Preparation and characterization of doxorubicin functionalized tiopronin-capped gold nanorods for cancer therapy

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Gold nanomaterials are emerging candidates in medical diagnosis and treatment. Among them, gold nanorods (Au NRs) are widely used for cancer treatment. Tiopronin as a novel thiol drug was used to stabilize Au NRs in this work. Doxorubicin (DOX), a chemotherapeutic drug which works by interacting with DNA to arrest the cell cycle and induce apoptosis, was linked to Au NRs through electrostatic reaction with tiopronin, to obtain Au-TIOP-DOX NRs. Au NRs are also regarded as hyperthermia agents for photothermal cancer treatment. This delivery system (Au-TIOP-DOX NRs) was designed for passively targeting tumor cells in cancer therapy. More importantly, the carboxyl groups of tiopronin can be modified with some biological molecules (DNA/RNA, peptides, or drugs) to make Au NRs as a novel drug-delivery system for cancer treatment.

gold nanorods, tiopronin, doxorubicin, cancer therapy

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Gold nanorods (Au NRs) are promising nanomaterials as therapeutics, imaging probes, contrast agents and drug vehicles in cancer treatment [1–4]. The unique properties, such as tunable surface plasmon resonance (SPR), two-photon luminescence and ease of functionalization, make them suitable in thermal therapy, cell imaging, gene silencing and targeted drug delivery [5–8]. PEGylated Au NRs are reported to be more stable in physiological condition and obtain a longer circulation time *in vivo* [9,10]. In addition, Dreaden et al. [11] synthesized a small molecule-Au NRs conjugate which can selectively target tumor-associated macrophage cells and induce cytotoxicity to breast cancer cells.

Cetyltrimethylammonium bromide (CTAB), a cationic detergent, is used as stabilizer and surface coating in the preparation of Au NRs and it is highly toxic to cells. Due to the free CTAB in the Au NRs solution which can hardly be

removed totally, the biological application of Au NRs is limited because of the cell toxicity [12,13]. Much work has been done to optimize the surface coating of Au NRs and then to reduce the cytotoxicity, improve the biocompatibility and increase the drug loading efficiency [14–16]. Among these work, PEGylation seems to be a good choice as it could prolong the circulation time of Au NRs in the circulatory system, escape from the clearance of reticulo-endothelial system (RES) [17]. However, the cellular uptake of PEG-Au NRs is not that satisfactory to people's expectation and we are looking for a better surface coating of Au NRs. In this work, we replace the CTAB on the Au NRs surface with tiopronin, a novel thiol-group drug which could form Au-S bond, a covalent bond stronger than the interaction between CTAB and Au atom. Thus, the replacement of CTAB is highly efficient and biocompatibility is improved. Tiopronin also has a carboxyl group which is capable of lots of chemical reactions; therefore, much therapeutics can be loaded on Au NRs. Here, we functionalized

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tiopronin-coated Au NRs with a clinical used cancer therapeutic, doxorubicin (DOX), and the treatment efficacy of Au-TIOP-DOX NRs were assessed initially [18,19].

1 Materials and methods

(i) Chemicals. Gold chloride trihydrate ($\geq 99.9\%$, $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), cetyltrimethylammonium bromide ($\geq 98\%$, CTAB, $\text{C}_{19}\text{H}_{42}\text{BrN}$) and N-(2-mercaptopropionyl) glycine (tiopronin, $\text{C}_5\text{H}_9\text{NO}_3\text{S}$) were purchased from Sigma-Aldrich (St Louis, USA). L(+)-Ascorbic acid (99.0%) was supplied by Acros (USA). Sodium citrate ($\geq 99.5\%$, $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) was obtained from Solarbio (Beijing, China). Silver nitrate ($\geq 99.8\%$, AgNO_3) was purchased from GuangFu (Tianjin, China). Doxorubicin ($\geq 99\%$, DOX) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. All glass wares used for the preparation and storage of gold nanoparticles were cleaned with aqua regia ($\text{HCl}:\text{HNO}_3 = 3:1$, v:v). All chemicals were used without further purification and Milli-Q water was used throughout this study.

(ii) Synthesis of CTAB capped gold nanorods. CTAB capped Au NRs (Au-CTAB NRs) were prepared according to the seed-mediated growth method [20]. Briefly, a seed solution was prepared by mixing 9.15 mL of CTAB (0.1 mol/L) and 0.25 mL of HAuCl_4 (0.01 mol/L) with 0.6 mL freshly prepared ice-cold NaBH_4 solution (0.1 mol/L). The color of the solution changed from dark yellow to brownish yellow under vigorous stirring, indicating the formation of the seed solution. This seed solution was used within 2–6 h for the synthesis of the Au-CTAB NRs. Briefly, 28.27 mL of 0.1 mol/L CTAB was mixed with 1.2 mL of 0.01 mol/L HAuCl_4 and 0.18 mL of 0.01 mol/L silver nitrate aqueous solution. After gentle mixing of the solution, 0.2 mL 0.1 mol/L ascorbic acid was added, and then the solution changed to colorless transparent. With continuously stirring, 0.15 mL of the seed solution was added to initiate the growth of the Au-CTAB NRs.

