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Selective inhibition of breast cancer stem cells by gold nanorods mediated plasmonic hyperthermia

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ABSTRACT

Cancer stem cells (CSCs) have been identified in a variety of cancers and emerged as a new target for cancer therapy. CSCs are resistant to many current cancer treatments, including chemotherapy and radiation therapy. Therefore, eradication of this cell population is a primary objective in cancer therapy. Here, we report gold nanorods (AuNRs) mediated photothermal treatment can selectively eliminate CSCs in MCF-7 breast cancer cells. It significantly reduced the aldehyde dehydrogenase positive ($ALDH^+$) cells subpopulation and the mammosphere formation ability of treated cells. Also, the gene expression of stem cell markers was decreased. Cellular uptake assay revealed that polyelectrolyte conjugated AuNRs could be internalized by CSCs much more and faster than non cancer stem cells (NCSCs), which might be the main reason for the selective elimination of CSCs. We further loaded salinomycin (SA), a CSCs inhibitor with polyelectrolyte conjugated AuNRs to get a synergistic CSCs inhibition. Enhanced inhibition of CSCs was obtained by NIR light triggered drug release and hyperthermia. This CSCs-targeted thermo-chemotherapy platform provides a new combinatorial strategy for efficient inhibition of CSCs, which is promising to improve cancer treatment and may overcome the chemoresistance and recurrence of cancer.

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women of all female cancers [1]. Albeit significant advances have been achieved in the diagnosis and treatment of breast cancer, as many as 40% of patients are still experiencing relapse and metastatic disease [2]. Extensive research has elucidated that a small subpopulations of cells within tumors, termed cancer stem cells (CSCs), are responsible for tumor growth and recurrence [3–6]. CSCs are defined as cells that have the capacity to self-renew and can generate the heterogeneous lineage of cancer cells that comprise the whole tumor [7]. There is increasing evidence that both hematopoietic and solid tumors contain populations of CSCs [8]. CSCs are resistant to current chemotherapy and radiotherapy and therefore responsible for tumor recurrence [9–11]. Thus, development of CSCs-targeted cancer therapy is pivotal for the prevention of tumor recurrence and metastasis. Gupta et al. have screened a series of chemicals to discover compounds selective targeting and inhibiting breast CSCs. They found that salinomycin (SA), a polyether antibiotic, selectively inhibited CSCs both *in vitro* and *in vivo*. It reduces the proportion of CSCs by >100 fold relative to paclitaxel, a commonly used breast cancer chemotherapeutic drug [12].

The unique physicochemical properties of nanomaterials have provided opportunities for multifunctional cancer therapy and diagnosis. In particular, gold nanostructures have been extensively investigated as a promising and versatile platform for cancer theranostics [13,14]. Among them, gold nanorods (AuNRs) showed many advantages in cancer cell imaging and photothermal therapy based on their localized surface plasmon resonance (SPR) [15–18]. Furthermore, the heat generated by photothermal conversion could trigger drug release for chemotherapeutics, which endowed AuNRs as promising light-responsive drug delivery systems [19,20]. In our previous study, we found that primary adult mesenchymal stem cells isolated from rat bone marrow were able to uptake more AuNRs than cancer cells without obvious cytotoxicity [21]. It therefore inspires us to explore this high uptake ability to kill CSCs

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via photothermal effect and light-driven release of CSCs-specific inhibitor. Previous researches have kindled hopes for overcoming the resistance of breast CSCs based on NPs photothermal effect, which is ineffective via conventional hyperthermia [22–24].

Our previous work indicated that coating AuNRs with polyelectrolytes bearing quaternary ammonium groups can greatly enhance their uptake without causing obvious cytotoxicity [25], we therefore constructed the outmost layer of the AuNRs with PDC (polydiallyldimethylammonium chloride). In order to investigate the photothermal effect, Au@PAA@PDC NRs were fabricated via layer-by-layer assembly. Their effects on CSCs in MCF-7 breast cancer cell were investigated. Three key parameters, the proportion of aldehyde dehydrogenase positive ($ALDH^+$) stem cells, gene expression of stem cell markers, as well as the mammosphere formation ability of the treated cells were investigated. Cellular uptake of Au@PAA@PDC by CSCs and non cancer stem cells (NCSCs) was evaluated. Furthermore, the CSCs inhibitor salinomycin (SA) was loaded in polyelectrolyte conjugated AuNRs (Au/SA@PDC) to achieve combined thermo-chemotherapy to CSCs.

