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# Enhanced cellular uptake and phototoxicity of Verteporfin-conjugated gold nanoparticles as theranostic nanocarriers for targeted photodynamic therapy and imaging of cancers

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ABSTRACT

Activatable theranostics with the capacity to respond to a given stimulus have recently been intensively explored to develop more specific, individualized therapies for various diseases, and to combine diagnostic and therapeutic capabilities into a single agent. In this work, we designed tumor-targeting ligand-conjugated block copolymer-gold nanoparticle (AuNP) conjugates as multifunctional nanocarriers of the hydrophobic photosensitizer (PS), verteporfin (Verte), for simultaneous photodynamic therapy and imaging of cancers. Folic acid (FA)-conjugated block copolymers composed of polyethylene glycol (PEG) and poly-β-benzyl-L-aspartate (PBLA) were attached to citrate-stabilized AuNPs through a bidentate dihydrolipoic acid (DHLA) linker. The resulting AuNP conjugates (FA-PEG-P(Asp-Hyd)-DHLA-AuNPs) were significantly more stable than unmodified AuNPs, and their optical properties were not affected by pH. The hydrophobic PS, Verte, was covalently incorporated onto the surfaces of the AuNP conjugates through a pH-sensitive linkage, which increased the water solubility of Verte from <1 μg/ml to >2000 μg/ml. The size of FA-PEG-P(Asp-Hyd)-DHLA-AuNPs-Verte as determined by light-scattering measurements was about 110.3 nm, and FE-SEM and FE-TEM images showed that these nanoparticles were spherical and showed adequate dispersivity after modification. In particular, an *in vitro* cell study revealed high intracellular uptake of FA-PEG-P(Asp-Hyd)-DHLA-AuNPs-Verte (about 98.62%) and marked phototoxicity after laser irradiation compared with free Verte. These results suggest that FA-PEG-P(Asp-Hyd)-DHLA-AuNPs-Verte has great potential as an effective nanocarrier for dual imaging and photodynamic therapy.


## 1. Introduction

Nanoparticle-based imaging and therapy have been investigated separately, but the emergence of nanotechnology has provided the opportunity to unite these two research areas. Since the term "theranostic" was first coined by Funkhouser [1], nanoscale theranostic systems that can deliver therapeutic drugs and diagnostic imaging agents simultaneously have been actively investigated to treat oncological, cardiovascular, dermatological, and ophthalmic diseases. In particular, a nanoscale theranostic system facilitates delivery of an anticancer agent with triggered or controlled release coupled to tumor-specific targeting of the micro-environment, while simultaneously allowing imaging of the tumor [2–7].

In this study, we focused on development of gold nanoparticle (AuNP)-based theranostic nanocarrier for photodynamic therapy (PDT) and imaging of cancer. PDT is a minimally invasive cancer treatment modality in which photoactivation of photosensitizer (PS) molecules accumulated within a tumor leads to energy transfer cascades, ultimately resulting in the conversion of molecular oxygen to cytotoxic reactive oxygen species (ROS), particularly singlet oxygen ($^1O_2$), which causes irreversible destruction of target tissues [8–13]. Compared to current treatments, including surgery, radiation therapy, and chemotherapy, PDT is an effective and selective method of destroying diseased tissue without damaging surrounding healthy tissue. However, conventional PS molecules are often randomly distributed *in vivo* and lack tumor selectivity [14]. A significant challenge that needs to be overcome for most treatments is the hydrophobic nature of PS molecules, which severely hampers intravenous administration through the blood stream. To overcome these limitations, various nanoscale drug carriers such as polymer-based nanoparticles, liposomes [15], polymeric micelles [16], dendrimers [17], silica nanoparticles [18], magnetic

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nanoparticles [19], and gold nanoparticles [20] have been explored as PS delivery systems in cancer therapy. Among these nanoscale drug carriers, AuNPs have received significant attention as a platform for drug delivery due to their chemical inertness, good biocompatibility, tunable size and shape, and well-established surface chemistry [21]. In particular, due to their surface plasmon resonance (SPR) and novel photoluminescence, AuNPs have been investigated as novel contrast agents in cancer cell imaging [22]. Thus, AuNPs are multifunctional, and can be used to combine different desired functionalities in one molecular-sized package.

