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Conjugation of the photosensitizer Chlorin e6 to pluronic F127 for enhanced cellular internalization for photodynamic therapy

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ABSTRACT

To improve the cellular internalization and tumor-specific targeting efficiency of a photosensitizer, Chlorin e6 (Ce6), was conjugated to Pluronic F127® (PF127) by esterification between the carboxylic groups of Ce6 and the two hydroxyl groups of PF127 (PCe6). After conjugation, two different PCe6 conjugates, PF127/Ce6 (PCe6-1) and Ce6/PF127/Ce6 (PCe6-2), were obtained and separated by hydrophobic interaction chromatography. In this study, PCe6-1 was employed because it has good solubility in aqueous media. The singlet oxygen generation (SOG) efficiency and fluorescence activity of PCe6-1 in aqueous media dramatically increased compared to free Ce6. Moreover, confocal imaging and fluorescence-activated cell sorting (FACS) analysis confirmed its enhanced internalization rate in mouse colon tumor (CT-26) cells. After light irradiation, the cellular phototoxicity of PCe6-1 against CT-26 was approximately 3 times higher than that of free Ce6 *in vitro*. Furthermore, PCe6-1 exhibited enhanced tumor-specific distribution and tumor growth inhibition after intravenous injection into tumor-bearing mice. These results suggest that PCe6-1 is a good candidate as a photosensitizer due to its high cellular internalization rate and tumor targeting efficiency.

1. Introduction

Photodynamic therapy (PDT) is an effective medical tool for the treatment various cancers by inducing apoptosis or necrosis. This type of therapy uses chemical photosensitizers (PSs) and light irradiation at specific wavelengths to eradicate target tumor tissues. Although PSs are nontoxic to cells in the absence of light, when irradiated with specific activating wavelengths, PSs generate cytotoxic singlet oxygen that destroys cells [1–3]. Several PSs such as Photofrin® and Laserphyrin® have been widely used in clinical cancer therapy as approved by the U.S. Food and Drug Administration (FDA) [4,5]. However, the use of PSs has been limited in clinical applications due to their poor solubility in aqueous media, cellular internalization and low tumor-targeting efficacy [6–10].

To overcome these problems in conventional PSs, nanoparticle based PS systems, such as liposome, solid lipid nanoparticle and graphene oxide have been reported for enhancing the tumor specificity of PS through enhanced permeation and retention (EPR) effect [11–13]. On the other hand, various polymeric PS systems, such as polyethylene glycol (PEG)/PS conjugates, have been also investigated [14–18]. This system did not enhance cellular internalization or site-specific distribution but did increase the solubility of PS in aqueous media. Recently, our group reported that a cationic polymeric PS (PEG-polyethylenimine-Chlorin e6), effectively increased ROS production at extremely high concentrations in aqueous media [19]. However, this cationic polymer is notorious for causing cell membrane damage due to the high density of positive charges on its surface [20].

Herein, Chlorin e6 (Ce6) was chosen as a PS in the present study. Ce6 is an effective PS given its several advantages for clinical use, such as activation by near infrared wavelengths, relatively deep penetration through layers of tissues and potency against a broad spectrum of cancers, especially lung cancer, metastatic breast cancer and refractory ovarian cancer [9,21]. Moreover, Pluronic F127® (PF127) was employed to make a water soluble polymeric PS with enhanced cellular internalization and tumor-targeting efficacy via conjugation of Ce6 (PCe6). PF127 (FDA approved, poly(ethylene oxide)-block-poly (propylene oxide)-block-poly(ethylene oxide) (PEO–PPO–PEO triblock copolymer), is has been widely used as a pharmaceutical adjuvant [22–26]. Moreover, this polymer is able to interact with cell membranes, leading to decreased microviscosity, pore formation on the membrane and accelerated “flip-flop” of the membrane component [22,27–30]. However, according to previous

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report, the cellular membrane binding property of PF127 is limited owing to their low HLB value [31]. We hypothesized that the conjugation of Ce6 with PF127 could decrease the HLB value. Thus, Ce6 conjugated with PF127 would be easily internalized into tumor cells.

Therefore, PF127/Ce6 (PCe6) conjugate has the following several advantages for PDT: i) increased solubility in aqueous media, ii) enhanced internalization into cells, and iii) high tumor-targeting efficiency via EPR effect.