(iii) Tiopronin coating of Au NRs. A surface ligand exchange reaction was adopted to obtain tiopronin coating of Au NRs (Au-TIOP NRs). Briefly, 1 mL of Au-CTAB NRs was centrifuged at 9000 r/min for 10 min, and the precipitate was re-dispersed in 1 mL deionized water. Under vigorous stirring, 1 mL Au-CTAB NRs and 1 mL tiopronin solution (2 mg/mL) were mixed, sonicated for 60 s and stirred overnight at room temperature. The resulting Au NRs were then collected by centrifugation at 9000 r/min for 15 min and the tiopronin coated Au NRs were obtained.

(iv) Preparation of DOX-conjugated Au NRs. DOX loading onto Au-TIOP NRs was done by simply mixing 1 mL 0.5 mg/mL DOX with 1 mL Au-TIOP NRs. The resulting solution was kept stirring overnight at room temperature. Excess DOX molecules were removed by centrifugation at 10000 r/min for 10 min. To evaluate the DOX-loading efficiency, the content of the residual DOX in the supernatant

was determined by UV-Vis measurements at 480 nm [18]. The DOX-loading efficiency was calculated by

Loading efficiency (%)

$$= \frac{m(\text{total DOX}) - m(\text{DOX in supernatant})}{m(\text{total DOX})} \times 100\%. \quad (1)$$

(v) Cell culture. The human breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C.

(vi) Cell viability assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the viability of MCF-7 cells. MCF-7 cells were seeded in 96-well plate, and after 24 h, Au-TIOP-DOX NRs and free DOX were added at the concentration of 0.08, 0.4, 2, 10 $\mu\text{g/mL}$ based on DOX into the plate. As negative controls, cells were cultured with Au-CTAB NRs and Au-TIOP NRs at the corresponding concentration of Au NRs with the Au-TIOP-DOX NRs added. Another 24 h later, 10 μL MTT solution was added per well, and after 4 h of incubation, formazan crystals were dissolved in 150 μL DMSO. The absorbance was measured at 570 nm with a reference wavelength of 630 nm using an Infinite M200 microplate reader (Tecan, Durham, NC, USA).

(vii) Characterization. Morphology characterization of the Au NRs was performed using a Tecnai G² 20 S-TWIN transmission electron microscope (TEM, Philips, Netherlands) with 200 kV acceleration voltage. Optical absorption spectra were measured with a Lambda 950 UV/Vis/NIR spectrophotometer (Perkin-Elmer, USA). Zeta potential distribution was determined by a Nano ZS Zetasizer (Malvern, England). The concentration of gold was determined via an Optima 5300DV Inductively Coupled Plasma Optical Emission Spectrometer, or an ELAN DRC-e Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer).

2 Results and discussion

The preparation of DOX-conjugated Au NRs (Au-TIOP-DOX NRs) is shown in Figure 1. CTAB stabilized Au NRs (Au-CTAB NRs) with aspect ratios ~ 4 were prepared firstly using a seed-growth method, the most popular method for the synthesis of colloidal Au NRs [20]. Tiopronin stabilized Au NRs (Au-TIOP NRs) were obtained via a well-developed ligand exchange method [21]. As we all know, thiol group exchange is the most common way to replace the original capping molecules since the metal-sulfur bond is known to be the strongest bond compared to other general functional groups. Tiopronin is a thiol drug with good biocompatibility used to control the rate of cysteine precipitation and excretion in the disease named cystinuria [22,23]. It is commonly used as a stabilizing agent for metal nanoparticles, as its thiol groups can bind to the surface of nanoparticles, preventing aggregation and sedimentation

Figure 1 Illustration of the procedure for preparing the doxorubicin functionalized tiopronin-capped Au NRs.

[24]. In this work, tiopronin not only makes Au NRs stable in various physiological relevant media but also makes the outside of the nanorods bearing carboxyl groups. The resulting Au-TIOP NRs exhibited reversed surface charge and allowed for the electrostatic adsorption of DOX.

As shown in Figure 2, the transmission electron microscopy (TEM) images showed Au NRs in each step with a uniform morphology with aspect ratios ~ 4 . It is also found that compared with CTAB capped Au NRs, Au-TIOP NRs have no change on shape and size after ligand-exchange reaction. From the negative staining TEM images of Au-TIOP NRs and Au-TIOP-DOX NRs shown in Figure 3, after DOX adsorption reaction, an about 2 nm thin coating can be seen around the surface of Au-TIOP-DOX.

