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Beta-carotene-bound albumin nanoparticles modified with chlorin e6 for breast tumor ablation based on photodynamic therapy

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ABSTRACT

Chlorin e6 (Ce6) has attracted considerable interest as a promising second-generation photosensitizer for photodynamic therapy (PDT). However, the *in vivo* availability of Ce6 is significantly restricted by its low water solubility and poor ability to target tumors. We sought to overcome the limitations of Ce6 by using albumin nanoparticles with nab™ (nanoparticle albumin-bound) technology. The fabricated albumin nanoparticles consisted of bovine serum albumin (BSA), Ce6-BSA, and beta-carotene as a carrier, photosensitizing agent, and cross-linker, respectively. These albumin nanoparticles (Ce6-BSA-BC-NPs) did not include any toxic chemotherapeutics but instead contained naturally safe beta-carotene and Ce6, which was activated only upon irradiation with 660-nm laser light. Ce6-BSA-BC-NPs were ~120 nm in size and spherical, similar to Abraxane®, and showed good physicochemical stability. The nanoparticles showed significant cytotoxicity toward 4T1 cells as evaluated by MTT, Live/Dead, and TUNEL assays. In particular, results of the TUNEL assay demonstrated that cell death induced by Ce6-BSA-BC-NPs and light irradiation (660 nm) occurred through the apoptotic pathway. Ce6-BSA-BC-NPs displayed a remarkably enhanced tumor suppression effect when irradiated by 660-nm light compared with free Ce6 (tumor volume 90 ± 39 versus 487 ± 69 mm^3 respectively). Overall, this improved *in vivo* antitumor efficacy seemed to be due to the targetability of albumin nanoparticles. We believe that our Ce6-BSA-BC-NPs with PDT offer a promising new potential therapeutic platform for breast cancer treatment.

1. Introduction

Photodynamic therapy (PDT) has been considered an effective means of ablating tumors. The process of PDT involves administration of a tumor-localizing photosensitizer (PS) and timely local irradiation of tumor regions using light appropriate for the PS [1,2] Under appropriate levels of tissue oxygen and intensity of light the PS generates reactive oxygen species (ROS) such as singlet oxygen or free radicals [3], which induce cancer cell death through apoptosis, necrosis or autophagy [1,4,5]. Three mechanisms of tumor ablation by PDT have been suggested: (i) direct tumor cell killing, (ii) damage to tumor vasculature, and (iii) tumor suppression via the immune response [3]. PDT shows many advantages such as alleviated invasiveness, short treatment time, safety even after repetition, minimal scarring after healing, and low cost compared with other treatments [1,6,7]. PDT has been tested clinically for the treatment of various cancer types including head and neck, brain, lung, pancreas, skin, prostate and breast cancer [3].

Chlorin e6 (Ce6) is one of the most outstanding PSs developed to date and its derivatives have been used clinically for PDT. Compared with the first-generation PS, Ce6 responds to a greater range of wavelengths, between 600 and ~900 nm, which enables higher penetration of laser light into deep tissues. Furthermore, it shows strong intrinsic fluorescence over the wavelength range and can be effectively applied for diagnostic imaging of tumors without interference from endogenous chromophores [8]. However, the availability of Ce6 is restricted by its poor water solubility and low targetability.

Efficient localization of PS in tumors is essential for successful PDT because the amount of PS in tumors is directly related to the efficiency of ROS generation upon irradiation. Many studies have used the tumor

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targetability of nanoparticles to address the limited availability of PSs in tumors for PDT. Among a number of nanoparticle platforms, albumin nanoparticles have attracted considerable interest in both academia and industry due to noticeable advantages of albumin protein as a pharmaceutical carrier, such as, biodegradability, biocompatibility, non-immunogenicity (human source), high chemical stability, and versatility. [9–11]. Moreover, albumin preferentially accumulates in tumor tissues due to its excellent targetability to tumors. It permeates leaky blood vessels via efficient extravasation process [12] and is transported across endothelial walls around tumor tissues through gp60 (60-kDa glycoprotein) receptors expressed on the tumor vasculature. Furthermore, SPARC (secreted protein, acidic and rich in cysteine) binds to albumin in the tumor interstitium and facilitates the uptake of albumin into tumors, providing the basis of targeted delivery by albumin nanoparticles [13–17]. Additionally, albumin itself plays a role as a dysopsonin that prevents the adsorption of various plasma proteins and protects macrophage recognition, leading to extended circulation time of nanoparticles [18,19]. In practice, the nab™ (nanoparticle albumin-bound) technology-based formulation, Abraxane®, has outperformed other existing formulations of paclitaxel, including Taxol®, by virtue of several significant advantages such as reduced toxicity, tolerated dose increase, and excellent tumor targetability. In this albumin nanoparticle structure, hydrophobic drugs play a significant role in assembling albumin by acting as physical cross-linkers.

Beta-carotene (BC) is a strongly red-orange-colored terpenoid that is synthesized biochemically from eight isoprene units in nature [20]. BC from many natural vegetable products, such as carrot, spinach, lettuce, and broccoli, is used as a safe natural dietary agent [21]. Some studies have reported the preventive effect of BC as a pro-vitamin by stimulating the immune system [22,23], showing an inverse relationship between BC uptake and risk of occurrence of estrogen receptor-negative [24,25] or –positive [26] breast cancers. BC has a high binding affinity to albumin, and its identical left-right symmetric structure has great potential for association of albumin molecules. On account of its high organic solubility it can be used as a physical cross-linker in the preparation of nab™ technology-based albumin nanoparticles in a similar manner to hydrophobic drugs.

