, can be delivered using nanogel by chemical bond conjugating or noncovalent interactions[10-13]. With the deep understanding of metal particles, various nanogels are designing to expend the scope of application in cancer diagnosis and treatment by coujugating nanogel with metal nanoparticles.

Gold nanoparticles (AuNPs), with surface plasmon resonance (SPR) enhanced light scattering and absorption, have been widespread used in cancer diagnosis and therapy, including sensing, delivering, labelling and heating[2, 14]. After conjugated with targeting ligands to biomarkers on cancer cells, AuNPs were used in molecular-specific imaging and detection of cancer. Furthermore, spherical AuNPs are used as platform to synthesize mixed monolayer-protected AuNPs. Polymer used for drug delivery can conjugate with AuNPs through non-covalent conjugation via different interactions including specific binding affinity, hydrophobic interactions and electrostatic interactions[15-17]. The mixed complexes have widely application in construction delivery system and sensing areas due to their ease of release[18]. As a basic physicochemical property of gold nanoparticles, the rapid conversion of absorbed light into heat induced by SPR absorption of AuNPs has been used in the photothermal therapy of cancer[19, 20].

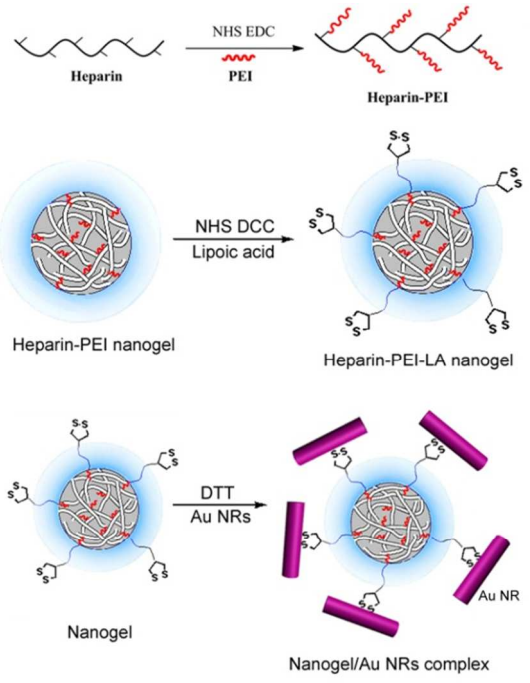

Fig. 1 The scheme of preparation Heparin-PEI-LA nanogel used for the adsorption of Au nanorods

Gold nanoparticles adsorption with polymer is usually achieved through specific adsorption of chemical bonds by thiol through a place ligand exchange reaction. In order to complete an effective delivery of the gold nanoparticle, Heparin-PEI nanogel was synthesized with lipoic acid (LA) modification on its surface (Fig. 1).

Heparin was connected with PEI through amide bond. The carboxyl group on heparin were activated by NHS and linked with polyethyleneimine via amide bond (Fig. S1). After negatively stained by phosphotungstic acid, the morphology of nanogel was observed by using transmission electron microscopy. As presented in Fig. 2A and 2B, it was clearly observed that the copolymer heparin-PEI could self-assemble into nanogel with homogeneous nanoscales.

TEM images of heparin-PEI-LA nanogel were shown in Fig. 2C. Heparin-PEI nanogels could not be observed without negative staining, while heparin-PEI-LA nanogels can be directly observed using an electron microscope with clear morphology. The size distribution of heparin-PEI nanogels was detected. As shown in Fig. 2D and Table 1, most nanogels had homogeneous nanoscales in a range of 80-100 nm. All these results indicated the developed heparin-PEI and heparin-PEI-LA nanogel showed narrow dispersibility in aqueous solution. Furthermore, the data in table indicated that the molecular weight of PEI did not significantly affect the size distribution of various heparin-based nanogels.