In order to confirm each step of the surface-modification of Au NRs, zeta potential study was conducted. As shown in Figure 4, the zeta potential value of the as-prepared Au-CTAB NRs is +30.5 mV. After ligand exchange reaction with tiopronin, the value changes to -18.9 mV, indicating the charge reverse of the Au NRs because of the free negatively charged carboxy groups of tiopronin exposed on

the outside surface of Au NRs. The successful adsorption of DOX makes the zeta potential to a value of -3.6 mV. Meanwhile, the photos in Figure 4 showed the color change in each step of different capped Au NRs. Moreover, after DOX binding to Au-TIOP NRs, the zeta potential remains a little negative, which may diffuse more quickly and perform better when delivering drugs deep into tissues [25].

In addition, to further determine whether the Au-TIOP-DOX NRs is successfully obtained, the UV-Vis absorption spectra of Au NRs with different coatings in each step were carried out. Au NRs showed two absorption bands: a strong longitudinal band in the near-infrared region corresponding to electron oscillation along the long axis and a weak transverse band, similar to that of gold nanospheres, in the visible region corresponding to electron oscillations along the short axis [26–28]. Compared with CTAB capped Au NRs, the absorption peak of Au-TIOP NRs shifted from 734 to 744 nm as shown in Figure 5. After electrostatic adsorption of DOX, Au-TIOP-DOX NRs showed two absorption peaks at 480 and 760 nm; 480 nm is the absorption peak of free DOX, and this demonstrated the attachment of DOX on the surface of Au-TIOP NRs. Because of the absorbance at 480 nm, Au-TIOP-DOX NRs can be utilized as fluorescence probe for imaging. Current plasmonic nanoparticles for

Figure 2 TEM images of Au-CTAB NRs, Au-TIOP NRs and Au-TIOP-DOX NRs.

Figure 3 Negative stained TEM images of Au-TIOP NRs and Au-TIOP-DOX NRs.

Figure 4 Zeta potential of Au-CTAB NRs, Au-TIOP NRs and Au-TIOP-DOX NRs. Corresponding pictures were shown on or under the zeta potential columns.

Figure 5 UV-Vis absorption spectra of Au-CTAB NRs, Au-TIOP NRs, Au-TIOP-DOX NRs and free DOX.

Figure 6 Cell viability of MCF-7 cells after 24 h treatment.

photothermal therapy of cancer are gold nanospheres, nanorods, nanoshells, and nanocages [3]. The last three types of plasmonic nanoparticles are more popular as they absorb light in the NIR window (650–900 nm [29]) where light penetration is optimal due to the minimal absorption by water and hemoglobin in the tissue. For their facile synthesis, excellent stability, small size, high photothermal conversion efficiency and tunable absorption in the biological window, Au NRs have become one of the most favorite nanoparticles for photothermal therapy. As UV-Vis absorption spectra shown in Figure 5, Au-TIOP-DOX NRs have a strong longitudinal band in the near-infrared region, so they also meet the conditions used for photothermal therapy.

In order to ensure sufficient DOX-loading onto the surface of gold nanorods by electrostatic adsorption, excess of DOX was added into the reaction. To evaluate the DOX-loading efficiency, the content of the residual DOX in the supernatant was determined by UV-Vis measurements at 480 nm [18]. The DOX-loading efficiency was calculated by eq. (1) is about 20% and the DOX-loading concentration is about 130 $\mu\text{g/mL}$.

To evaluate the cell toxicity of each different capped Au NRs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the cell viability after incubation with these nanomaterials. As shown in Figure 6, MCF-7 cells were cultured with Au-CTAB NRs, Au-TIOP NRs, Au-TIOP-DOX NRs and free DOX at different concentrations. The results showed that the cytotoxicity of Au-TIOP NRs is the lowest one, indicating the successful exchange of CTAB. Due to the free CTAB in the Au NRs solution which can hardly be removed totally, the Au-CTAB NRs had relatively strong cell toxicity. The toxicity test indicated the replacement of CTAB was highly efficient. Compared with DOX, the Au-TIOP-DOX NRs had a better effect in killing cancer cells from the cell viability test. The high cytotoxic effect of Au-TIOP-DOX NRs is considered as a result of the release of DOX into MCF-7

cells after the cellular uptake. Hence, we expect that Au-TIOP-DOX NRs have promising potential as drug loading and delivery carriers in cancer therapy.

3 Conclusions

In summary, Au-TIOP-DOX NRs were prepared through thiol exchange and electrostatic adsorption method. Tiopronin, a thiol drug with good biocompatibility, was used to replace the CTAB on the surface of Au NRs, and the reversed surface charge of Au-TIOP NRs allowed for the further electrostatic adsorption of DOX. Characterizations were carried out to verify each step of different coatings of Au NRs. Furthermore, the cell viability was tested to determine the cancer-killing effect and the result was that the Au-TIOP-DOX NRs have a better cytotoxicity compared to free DOX. More importantly, the carboxyl groups of tiopronin not only can be used for electrostatic adsorption but also can be modified with molecules, peptides or drugs for active targeting purposes. The prepared doxorubicin functionalized tiopronin-capped Au NRs can be promising in drug-delivery, fluorescence imaging and localized hyperthermia probe for cancer therapy.

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