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Specific tumor delivery of paclitaxel using glycolipid-like polymer micelles containing gold nanospheres

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ABSTRACT

It is difficult for most of the drug delivery systems to really display a temporal and spatial release of entrapped drug once the systems are iv administrated. We hypothesized that the photothermal effect, mediated by a near-infrared (NIR) laser and hollow gold nanospheres (HAuNS), can modulate paclitaxel (PTX) release from polymer micelles, and further result in the enhanced antitumor activity of the micelles. We loaded PTX and HAuNS, which display strong plasmon absorption in the NIR region, into glycolipid-like polymer micelles with an excellent cell internalization capability. The surface of the micelles was conjugated successfully with a peptide, which has the specific-binding with EphB4, a member of the Eph family of receptor tyrosine kinases overexpressed on cell membrane of numerous tumors, to increase the delivery of PTX into tumor cells. Rapid and repetitive drug release from our polymer (HP-TCS) micelles could be readily achieved upon NIR laser irradiation. Our data demonstrated the specific delivery of HP-TCS micelles into positive-EphB4 tumors using a duel-tumor model after iv administration during the whole experiment process (1–48 h). Interestingly, significantly higher uptake of the micelles by SKOV3 tumors (positive-EphB4) than A549 tumors (negtive-EphB4) was observed, with increased ratio on experiment time. However, the specific cell uptake was observed only during the short incubation time (1–4 h) *in vitro*. Our data also indicated the treatment of tumor cells with the micelles followed by NIR laser irradiation showed significantly greater toxicity activity than the treatment with the micelles alone, free PTX and the micelles (without PTX loading) plus NIR laser irradiation. The enhanced toxicity activity to tumor cells should be attributed to the enhanced drug cellular uptake mediated by the glycolipid-like micelles, chemical toxicity of the released drug from the micelles due to the trigger of NIR laser, and the photothermal ablation under NIR laser irradiation.

1. Introduction

Controlled drug delivery systems have advanced over the last 60 years [1]. Numerous delivery systems have been developed, which in nanotechnology-based systems for targeting delivery to tumor have received great attention recently, including drug–polymer conjugates, drug–protein conjugates, liposomes, polymer micelles, dendrimers, drug nanocrystals and inorganic nanoparticles [2,3]. Of there, polymer micelle is a very promising drug delivery system due to many obvious advantages, such as low cytotoxicity, nanoscale size, high bioavailability and drug-encapsulated ability. It is well known that only the released drug molecular can play the therapeutic activity after the drug is loaded into a carrier system. However, like most of drug delivery systems, polymer micelle also faces the defect, which is the difficulty to decide when or where the encapsulated drugs should be released once the systems are iv administrated.

We developed glycolipid-like polymer micelles in the previous study, which was synthesized by a coupling reaction between the amino groups of chitosan oligosaccharide (CSO) and carboxyl group of stearic acid (SA) [4]. The obtained polymer (CSO–SA) micelles showed excellent internalization into tumor cells, attributed to their components and special spatial structure (so-called "minor-cores", hydrophobic microdomains, near the surface of the micelles' shell) [5–7]. Therefore, the micelles were employed to deliver the antitumor drug, paclitaxel (PTX), into tumor site. The tumor-cell killing efficiency of PTX encapsulated in micelles was improved sharply due to the increased intracellular delivery of the

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drug. However, it was found that only 13% of PTX was released from the micelles after 9 h incubation under *in vitro* physiological environment. Even thinking of the decreasing pH value in the tumor site, the accumulative PTX release was still below 40% after 9 h in the simulative tumor environment [5].

Hollow gold nanospheres (HAuNS) are a class of gold nanoparticles that have plasmon absorption in the near-infrared (NIR) region and that display a strong photothermal conducting property. HAuNS' unique combination of small size (30–50 nm in diameter) and strong and tunable (520–950 nm) absorption band suggests that HAuNS are a promising photothermal conducting agent for a variety of biomedical applications, including imaging [8,9] and cancer therapy [10]. Our previous studies have explored the potential utility of HAuNS as a delivery vehicle to shuttle biomolecules or to trigger drug release under NIR light irradiation [11–13].