	Particle Size (nm)	PDI	Zeta potential (mV)
Heparin-PEI_{800}	93.02±0.37	0.214±0.022	2.5±0.91
Heparin-PEI_{1800}	70.48±5.1	0.42±0.32	7.71±0.49
Heparin-PEI_{800}-LA	40.48±2.06	0.48±0.08	1.28±0.49
Heparin-PEI_{1800}-LA	76.49±3.89	0.284±0.017	38.75±0.05

Table 1. Size distribution and zeta potential of various heparin-PEI and heparin-PEI-LA nanogels.

The cytotoxicity of various nanogels containing various types of PEI was tested *in vitro* using L929 cell line (Fig. 2E-F). With increasing dosage of nanogels, there were no significant changes in cell viability. These two developed nanogels were low toxicity with acceptable range. Therefore, we assumed that the heparin as a good biocompatibility and biodegradable polymeric material could be used in the synthesis of nanogels. On the other hand, pilot studies have been demonstrated that the small molecular polyethyleneimine could be quickly metabolized, while it was uptaken into cell and biodegradated from the heparin-PEI nanogel. In addition, as a nutrient component, lipoic acid has no toxicity to cell.

Gold nanorods were synthesized and their characterizations were shown in Fig. 3. As shown in Fig.3A-B, the gold nanorod (AuNRs) with a strong absorption spectrum in the near infrared region (780-900 nm) was successfully synthesized by seed-mediated growth method. After a surface modification using lipoic acid, the exposed disulfide linkage could be used to adsorb gold nanoparticles. To confirm this assumption, images of nanogel product were taken by an electron microscopy (Fig.3C). However, the LA-modified nanogel could not adsorb Au-rod efficiently without adding reducing agent. In the process of nanorod/heparin-PEI-LA nanogel compound preparation, reducing agent DTT (0.1 mM) was added to improve the Au nanorod adsorption efficiency. UV-Vis spectra were collected using a Lambda 25 UV/Vis spectrometer (Perkin Elmer, USA). The data shown in Fig S2 indicated that the adsorption between AuNRs and nanogels had no effect to the UV absorption of gold nanorods. The biocompatibility of the nanogel/Au complex was tested via an effect of nanogel/Au complexes on cell proliferation. The obtained result indicated that the nanogel/Au complex had low toxicity in the function of concentration (Fig. S3).

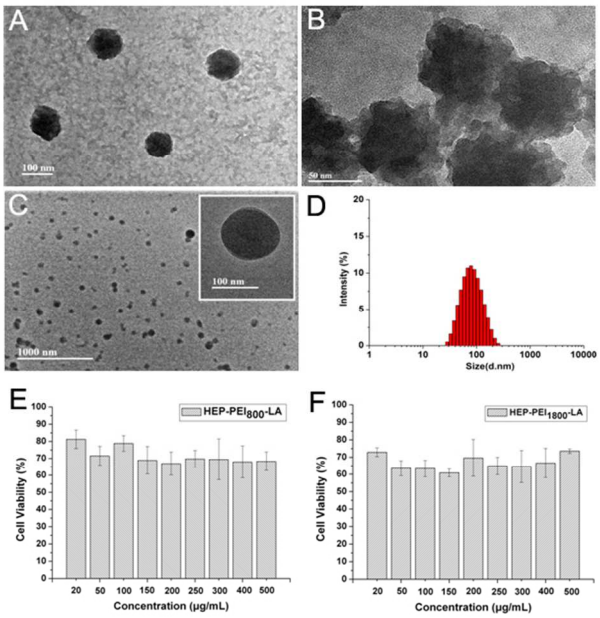

Fig. 2 The characterization of the Heparin-PEI and Heparin-PEI-LA nanogels. (A) and (B) the TEM images of the Heparin-PEI nanogel in different enlargement. (C) TEM images of the Heparin-PEI-LA nanogel; (D) The size distribution of obtained Heparin-PEI_{1800}-LA nanogels; (E) and (F) cell viabilities of L929 cells treated with Heparin-PEI_{800}-LA nanogels and Heparin-PEI_{1800}-LA nanogels.