Nanoparticles with pH-triggered drug release properties have been reported to be promising platforms for tumor-targeted intracellular drug and gene delivery [17,23]. Interstitial fluid in tumors is known to have a lower pH (pH 6.75) than that of normal tissue (pH 7.23) [24–27]. In addition, nanoparticles internalized by endocytosis are found in the acidic environments of endosomes (pH 5.0–6.0) and lysosomes (pH 4.5–5.0) [28]. If such environments accelerate the degradation of pH-sensitive nanoparticles, the conjugated drug will be released. In our previous study, we reported the pH-dependent release behaviors of PS from nanocarriers induced by the hydrolysis of hydrazone linkages under acidic conditions [29]. Therefore, use of pH-sensitive nanoparticles may overcome the intracellular barriers of endosomal or lysosomal membranes that prevent drugs from reaching their targets. Several recent studies have reported PS-loaded AuNPs for PDT [30–32], but a AuNP-based theranostic system involving PS molecules chemically conjugated through pH-sensitive linkages has not previously been reported.

Therefore, we aimed to develop a tumor-targeting theranostic system based on AuNPs with pH-sensitive cleavage linkages for PDT and imaging of cancer. We employed FA-conjugated biocompatible and biodegradable polyethylene glycol (PEG) and poly(β-benzyl-L-aspartate) (PBLA) block copolymer for AuNP modification. We previously reported the Au-based nanocarrier system which was chemically conjugated with pheophorbide a (Pheo) as a PS [33]. However, there was only minor difference in the cellular uptake efficiency of free Pheo and Au-based nanocarrier due to the slightly high water solubility of Pheo (about 15 μg/ml). Therefore, to improve the selective intracellular uptake and phototoxicity, we used a benzoporphyrin derivative, verteporfin (Verte), as a hydrophobic PS in this study. Verte has a long excitation wavelength (708 nm) and has been used as a highly efficient PS for PDT to eliminate abnormal blood vessels in the eye associated with conditions such as macular degeneration [34–36]. It also is an ideal fluorescent marker for bioassays and cell imaging. Verte was conjugated to the side chain of the core-forming segment *via* an acid-labile hydrazone bond that is stable at physiological pH (7.0–7.4), but cleavable at a lower pH (4.0–6.0) such as that encountered in the vicinity of tumor tissues or within endosomal/lysosomal compartments. Physicochemical properties of Verte-conjugated block copolymer-AuNP conjugates were characterized by determining particle size, size distribution, morphology, UV–visible absorption spectra, and XPS spectra. Stability of Verte-conjugated block copolymer-AuNP conjugates at various pH values was also investigated. In particular, *in vitro* cellular localization was examined in HeLa cells by confocal microscopy, flow cytometry, and transmission electron microscopy (TEM). In addition, the phototoxicity of Verte-conjugated block copolymer-AuNP conjugates was evaluated and compared with that of free Verte.

## 2. Materials and methods

### 2.1. Materials

PEG-bis(amine) (molecular weight: 3.350 kDa), β-benzyl-L-aspartate (BLA), triethylamine (TEA), FA, hydrazine monohydrate, sodium bicarbonate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC), α-lipoic acid (LA), sodium citrate tribasic dihydrate, and verteporfin (Verte) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium borohydride, *N*-hydroxysuccinimide (NHS), and N, N′-dicyclohexylcarbodiimide (DCC) were obtained from Fluka (Buchs, Switzerland). Chloroauric acid, triphosgene, and 4-(dimethylamino) pyridine (DMAP) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). 4-Hydroxy-2-butanonewas purchased from TCI (Tokyo, Japan). Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), *n*-hexane, benzene, *N*, *N*-dimethylformamide (DMF), chloroform, diethyl ether, 1, 4-dioxane, methanol, dichloromethane (DCM), and acetic acid were obtained from Samchun Pure Chemical Co., Ltd. (Gyeonggi-do, Korea). RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco's phosphate buffered saline (DPBS) were obtained from Gibco BRL (Invitrogen Corp., Carlsbad, *CA*, USA). Spectra/Por membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, *CA*, USA). All other chemicals were analytical grade and used as-received without further purification.

