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Integrated self-assembling drug delivery system possessing dual responsive and active targeting for orthotopic ovarian cancer theranostics

Chun-Jui Lin [a], Chen-Hsiang Kuan [b], Li-Wen Wang [c], Hsi-Chin Wu [d], Yunching Chen [a], Chien-Wen Chang [e], Rih-Yang Huang [e], Tzu-Wei Wang [c, *]

[a] Institute of Biomedical Engineering, National Tsing Hua University, Hsinchu, Taiwan
[b] Department of Plastic Surgery, National Taiwan University Hospital, Taiwan
[c] Department of Materials Science and Engineering, National Tsing Hua University, Hsinchu, Taiwan
[d] Department of Material Engineering, Tatung University, Taipei, Taiwan
[e] Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu, Taiwan

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ABSTRACT

Ovarian cancers are the leading cause for mortality among gynecologic malignancies with five-year survival rate less than 30%. The purpose of this study is to develop a redox and pH-sensitive self-assembling hyaluronic acid nanoparticle with active targeting peptide for anticancer drug delivery. Anti-cancer drug is grafted onto hyaluronic acid (HA) via *cis*-aconityl linkage and disulfide bond to possess pH sensitivity and redox property, respectively. This conjugate is amphiphilic and can self-assemble into nanoparticle (NP) in aqueous solution. The results show that the nanoconjugate is successfully developed and the grafting ratio of cystamine (cys) is 17.8% with drug loading amount about 6.2% calculated by ^{1}H NMR spectra. The particle size is approximately 229.0 nm using dynamic light scatting measurement, and the morphology of nanoparticles is observed as spherical shape by transmission electron microscope. The pH and redox sensitivities are evaluated by changing either pH value or concentration of dithiothreitol in the medium. It is proved that the drug carrier is capable of achieving sustained controlled release of anti-cancer drug to 95% within 150 h. The intracellular uptake is observed by fluorescent microscope and the images show that conjugating luteinizing hormone-releasing hormone (LHRH) peptide can enhance specific uptake of nanoparticles by OVCAR-3 cancer cells; thus, resulting in inhibitory cell growth to less than 20% in 72 h *in vitro*. Orthotopic ovarian tumor model is also established to evaluate the therapeutic and diagnostic efficacy using non-invasive *in vivo* imaging system. The representative results demonstrate that LHRH-conjugated NPs possess a preferable tumor imaging capability and an excellent antitumor ability to almost 30% of original size in 20 days.

1. Introduction

Self-assembled nanoparticles have been regarded an advanced system for hydrophobic drugs or nucleic acids delivery [1]. After self-assembly, the hydrophilic segments serve as protective shell to avoid being removed by the reticuloendothelial system (RES). On the other hand, the hydrophobic segments play the major role as a reservoir for water-insoluble drug [2]. In cancer therapy, nanocarriers also possess passive targeting effect because of the enhanced permeability and retention effect (EPR effect) in tumor sites, causing ten to thirty-fold increase of tumor-to-blood drug concentration [3,4].

For controlled release, drug delivery system can be designed to possess stimuli-sensitivity [5,6]. It is suggested that *cis*-aconityl linkage showed a 100-fold increase in the rate of cleavage from pH 7.5 to pH 5.5, allowing rapidly release of drug in acidic pH such as in endosome or lysosome [7–9]. By introducing the disulfide linkages, drug-polymer conjugate maintains stable within extracellular low glutathione concentration (2–20 μM). After encountering intracellular high glutathione concentration (2–20 mM), cleavage of

* Corresponding author. Department of Materials Science and Engineering, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, Hsinchu, 30013, Taiwan.
E-mail address: twwang@mx.nthu.edu.tw (T.-W. Wang).

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disulfide bonds results in drug release [10–13].

To increase *in vivo* specificity, ligands such as small molecule, peptide or antibody are grafted on the surface of drug carrier. Luteinizing hormone-releasing hormone (LHRH, also known as gonadotropin-releasing hormone (GnRH) and luliberin), is one of targeting ligands. The receptors for LHRH (LHRH-R) are overexpressed in ovarian cancer cells, while not expressed in healthy tissues or organs [14,15]. Moreover, the overexpression of receptors of hyaluronic acid (HA) in many cancer cell lines has been reported [16,17].

The word "theranostics" means a combination of "therapy" and "diagnosis" as an integrated system [18]. Nanoparticles designed for theranostics have been investigated widely for simultaneous delivery of diagonostic imaging agents and therapeutic drugs [19,20]. Low absorption and autofluorescence from organisms and tissues of near-infrared (NIR) fluorescent dye prevent background interference, improve tissue depth penetration and image sensitivity [21]. Among the common NIR fluorescent dyes, some are suitable probes (such as Cy5, Cy5.5, Cy7 and their derivatives) for *in vivo* imaging due to their high molar absorption coefficient (often reaching 200,000 $mol^{-1}cm^{-1}L$) and fluorescence quantum yield [22,23].

Ovarian cancers are the leading cause for mortality among gynecologic malignancies with five-year survival rate less than 30% [24]. Currently, the standard protocol for ovarian cancer treatment is surgical resection followed by chemotherapy. However, administration of chemotherapy agents would cause severe side effects [25]. Moreover, the metastasis of tumor cells through lymphatics and blood vessel is promoted because of absence of an anatomical barrier around ovaries [26]. It remains a major challenge to provide effective therapeutic and diagnostic strategies against the disease. In addition, *in vivo* model is critical for preclinical trial of the system. Compared with subcutaneous model, orthotopic model provides novel insights into the pathophysiology of ovarian cancer [27].