At preliminary study, we attempted to use sodium borohydride as the reducing agent to prepare AuNRs-nanogel complexes. Sodium borohydride has been commonly used in preparing Au nanorods. However, its reduction effect was so violent that a large purple flocculent precipitate occurred in our small scale preparation. Therefore, DTT, as a mild reducing agent, was used in the reduction of disulfide, which induced a specific adsorption between heparin-PEI-LA nanogel and gold nanorods. In preparing nanogel-AuNRs, the morphologies of nanogel-AuNRs complexes obtained from DTT group and sodium borohyide group were different. A strong and transient reduction induced by

sodium borohydride caused a precipitation of heparin-PEI-LA nanogel.

The temperature increase induced by photothermal conversion with 808 nm laser irradiation was measured by a thermometer. The concentration of AuNRs used in each group was 0.2 mM. Results shown in Fig. 3D indicated that the temperature of gold nanorod water solution rose from 27°C to 65°C by an unremitting excitation light irradiation. On the other hand, after continuous irradiation of 808nm laser, the temperature of Au nanorod-nanogel complexes solution has also increased from 27°C to 53°C, but the increase efficiency was lower than that in single gold nanoparticles in water solution. In actually, we cannot accurately explain the different temperature increasing between the group of single AuNRs and AuNRs/nanogel complexes. Possible reason of our speculation was below. After been adsorption by heparin-PEI-LA nanogel, the particle size induced photothermal conversion efficiency of Au nanorods was changed, and thus resulted in a little lower temperature in AuNRs/nanogel complexes group.

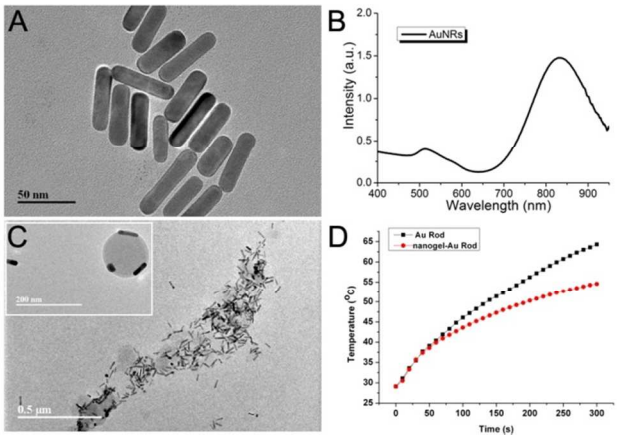

Fig. 3 Characterizations of Au nanorods and heparin-PEI_{1800}-LA nanogel complexes. (A) TEM (120 kV, JEM-1230, Japan) images of gold NRs synthesized using 0.2M CTAB and 0.078 M antiscorbic acid in the growth solution. (B) Absorption spectra of obtained gold nanorods. (C) TEM images of gold NRs/heparin-PEI-LA nanogel complexes. (D)Record of temperature increment induced by photothermal conversion under 808 nm laser irradiation.

AuNRs was composited with heparin-PEI-LA nanogel, and then the photothermal treatment effect of AuNRs-nanogel complexes was tested *in vitro* by using free AuNRs as control. MCF-7 cells were co-culture with AuNRs-nanogel complexes and free AuNRs respectively for 4 hours and then exposed to an 808 nm laser at 1 W/cm^2 for 3 min. the survival and dead cells were discriminated by both propidium iodide and calcein AM staining. The AuNRs concentration used in each group was 1 nM. Fluorescence images of MCF-7 cells with the treatments of free AuNRs or AuNRs/nanogel complexes shown in Fig. S4, the efficiency of survival cell in free Au NRs treated group was much higher than that in AuNRs-nanogel treated group. A quantitative data shown in Fig.4 indicated the *in vitro* photothermal toxicity of nanogel-delivered AuNRs. The MTT assay was agree well with the fluorescence staining of live/dead cells shown in Fig, S4. Both the two results indicated the photothermal toxicity of AuNRs in AuNRs/nanogel group was higher than it in the single AuNRs group. Thus, these photothermal effects under different treatments conclude an increasing cell uptake to gold nanorod delivered via heparin-PEI-LA nanogel. The improved photothermal treatment effect might be induced via enhancement of AuNRs cellular uptake. These results indicated that delivering nanorod using heparin-PEI-LA might be an advanced method to realize integration of tumor diagnosis and treatment.

