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GSH-mediated photoactivity of pheophorbide a-conjugated heparin/gold nanoparticle for photodynamic therapy

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ABSTRACT

In this study, we developed a new photosensitizer (PS)-conjugated hybrid nanoparticle comprised of gold nanoparticle (AuNP) as an efficient energy quencher, polysaccharide heparin and a second generation PS, pheophorbide a (PhA) for PDT. The hybrid nanoparticles (PhA-H/AuNPs) with an average size of 40 nm were prepared by surface coating of AuNPs with PhA conjugated heparins via gold-thiol interaction. The glutathione (GSH)-mediated switchable photoactivity of the PhA-H/AuNPs was observed by fluorescence quenching and dequenching behaviors in the absence and presence of GSH. The photoactivity was significantly suppressed in aqueous media, but instantaneously restored at the GSH-rich intracellular environment to generate a strong fluorescence signal together with active production of singlet oxygen species with light treatment. *In vitro* cell tests revealed marked phototoxicity and high intracellular uptake of PhA-H/AuNPs in contrast with free PhA. The PhA-H/AuNPs also exhibited a prolonged circulation characteristic, enhanced tumor specificity, and improved photodynamic therapeutic efficacy compared with free PhA in tumor-bearing mice. As a result, the PhA-H/AuNPs may serve as an effective smart nanomedicine platform for PDT and have great potential for the clinical treatment of various tumors.

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1. Introduction

Photodynamic therapy (PDT) is a revolutionary treatment aimed at detecting cancers and treating them without surgery or chemotherapy [1,2]. It is based on the discovery that certain chemicals known as photosensitizing agents can kill one-celled organism when the organisms are exposed to an appropriate laser light. Compared with conventional therapies, PDT has several advantages: (i) a non-invasive nature, (ii) the ability to precisely treat a tumor site through site-specific light treatment, (iii) the ability to treat patients with repeated doses and in an outpatient setting, and (iv) a fast healing process [3].

PDT agents, named photosensitizers (PSs), essentially function as catalysts when they absorb visible light and transfer the energy to surrounding oxygen molecules, resulting in the generation of reactive oxygen species (ROS), such as singlet oxygen and free radicals. Singlet oxygen, the predominant cytotoxic agent produced during PDT, is a highly reactive form of oxygen that is produced by inverting the spin of one of the outermost electrons, which can cause irreversible damages to proteins, lipids, nucleic acids and other cellular components [4,5]. Although PS demonstrates a high sensitivity in treating small tumor lesions through precisely site-specific light treatment, a major challenge is its low specificity when using a poorly water-soluble and non-targeted PS, such as pheophorbide a (PhA), causing accidental damage to adjacent healthy tissues and blood cells, or long-lasting cutaneous photosensitivity [6,7]. Therefore, there have been continuous efforts for developing tumor-sensitive PSs to increase the specificity of treatment and imaging as well as provide information on the level of biomarker expression in cancers.

To overcome such limitations, several strategies have been applied to improve the tumor-targeted PDT, including passive delivery of PS to the tumor site using nanostructured drug carriers based on the enhanced permeability and retention (EPR) effect [8–10] and active targeting approaches, such as antibody [11] and targeting-ligands conjugation [12]. Recently, gold nanoparticles (AuNPs) have received significant attention as a platform for drug delivery [13–15] due to their chemical inertness, good biocompatibility, and tunable size (2–100 nm). Furthermore, their unique optical properties provide several attractive advantages for biomedical applications [16]. First, their strong light scattering property enables AuNPs to serve as cell imaging agents. Second, AuNPs can be useful as photothermal therapy agents due to their photo-thermal conversion property. Furthermore, AuNPs produce a strong surface plasmon absorption band and have utility as efficient energy quenchers through fluorescence resonance energy transfer (FRET), which can quench the excited energy of fluorochromes even at a distance of around 40 nm [17]. By surface coating with water-soluble polymers, AuNPs can be solubilized and stabilized in physiological

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conditions without colloidal aggregation for extended period. Through combining with PDT agent, AuNPs could serve as a multifunctional theranostic nano-agent for cancer treatments and hold the promise to be an efficient PS delivery platform for PDT [18].

Glutathione (GSH)-responsive nano-vehicles based on disulfide linkage have been reported as a promising platform for targeted intracellular drug and gene delivery [19]. This strategy represents an alternative nonenzymatic approach to the release of therapeutic agents in a triggered fashion after delivery to the cells. It relies on the fact that several intracellular compartments, such as cytosol, mitochondria, and cell nucleus, contain a much higher concentration of GSH (~1–10 mM) than extracellular fluids (~2–20 μM) [20,21]. It has been also reported that some tumor cells have a significantly higher concentration of GSH than normal cells [22,23]. However, a difficulty in tuning the reactivity of the disulfide linkage presents a challenge for this approach. In addition, thiol-disulfide exchange can occur with surface cysteines of proteins in the bloodstream, resulting in the altered bioavailability and drug carrier's pharmacokinetic profiles [24]. Delivery and release of therapeutic agents using polymer-coated AuNPs that are based on the GSH-mediated ligand exchange reaction of thiol-gold can be expected to resist the exchange with proteins because of the steric shielding of the gold-thiol interface [25]. Moreover, the tunable chain length of surface immobilized polymers can be utilized to manipulate the release of payloads in response to GSH.