In this study, we developed a drug delivery system, which in glycolipid-like polymer micelles were used to encapsulate PTX and HAuNS. HAuNS was modified hydrophobically to increase its encapsulation efficiency into the micelles. We hypothesized that the photothermal effect mediated by a NIR laser and HAuNS could modulate PTX release from the micelles. By the local irradiation in tumor site after iv administration of the micelles, we may optionally decide when and where the anticancer agent is released from the system. We also hypothesized the antitumor activity of the system can be significantly enhanced under the NIR irradiation. In order to enhance the interaction between the micelles and tumor cells, we conjugated a peptide TNYLFSPNGPIARAW (designated as TNYL) on the surface of the micelles, which displayed high binding affinity to EphB4, with an equilibrium dissociation constant (Kd) values of 1.98–23 nmol/L [14,15]. The Eph receptors constitute the largest known family of receptor tyrosine kinases and have been reported to control various pathological processes, including tumor progression and angiogenesis [16–18]. Overexpression of EphB4 has also been observed in numerous tumor types [16,17,19–21]. Therefore, EphB4 is a particularly promising target for tumor-specific delivery of our system.

2. Materials and methods

2.1. Materials

Sodium citrate (>99%), cobalt chloride hexahydrate (99.99%), sodium borohydride (99%), and chloroauric acid trihydrate (American Chemical Society reagent grade) were purchased from Thermo Fisher Scientific (Waltham, MA) and were used as received. Octadecyl-3-Mercaptopionate (OMP) was from Chemical Industry Co. (Japan). Chitosan oligosaccharide (CSO) with about 18.0 kDa weight average molecular weight was obtained by enzymatic degradation of 95% deacetylated chitosan (M_w = 45.0 kDa), which was supplied by Yuhuan Marine Biochemistry Co., Ltd. (Zhejiang, China); stearic acid (SA) was from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China); PTX was gifted from Zhejiang Hisun Pharmaceutical Co, Ltd, (Taizhou Zhejiang, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N,N-Disuccinimidyl carbonate (DSC), and 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO). NH_2-PEG_{2000}-NH_2 was purchased from Sigma-Aldrich Inc, (St Louis, MO). TNYL (sequence: TNYLFSPNGPIARAW) was provided from Baiaotai biotechnology Inc. (Guangzhou, China). All other solvents were of analytical or chromatographic grade.

2.2. Cell culture

SKOV3 (human ovarian carcinoma), and A549 (human lung adenocarcinoma) cell lines were obtained from Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation and hydrophobic modification of HAuNS

HAuNS were synthesized according to a previously reported method [11]. Briefly, cobalt nanoparticles were first synthesized by deoxygenating deionized water containing 4.5 mL of 1 mol/L sodium borohydride, 2.8 mL of 0.1 mol/L sodium citrate, and 1.0 mL of 0.4 mol/L cobalt chloride. After chloroauric acid was added into the solution containing cobalt nanoparticles, the cobalt immediately reduced the gold ions onto the surface of the nanoparticles and was simultaneously oxidized to cobalt oxide. Any remaining cobalt was further oxidized by air, resulting in the final product, HAuNS. The size of the HAuNS was determined using dynamic light scattering on a Zetasizer (3000 HS; Malvern Instruments, UK). The UV–visible spectra were recorded on a Beckman Coulter DU-800 UV–visible spectrometer.

HAuNS was further modified hydrophobically by OMP. Briefly, 0.2 mL HAuNS [200 optical density (OD), 10.0 mg/mL] was centrifuged at 8000 rpm for 10 min. The pellet of HAuNS was mixed with OMP (0.1 mmol) in 1 mL DMF solution. After overnight stirring, OMP modified HAuNS (OMP-HAuNS) was purified by the centrifugation (8000 rpm for 10 min), followed by washing twice with DMF, and then was identified by FT-IR analysis.

2.4. Synthesis of CSO–SA and TNYL conjugated CSO–SA

CSO–SA was synthesized via reaction of the carboxyl groups of SA with the amine groups of CSO in the presence of EDC [4]. Briefly, CSO (2.0 g) was dissolved in 50 mL of distilled water. SA (1.75 g) and EDC (10 mol/mol of SA) were dissolved in 40 mL of ethanol. CSO solution was heated to 80 °C under vigorous stirring accompanied by dropwise addition of SA solution. The reaction lasted 5 h. The byproducts were removed via ultrafiltration by Millipore Labscale TFF system [molecular weight cutoff (MWCO) 10 000, Billerica, MA]. The obtained product was lyophilized (Labconco, FreeZone 2.5 Plus, Kansas City, MO), further washed with ethanol, and separated by the centrifugation (3K30, Sigma Labrorzentrifugen GmbH, Germany).