2. Materials and methods

2.1. Materials

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium borohydride ($NaBH_4$), silver nitrate ($AgNO_3$), sulfuric acid (H_2SO_4), L-ascorbic acid (AA) and cetyltrimethylammonium bromide (CTAB) were purchased from Alfa Aesar. Poly(-acrylic acid) (PAA, 35%wt in H_2O, *M*w ~100,000 g/mol) and polydiallyldimethylammonium chloride (PDC, 35%wt in H_2O, *M*w ~100,000 g/mol) were purchased from Sigma–Aldrich. Salinomycin sodium salt 2,5-hydrate (SA) was purchased from Dr. Ehrenstorfer GmbH. 2,4-Dinitrophenylhydrazine (stabilized with 50% water) was purchased from J&K CHEMICA. Trichloroacetic acid, DMSO and NaCl were purchased from Beijing Chemical Plant. Ultrapure water (18 MΩ·cm) was used in all the preparation.

2.2. Preparation of Au@PAA@PDC

AuNRs were prepared using a seed-mediated silver-assisted approach with cetyltrimethylammonium bromide (CTAB) surfactant referring to the reported method [26]. Au@PAA@PDC was synthesized according to a published procedure with some modifications [27]. PAA and PDC stock solution (20 mg/mL) were prepared in 1 mM aqueous NaCl. First, 1 mL of PAA stock solution was added to 5 mL of the as-prepared AuNRs solution. After gentle mixing for 30 min, excess polymer was removed by centrifugation at 9400 rpm for 15 min. The precipitate of AuNRs conjugated with PAA (Au@PAA) was re-dispersed in 5 mL NaCl solution. Thereafter, 1 mL of PDC solution was added to the as-prepared solution. After gentle mixing for 30 min, excess polymer was removed by centrifugation. The precipitate of Au@PAA conjugated with PDC (Au@PAA@PDC) was re-dispersed in 0.5 mL NaCl solution.

2.3. Preparation of Au/SA@PDC

Stock solution of SA was prepared by adding 8 mg SA powder into 1 mL DMSO. First, 25 μL of SA stock solution was added into 5 mL as-prepared AuNRs solution. After gentle stirring for 30 min, the excess SA was removed by centrifugation at 9400 rpm for 15 min, and the precipitate was re-dispersed in 5 mL NaCl solution. Then 1 mL PDC solution was added and stirring for 30 min gently. After centrifugation the obtained Au/SA@PDC was re-dispersed in 0.5 mL NaCl solution. The residual of SA in the supernatant was measured and used for the calculation of drug loading efficiency.

Drug loading efficiency was calculated by subtracting the SA amount in the collected supernatant from the original drug solution. The amount of SA was measured by high performance liquid chromatography (HPLC) at 392 nm. SA was derived by 2,4-Dinitrophenylhydrazine before introduced to HPLC test according to the method in Chinese standard (GB/T 20196-2006). Loading efficiency was calculated by the following equations: Loading efficiency = (weight of SA in Au/SA@PDC)/(weight of Au/SA@PDC-weight of SA).

2.4. Characterization of Au@PAA@PDC and Au/SA@PDC

The morphology of Au@PAA@PDC and Au/SA@PDC was revealed by transmission electron microscope (TEM, FEI Tecnai G2 F20 U-TWIN). The UV–Vis–NIR absorption spectra were acquired from a Tecan Infinite M200 (Tecan, Switzerland). The zeta potential of the nanoparticles were measured with dynamic light scattering (DLS, Zetasizer, Malven Nano ZS90). Temperature rising of Au@PAA@PDC irradiated by femtosecond (fs)-pulse laser at 740 nm (beam diameter, 2.0 mm; power, 200 or 300 mW) was measured with a thermoelectric couple at the indicated time points.

2.5. In vitro drug release

In the *in vitro* drug release experiment, 0.5 mL concentrated Au/SA@PDC solution in two kinds of buffers (pH 5.0 or 7.4) was agitated at 37 °C and 48 °C, respectively. The mixture was centrifuged at the following time points: 5 min, 10 min, 15 min, 20 min, 30 min, 1 h, 3 h, 6 h, 12 h and 24 h. The supernatant was collected and the same volume of fresh buffers was added back to the residual mixture. The amount of released SA in the supernatant was measured by HPLC at 392 nm.