In this study, we described a rational design of heparin-coated AuNP (H/AuNP) as a GSH-responsive carrier for delivery of a model hydrophobic PS, PhA, for efficient PDT. Heparin, a highly sulfated polysaccharide, was used as a backbone polymer not only because of its water-solubility and biocompatibility, furnishing the AuNPs with good colloidal stability in a physiological environment, but also for its attractive biological functions, such as anti-inflammation, anti-angiogenesis, and anti-tumor cell proliferation [26–29]. When PhA molecules are introduced onto AuNP surface, efficient energy transfer from PhA to AuNP may occur, making the PhA nonfluorescent and photo-inactive. Thus, hybrid PhA-H/AuNPs are essentially photo-inactive nanoparticles with quenching in the circulatory system. However, when the PhA-H/AuNPs are passively localized in tumor tissue and internalized by tumor cells, GSH, the most abundant reducing agent in the cytoplasm, potentially serves as an effective trigger to facilitate the triggered release of PhA from AuNPs by breaking the gold-thiol linkage. The recovery of photoactivity of PhA may occur instantaneously, followed by exerting the significant photo-killing effect on tumor cells upon light irradiation. Targeted delivery and GSH-mediated photoactivity using AuNPs may favor the bioavailability and pharmacokinetics of PhA, and significantly reduce the side effects, such as accidental damage on blood cells during systemic circulation and long-lasting cutaneous light sensitivity.

Here, the physicochemical properties of PhA-H/AuNPs were characterized in terms of surface plasma absorption, particle size and size distribution, fluorescence intensity, and singlet quantum yield. In particular, to assay the potential of the GSH-mediated dequenching effect, changes in photoactivity in the presence and absence of GSH were examined. The *in vitro* phototoxicity and cellular internalization behavior were evaluated with A549 cells. Finally, the tumor specificity and therapeutic efficacy of PhA-H/AuNPs were evaluated *in vivo* using tumor-bearing (A549) SKH1 mice.

2. Materials and methods

2.1. Materials

Unfractionated heparin, with an average molecular weight of 12,000 Da, was obtained from Mediplex Co. (Korea). Pheophorbide a (PhA) was purchased from Frontier Scientific Inc. (Logan, USA). Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium citrate, cysteamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 9,10-dimethylanthracene (DMA), ethylenediamine, N-hydroxysuccinimide (NHS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione (GSH), and an *in vitro* toxicology assay kit (MTT based) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Penicillin–streptomycin, fetal bovine serum (FBS), and 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution were purchased from American Type Culture Collection (Rockville, MD). The RPMI 1640 medium was obtained from Invitrogen (Carlsbad, CA). Spectra/Pro membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). All the chemicals were analytical grade and used as received without further purification.

2.2. Fabrication of hybrid nanoparticles (PhA-H/AuNPs)

a. *Synthesis of thiolated heparin*. Heparin (200 mg) was dissolved in 10 mL of MES buffer (0.1 M, pH 5.5) and reacted with cysteamine (15.4 mg) and EDC (95.8 mg) at room temperature for 24 h. The resultant solutions were dialyzed for 2 days to remove byproducts and uncoupled compounds and then lyophilized. The chemical structure of thiolated heparin was confirmed by FT-IR. The coupling ratio of cysteamine was determined by Ellman's assay.
b. *Synthesis of PhA-conjugated heparin (PhA-H)*. Aminated PhA synthesized as described in our previous report [30] was conjugated to thiolated heparin through the coupling reaction between the amino group of animated PhA and the carboxyl group of heparin (Scheme 1). Briefly, 150 mg of thiolated heparin, and 40 mg of aminated PhA were dissolved in formamide (5 mL) and DMF (5 mL), respectively. EDC was added to the heparin solution, followed by mixing with PhA solution. The reaction was performed at room temperature under nitrogen gas for 36 h. After the coupling reaction, the solution was dialyzed for 2 days and then filtered through a 0.2-μm syringe filter. The crude product was obtained after freeze-drying and then washed with methanol to remove the uncoupled PhA. The final product was collected by centrifugation and drying under vacuum. The coupling ratio of PhA was characterized using the colorimetric method as described in our previous study [30].
c. *Preparation of AuNPs*. Citrate-stabilized AuNPs were prepared using published procedures [31]. Briefly, all the glass wares were first washed with an aqua regia solution, then rinsed with high-purified deionized water several times, and dried. $HAuCl_4$ stock solution (0.01 g/mL, 2 mL) was added to 100 mL of deionized water, and the solution was heated until boiling under vigorous stirring. Next, 5 mL of 50 mM sodium citrate solution was introduced rapidly using a syringe. The solution was boiled for further 30 min with vigorous stirring, and then stored at 4 °C.
d. *Fabrication of PhA-H/AuNPs*. AuNPs were surface-coated with PhA-H via the thiol-gold interaction to obtain the final hybrid nanoparticles, PhA-H/AuNPs. An excess amount of PhA-H (10 mg) was added to 20 mL of AuNP stock solution and reacted overnight to fabricate PhA-H/AuNPs. The resultant PhA-H/AuNP solution was centrifuged at 12,000 rpm for 10 min. The precipitated PhA-H/AuNPs were resuspended in PBS (0.01 M, pH 7.4) and sonicated for 2 min to disperse the nanoparticles, followed by dialysis against deionized water (MWCO = 50,000 Da) for 2 days to further completely remove the unreacted PhA-H. The final products were collected by freeze-drying.

2.3. Characterization of PhA-H/AuNPs

The average number of surface bound PhA-H molecules on each AuNP was determined by measuring the fluorescence intensity of the unreacted PhA-H using a fluorescence spectrometer. The excitation wavelength was fixed at 405 nm, and the emission spectra were recorded from 600 to 700 nm using a spectrofluorometer (FP6500,

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