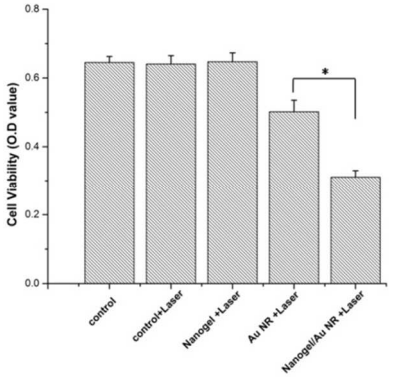

Fig. 4 The cell viability of MCF-7 cells after a laser irradiation (n=3).

A targeting biodistribution of functional molecule is a basic property for delivering system. Using fluorescent agent Cy7, these heparin-PEI-LA nanogels were labelled. The biodistribution was imaged in Fig. 5. The fluorescent signal was traced by NIR imaging.

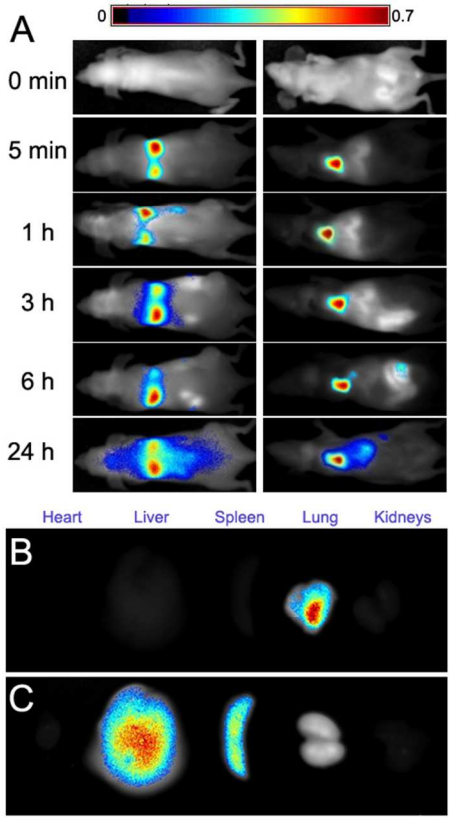

Fig. 5 The biodistribution of Cy7-labled heparin-PEI-LA nanogel. (A)

In vivo imaging of Cy-7-labled nanogels delivered systemically via tail vein injections in nude mice. (B) Fluorescence image of organs after 24 h post-injection of Cy7-labled heparin-PEI-LA nanogels. (C) Fluorescence image of organs after 24 h post-injection of free Cy7.

The fluorescence and intensity distributions as a function of time for free Cy7 and Cy7-labled nanogels (Fig. 5A). The fluorescence signal was enrichment in the lung quickly after tail vein injection. With time increasing, there was no significant weakening of the fluorescence signal in lung. For nude mice treated with free Cy7, fluorescence signal appeared in liver and spleen at 24 h post injection. At the same time, fluorescence signal from Cy7-marked nanogel in the lung was strongest (Fig.5B and C). The result indicated that a certain enrichment of Heparin-PEI-LA nanogel appeared in lung. It was different with that distribution of free stain Cy7.

As everyone knows, particle size, zeta potential and chemical modification are key agents for affecting the biodistribution of drug delivery system. Compared with free Heparin-PEI-LA nanogel, complexes formulated by positive nanogel and negative fluorescent molecular have different biodistribution. The different distribution might induce by zeta potential on its surface. Further study needs to be done to confirm the relationship between zeta potential and biodistribution.

Conclusions

In summary, an Au-nanorod delivery system based on Heparin-PEI-LA was developed through specific adsorption. Heparin-PEI was synthesized by chemical grafting method. By a surface modification using the small molecule compound lipoic acid, the developed heparin-PEI-LA nanogel could effectively adsorb gold nanoparticles without affecting the light-heat conversion. The Cy7-labled biodistribution of nanogels indicated that the blank Heparin-PEI-LA nanogel can be concentrated in lung *in vivo*. In addition, the photothermal efficiency of gold nanoparticle can be improved by the nanogel delivery system *in vitro*, which showed a great application in tumor therapy. Subsequent mouse model as well as the effect of photothermal therapy remains to be verified *in vivo*.