In this study, a dual-responsive (redox and pH-sensitive) self-assembling nanoparticle from hyaluronic acid for drug delivery was synthesized as shown in Scheme 1. This system is designed as a multifunctional drug delivery system. First of all, HA and LHRH peptide serve as the ligands for the overexpressed corresponding receptors to enhance the specific endocytosis by cancer cells, while prevent cytotoxicity to normal tissue. Second, dual stimuli-sensitive spacers demonstrate synergistic controlled release ability which is triggered by intracellular reductive reagents and low pH value in endosome. Lastly, the combination delivery of therapeutic anticancer drug and NIR dye, which is so called "theranostics." Real-time tracking of nanoparticles *in vivo* is monitored by NIR fluorescent imaging using non-invasive *in vivo* imaging system (IVIS). In addition to the designed tactics of nanoparticles, orthotopic ovarian tumor model is successfully established to evaluate the antitumor effect *in vivo*.

2. Materials and methods

Hyaluronic acid (HA) was purchased from LIFECORE Biomedical Co. Ltd. (U.S.A.). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), doxorubicin (DOX) and ethyl acetate were purchased from Sigma-Aldrich Co. Ltd. (U.S.A). Cystamine dihydrochloride, sodium metaperiodate ($NaIO_4$) and *cis*-aconitic anhydride were purchased from Alfa Aeser Co. Ltd. (UK). Chloroform was purchased from JTBaker Co. Ltd. (U.S.A.). 1,4-Dioxane was purchased from Acros Organics Co. Ltd. (Belgium). Dimethylformamide was purchased from Merck Co. Ltd. (Germany). LHRH peptide (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was purchased from ProSpec Co. Ltd. (Israel). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Penicillin Streptomycin mixtures (PS) was purchased from Life Technology Co. Ltd. (U.S.A.). All materials were used as received without further purification unless otherwise noted.

OVCAR-3 human ovarian cancer cell line and 3T3 mouse fibroblast cell line were purchased from Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. OVCAR-3 cell line stably expressing a firefly luciferase gene (OVCAR-3/luc) was provided by Prof. Chien-Wen Chang, Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu, Taiwan. All above cell lines were cultured in DMEM (high glucose) containing 10% (v/v) FBS and 1% (v/v) PS. Cell culture was maintained at 37 °C and 5% CO_2 in a humidified incubator. Female athymic nude mice of 6–8 week were purchased from National Laboratory Animal Center (Taipei, Taiwan.).

3. Synthesis of LHRH-HA-cys-ADOX conjugate

3.1. Grafting of LHRH peptide on hyaluronic acid

Aldehyde-functionalized hyaluronic acid was synthesized before LHRH peptide was conjugated onto HA [28,29]. To prepare aldehyde-functionalized HA (HA-ALD), 0.1 g of HA (M.W. 16 kDa) was dissolved in 10 mL deionised water (1 wt%), and stirred to completely dissolved. 1 g of sodium metaperiodate was dissolved in 10 mL deionised water, and then added to the solution of HA. The solution was stirred in dark for 2 h. To stop the reaction, 1 mL ethylene glycol was added and stirred for 1 h. The resulting solution was dialyzed for one day against deionised water followed by lyophilized for further use. To conjugate LHRH peptide with HA-ALD (LHRH-HA), sodium acetate solution was prepared by adding sodium hydroxide solution into acetate solution. 20 mg of HA-ALD was dissolved in sodium acetate buffer (pH 5.5) at a concentration of 5 mg/mL. The number of LHRH peptide in the feed ratio of 0.1 was added. The reaction was performed at RT for 24 h with mild stirring. HA-LHRH was purified by dialysis against a large excess amount of PBS for 1 day. The HA-LHRH was obtained after being lyophilized. The aldehyde groups on HA backbone was quantified with Amplite™ Colorimetric Aldehyde Quantitation Kit (AAT Bioquest, Inc., USA).

3.2. Modification of hyaluronic acid with cystamine

To provide amino group on HA or LHRH-HA, 0.20 g of HA (MW 16K, 0.50 mmol) or LHRH-HA was dissolved in PBS solution (0.1 m, pH 7.4) for 2 h to obtain a 4 mg/mL polymer solution. EDC (2.50 mmol) and sulfo-NHS (1.0 mmol) were added to the solution of HA and stirred for 15 min. 1.12 g of cystamine dihydrochloride (5.0 mmol) was added and the reaction mixture was incubated for 6 h at room temperature under stirring. The resulting solution was dialyzed (MWCO 6000) against 0.1 M NaCl and then in distilled water for 1 day. The polymer solution was lyophilized and stored at 4 °C [30].

3.3. Preparation of HA-cys-ADOX or LHRH-HA-cys-ADOX conjugate

The first step was the ring-opening reaction of *cis*-aconitic anhydride with DOX as described in previous research report [31]. Doxorubicin (10 mg) was dissolved in 400 μL of deionised water. *Cis*-aconitic anhydride (13.46 mg) in 1 mL of 1,4-dioxane was slowly added and was stirred overnight at 4 °C. The solution was mixed with 5 mL of chloroform and 5 mL of 5% aqueous sodium bicarbonate solution. The chloroform phase was decanted and the residual solution was extracted with ethyl acetate. The resulting solution was concentrated using a rotary evaporator and dried at