### 2.2. Synthesis of AuNP-based multifunctional nanocarriers

#### 2.2.1. Synthesis of FA-conjugated block copolymers for AuNP modification

FA-conjugated PEG-P(Asp-Hyd) block copolymer used for surface modification of AuNPs was synthesized as reported previously [29,33]. Briefly, the copolymer was synthesized *via* several steps as follows (Fig. 1).

First, FA ligand was introduced into PEG-bis(amine) to synthesize FA-PEG-$NH_2$. FA (0.25 mmol) was dissolved in DMSO. After complete dissolution, DCC (0.32 mmol), NHS (0.32 mmol), PEG-bis(amine) (0.21 mmol), and TEA (2.1 mmol) were added. We maintained the molar feed ratio of FA to PEG-bis(amine) at 1.2:1 to obtain mono-substitution of the amino group in PEG-bis(amine) (FA-PEG-$NH_2$). After the reaction mixture was stirred for 24 h at 400 rpm and room temperature (25 °C) in the dark, the by-product, dicyclohexylurea, was removed by filtration. The resulting product was purified by dialysis against a $NaHCO_3$ solution (pH 8.4) for 2 days to remove the unconjugated FA, then dialyzed against deionized water to remove $NaHCO_3$, and freeze-dried.

Second, the β-benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) segment was synthesized. After BLA (32.3 mmol) was added to a round-bottom flask, triphosgene (14.5 mmol) dissolved in THF was added slowly into the flask. As the reaction proceeded with stirring at 60 °C for 2 h under nitrogen atmosphere, the mixture became clearer. The solvent of the reactant mixture was removed using a rotary evaporator, and the resulting product, BLA-NCA, was further purified by recrystallization using a mixed solvent composed of THF and *n*-hexane three times [37]. Finally, a white, powdery substance was obtained after vacuum-drying.

Third, FA-PEG-poly(β-benzyl-L-aspartate) (FA-PEG-$PBLA_{50}$) copolymers were prepared by ring-opening polymerization of BLA-NCA. BLA-NCA (5.5 mmol) dissolved in DMF and FA-PEG-$NH_2$ (0.11 mmol) dissolved in chloroform were mixed. Quantities of solvents were adjusted to 2 ml of DMF per 1 g of BLA-NCA, and the amount of chloroform added was 10-fold higher than the amount of DMF. The reaction was allowed to proceed at 40 °C for 48 h in the dark under nitrogen atmosphere. The reaction mixture was precipitated with excess cold diethyl ether, and the precipitate was dissolved in 1,4-dioxane and then freeze-dried [38].

Fourth, α-lipoic acid (LA) was introduced to FA-PEG-$PBLA_{50}$ to synthesize FA-PEG-$PBLA_{50}$-LA, and then the benzyl groups were removed to convert FA-PEG-$P(Asp\text{-}Hyd)_{50}$-LA. After FA-PEG-$PBLA_{50}$ (0.18 mmol) had dissolved completely in DMSO, EDC (0.32 mmol), NHS (0.32 mmol), LA (0.27 mmol), and TEA (2.1 mmol) were added. The reaction mixture was stirred at 400 rpm for 24 h at room temperature (25 °C) in the dark under a nitrogen atmosphere. Then, to substitute benzyl esters of the side chains of FA-PEG-PBLA with hydrazide groups, hydrazine monohydrate (90 mmol; 10-fold excess of hydrazine monohydrate relative to benzyl groups) was added. The reaction was allowed to proceed with 1000 rpm stirring for 4 h at room temperature