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Notes and references

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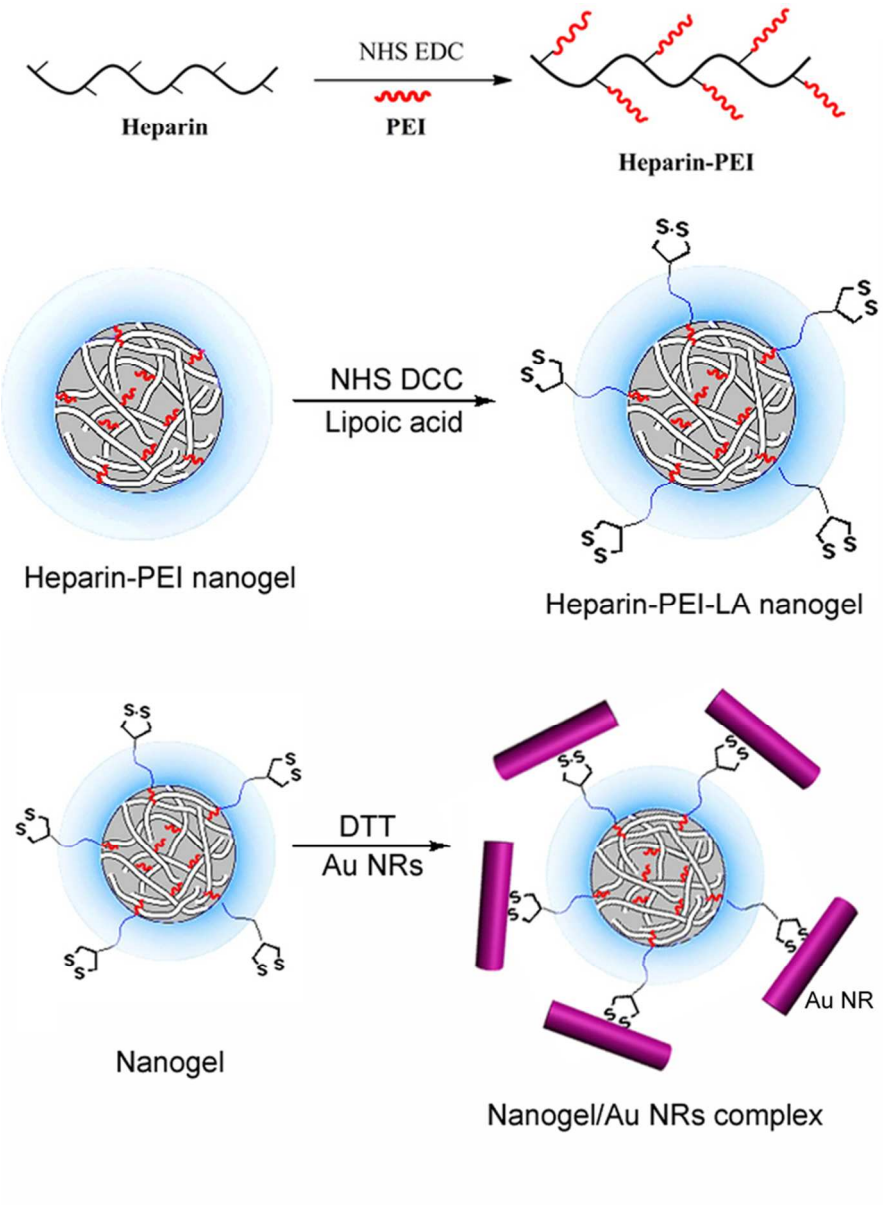

The scheme of preparation Heparin-PEI-LA nanogel used for the adsorption of Au nanorods
72x97mm (300 x 300 DPI)