CSO–SA was further conjugated with TNYL peptide. The reaction was performed at room temperature. Briefly, TNYL (10 mg) was mixed with $(Boc)_2O$ [TNYL : $(Boc)_2O$ = 1:5.2, mol/mol] in dried DMF, followed by stirring for 12 h. Then, NH_2-PEG_{2000}-NH_2 (11.9 mg) and EDC (11.4 mg) were added into above solution. Succinimidyl t-Boc-TNYL-PEG_{2000}-NH_2 was obtained by 9 h reaction after the addition of DSC (NH_2-PEG_{2000}-NH_2 : DSC = 1:1, mol/mol), and further reacted with CSO–SA (CSO–SA : succinimidyl t-Boc-TNYL-PEG_{2000}-NH_2 = 1:1, mol/mol) for 24 h. Finally, the protection of $(Boc)_2O$ to TNYL peptide was removed by adding hydrochloric acid into the solution. The pH value of the solution was adjusted back to 7.0 using sodium hydroxide. For purification, the solution was dialyzed using a membrane (MWCO 7 kDa, Spectrum Laboratories) against distilled water for 24 h. TNYL conjugated COS–SA (TNTL-CSO–SA) was obtained by the lyophilization, and identified by ^1H NMR spectroscopy.

2.5. Preparation of HAuNS and PTX loaded TNYL-CSO–SA micelles

HAuNS and PTX loaded TNYL-CSO–SA (HP-TCS) micelles were prepared by dialysis method [5]. Briefly, OMP-HAuNS (0.5 mL of 100 OD), PTX (2 mg) and TNYL-CSO–SA (20 mg) were dispersed in the mixture solution (water: DMSO = 1:9, v/v), followed by the stirring for 3 h. Then, the above solution was dialyzed using a membrane (MWCO 7 kDa, Spectrum Laboratories) against distilled water for 24 h, and was filtered through a 0.22 mm pore-sized membrane to remove free PTX and OMP-HAuNS. The powder of HP-TCS was obtained by lyophilization. The size distribution and zeta potential of HP-TCS micelles were measured by a Zetasizer. The concentration of PTX in the micelle solution was determined by high-performance liquid chromatography (HPLC).

2.6. NIR-light-triggered release of PTX from HP-TCS micelles

The sample was transferred into a dialysis membrane bag (MWCO 7 kDa), and then submerged into the medium containing 1 M sodium salicylate with pH 7.4 under the stirring (100 rpm) at 37 °C. For NIR laser irradiation, the sample was taken out from the dialysis bag at the designed time point, and put into a transparent glass tube. The samples were irradiated with the 808-nm NIR light at an output power of 3 or 6 W over a period of 5min (Diomed 15 plus, Cambridge, UK), as our previous reported [12]. Then, the sample was put back into the dialysis bag to continue the release study. The released PTX in the medium was quantified by HPLC.

NIR-light-triggered release from HP-TCS micelles was further confirmed by an *in vivo* imaging system (Cambridge Research & Instrumentation, Inc., Woburn, MA). Firstly, DIR, a near-infrared lipophilic fluorescent tracer, was used as the model drug to be encapsulated into HP-TCS micelles as the above method in section 2.4. Then, the agarose gel lump was prepared using the reported methods with a little modification [22]. The release study was carried out as the showed in fig. S1. Briefly, we made a circular hole (1 cm diameter and 0.5 cm depth) in the agarose gel lump. The sample was put into the hole, and irradiated by NIR laser (output power of 3 W for 10 min). Then, the gel was observed by the *in vivo* imaging system. NIR-light-triggered release of DIR from HP-TCS micelles was identified by the diffused degree of DIR molecule in agarose gel.

2.7. Cellular uptake of HP-TCS micelles

EphB4 expression in cells was analyzed according to our previously reported Ref. [15]. Briefly, for western blot analysis, cells were treated by CelLytic M cell lysis buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Roche Applied