Similar procedure was applied in the NIR laser-triggered drug release experiments. The concentrated Au/SA@PDC solutions was irradiated with a fs-pulse laser (wavelength, 740 nm; beam diameter, 2.0 mm; laser power: 100, 200, 300 and 400 mW) for 15 min, then the released SA was analyzed with HPLC.

2.6. Cell culture

Human breast cancer cell line MCF-7 was kindly presented by Professor Fuyu Yang from Institute of Biophysics, Chinese Academy of Sciences (Beijing, China). The cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine, in a humidified atmosphere of 5% CO_2 at 37 °C.

2.7. ALDH assay

Cells were suspended in DMEM and seeded in 6-well culture plate at the density of 3×10^5 and cultured in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The medium was changed and cells were incubated for another 24 h with DMEM containing 13 μg/mL Au@PAA@PDC. After that, cells were washed three times with PBS and trypsinized. The collected cells were centrifuged at 1500 rpm for 5 min. The pellets of the cells were irradiated by fs-pulse laser at 740 nm with a power of 100 mW (beam diameter, 2.0 mm) for 8 or 15 min, respectively. After laser irradiation, cells were re-suspended in DMEM and seeded in 6-well culture plate for further 24 h culture. Single-cell suspension was prepared for the aldefluor assay according to the manufacturer's instructions (Stem Cell Technologies). Briefly, 10^6 cells were suspended in 1 mL of assay buffer. Five microliter activated aldefluor substrate was added to the suspension, and an aliquot of 0.5 mL was immediately quenched with a specific ALDH inhibitor diethylaminobenzaldehyde (DEAB). After incubation at 37 °C for 40 min, the cells were centrifuged and re-suspended in 0.5 mL aldefluor assay buffer. $ALDH^+$ cells were assayed with flow cytometry (FACS Calibur, BD Bioscience, USA).

2.8. Mammosphere formation assay

For Mammosphere formation assay, the irradiated MCF-7 cells were washed with PBS and then suspended in DMEM/F12 serum free medium supplemented with human recombinant epidermal growth factor (EGF, 20 ng/mL) and basic fibroblast growth factor (bFGF, 20 ng/mL), 2% (v/v) B27, 5 mg/mL insulin and 0.4% bovine serum albumin. Cells were seeded in ultralow attachment 6-well plates (Costar, Corning Incorporated) with 5000 cells/well. After 7 days of culture, mammospheres were observed and quantified using an inverted phase contrast microscope (Olympus Co.). After trypsinized to single cell, the number of the mammosphere cells in each well was counted.

2.9. RT-PCR assay

After irradiation by NIR laser as described above, MCF-7 cells were suspended in DMEM and seeded in 6-well culture plate and cultured at 37 °C for 24 h. RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to standard protocol. cDNA was synthesized with Superscript III (Invitrogen) and random hexamers using 2 μg purified RNA. Real-time PCR was performed with SYBR-Green Mastermix (Applied Biosystems) in a total volume of 20 μL according to the manufacturer's instructions in a Realplex4 (Eppendorf, German) under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 40 s. All runs were performed in triplicate, and specific amplification was checked with melting curve analysis. The ΔCt formula was used to determine fold-change differences, and human GAPDH was used as the reference gene. The following primers synthesized by Sangon Biotech were used:

ALDH1 FORWARD: TCGTCTGCTGCTGGCGACAATG;
ALDH1 REVERSE: CCCAACCTGCACAGTAGCGCAA;
KLF4 FORWARD: ACATGGCTGTCAGCGACGCG;
KLF4 REVERSE: GCCAGCGGTTATTCGGGGCAC.

2.10. Cellular uptake of Au@PAA@PDC by ICP-MS

MCF-7 cells were cultured in 6-well plate at a density of 3×10^5 cells/well. After 24 h culture in a humidified atmosphere of 5% CO_2 at 37 °C, the medium was removed and fresh complete medium containing 13 μg/mL Au@PAA@PDC was added to each well for the cellular uptake experiment. For mammosphere cells uptake, mammospheres were digested with 0.25% trypsin containing 0.02% EDTA, and seeded in ultralow attachment 6-well plates with the same density as MCF-7 cells. In the next day, 13 μg/mL Au@PAA@PDC was added to each well. Cells were