In this study, a prototype of albumin nanoparticles consisting of naïve BSA, Ce6-BSA, and BC (Ce6-BSA-BC-NPs) was developed as a photodynamic therapy agent for the treatment of breast cancer in order to overcome the problems of poor solubility and tumor targetability. These albumin nanoparticles were fabricated using high-pressure homogenization based on nab™ technology and their physicochemical properties were examined. Also, the *in vitro* cytotoxicity and *in vivo* antitumor efficacy of Ce6-BSA-BC-NPs were examined in 4T1 cells (a triple-negative breast cancer cell line) and 4T1 cell-xenografted *nu/nu* mice after irradiation with 660-nm light (Fig. 1).

2. Materials and methods

2.1. Materials

Chlorin e6 (Ce6) was purchased from Frontier Scientific (Salt Lake City, UT, USA). Bovine serum albumin (BSA; 66.5 kDa and ~99%) and beta-carotene were from Sigma-Aldrich (St. Louis, MO, USA). 4T1 (ATCC® CRL-2539™) breast cancer cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were obtained from Corning (Corning, NY, USA). The LIVE/DEAD® viability/cytotoxicity kits for mammalian cells and singlet oxygen sensor green reagent (SOSG) were provided by Thermo Fisher Scientific (Rockford, IL, USA). The *in situ* cell death detection kit was obtained from Roche Diagnostics (Mannheim, Germany). All other reagents were supplied by Sigma-Aldrich unless otherwise specified.

2.2. Animals

BALB/c nu/nu mice (male, 6 weeks old) were purchased from Hanlim Experimental Animal Laboratory (Seoul, South Korea). Animals were cared for in accordance with the guidelines issued by the National Institutes of Health (NIH) according to the care and use of laboratory animals (NIH publication 80-23, revised in 1996). Mice were divided into groups corresponding to the treatment and provided with food and water under a 12-h light/dark cycle (lights on at 6 am). This study was approved by the Ethical Committee on Animal Experimentation at Sungkyunkwan University.

2.3. Preparation of Ce6-BSA conjugates

BSA was conjugated with Ce6 using a slight modification of previous methods [27,28]. In brief, aliquots of Ce6 (1 mmol), N-dicyclohexylcarbodiimide (DCC, 4 mmol), N- hydroxysuccinimide (NHS, 5 mmol) and triethylamine (TEA, 4 mmol) were dissolved in 10 ml dimethyl sulfoxide (DMSO, anhydrous) in a glass tube. The mixture was gently stirred and allowed to react in the dark at ambient temperature for 24 h. After removal of the precipitate, the Ce6-NHS produced was stored at −70 °C until use. Separately, different aliquots (1.5 and 4.5 ml) of Ce6-NHS (0.1 mmol) in DMSO, which correspond to feeding ratios of 20:1 and 60:1 (Ce6:BSA) respectively, were dropwise mixed with 50 ml BSA (0.0075 mmol) dissolved in 0.1 M sodium borate buffer (pH 8.5). The resulting suspension was continuously stirred at 450 rpm in the dark at room temperature for 24 h. Unreacted Ce6 and DMSO were removed using a dialysis membrane (MwCO: 10 kDa; amicon ultra, Millipore, Beverly, MA, USA) against 60% ethanol for 48 h and then against deionized water (DW) for 24 h. Finally, Ce6-BSA was concentrated in DW using a centrifugal concentrator (MwCO: 30 kDa, Amicon® Ultra, Millipore) and then lyophilized and stored at -20 °C for further experiments.

2.4. Characterization of Ce6-BSA conjugates

Ce6-BSA conjugates (feeding ratios of 20:1 or 60:1) were characterized by matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) mass spectrometry (UltrafleXtreme, Bruker, Coventry, UK) and reversed-phase high-performance liquid chromatography (RP-HPLC) on a PLRP-S column (150 × 4.6 mm, 8 μm, Agilent Technologies, CA, USA) [28].

2.5. Preparation of Ce6-BSA-BC-NPs and BSA-BC-NPs

Ce6-BSA-BC-NPs and BSA-BC-NPs were prepared using a slight modification of nanoparticle albumin bound (nab™) technology [9,29,30]. In brief, aliquots of 40 mg BSA/10 mg Ce6-BSA (feeding ratio of 20:1) or 50 mg BSA were dissolved in 5 ml DW. Beta-carotene (1 mg) was dissolved in 100 μl of a 9:1 solution of chloroform and ethanol. These solutions consisting of aqueous and organic phases were gently mixed and homogenized using a WiseTis HG15D homogenizer (DAIHAN Scientific Co, Seoul, South Korea) at 18,000 rpm. The resulting solutions were passed through a high-pressure homogenizer (EmulsiFlex-B15 device, Avestin, Ottawa, Ontario, Canada) for nine cycles at 20,000 psi. The dispersions were rotary evaporated to remove chloroform at 40 °C for 15 min under reduced pressure. Finally, the NPs were centrifuged at 6000 rpm and the supernatants were lyophilized and stored at −20 °C until required.

2.6. Characterization of Ce6-BSA-BC-NPs and BSA-BC-NPs

The average particle sizes and zeta potentials of Ce6-BSA-BC-NPs and BSA-BC-NPs (each 1 mg/ml in DW) were measured using dynamic light scattering (DLS) (Zetasizer Nano ZS90, Malvern Instruments, Worcestershire, UK) with a 633-nm He-Ne laser beam and a fixed 90°