In this study, the physicochemical properties and photoactivity of PCe6 were determined via fluorescence intensity and spectrofluorophotometry, respectively. In particular, the cellular internalization behavior and phototoxicity of PCe6 were compared to free Ce6 using confocal microscopy, fluorescence-activated cell sorting (FACS), and MTT assay, respectively. We selected the murine CT26 colorectal tumor model for these studies. Because the colorectal cancer model have been widely used in preclinical study of PDT [32]. Lastly, the time-dependent biodistribution and tumor growth inhibition efficiency of the PCe6 were investigated in tumor-bearing mice [33,34].

2. Materials and methods

2.1. Materials

Pluronic F 127® (PF127) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Chlorin e6 (Ce6) was purchased from Frontier Scientific, Inc. (Salt Lake City, UT, USA).

1, 3-dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), anhydrous dimethyl sulfoxide (DMSO) and 9,10-dimethylanthracene (DMA) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The dialysis membrane was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), and Dulbecco's phosphate buffer saline (DPBS) were obtained from Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). Singlet oxygen sensor green (SOSG) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay was performed using the DeadEnd™ Colorimetric Cell Death Detection kit (Promega, USA).

2.2. Preparation and characterization of PF127/Ce6 (PCe6)

The conjugation of PF127/Ce6 via DCC and DMAP-mediated ester formation was previously reported by our group [35]. Briefly, PF127 and Ce6 were conjugated in DMSO, as described below. PF127 powder (100 mg) was dissolved in anhydrous DMSO. Ce6 (30 mg, corresponding to a mole ratio of Ce6 to PF127 of 2.5) was dissolved in DMSO (10 ml), followed by the addition of 1.5 molar equivalent of DCC and DMAP while the PF127 was dissolved in DMSO via vigorous stirring. After allowing the PF127 and Ce6 solutions to completely dissolve, they were mixed slowly, and the reaction mixture was gently stirred for 24 h at room temperature. The reaction mixture was dialyzed (M_w cut-off: 1000 Da) for 3 days against distilled water to remove unconjugated Ce6 and DMSO. The final solution was flash-frozen dry and lyophilized.

For the purification, the following hydrophobic chromatographic columns were used: the crude products (PCe6) were chromatographed using an open column filled with Sephadex LH-20 (Sigma–Aldrich, Co.) as the stationary phase, and methanol served as the mobile phase. A 4.0 g portion of the Sephadex LH-20 was dissolved in 10 ml of methanol for activation, applied to a chromatographic column (2 × 60 cm) packed with LH-20 and eluted with 95% (v/v) methanol by gravity. After loading the crude products, the flow rate was 0.5 ml/min, with the mobile phase starting from 50% methanol (5:5 MeOH in water) (0–60 min), and proceeding to 80% methanol (8:2 MeOH in water) (61–110 min). Each fraction was collected, obtain pure products (PCe6-1 and PCe6-2) and their absorbance were measured at 664 nm. The ^{1}H NMR spectrum was recorded in deuterated dimethyl sulfoxide (DMSO) at room temperature using a Bruker NMR Spectrometer (Bruker, Germany).

The Ce6 content of PCe6-1 was measured using UV/Vis spectroscopy. Free Ce6 dissolved in DMSO was prepared at different concentrations (1,000, 500, 250, 125, 62.5, 31.3 and 15.6 μg/ml) to generate a standard curve, and the amount of Ce6 conjugated to PF127 was measured as the absorption at 404 nm using a UV/visible spectrophotometer (UV-2450, Shimadzu, Japan).

The PCe6-1 particle size was determined using dynamic light scattering (DLS; Series4700; Malvern Instruments Ltd., UK) with an argon ion laser system tuned to a wave-length of 488 nm. Each sample was filtered through a 0.45 mm filter directly into a precleaned 10 mm-diameter cylindrical cell. Intensity auto correlation was measured at a scattering angle (θ) of 90° at 25 °C.

PCe6-1 morphology was observed by field emission scanning electron microscopy (FE-SEM; S-4700; Hitachi, Japan). 1 mg/ml of PCe6-1 solution was dropped onto a cover slip and placed on a graphite surface that was coated with Pt by sputtering for 4 min at 20 mA.

To estimate the photoactivity of the PCe6-1 in distilled water (1 mg/ml), fluorescence spectra (RF-5301PC; Shimadzu, Japan) were used.

2.3. The evaluation of the singlet oxygen generation (SOG) efficiency

To evaluate the SOG of samples under different conditions (in distilled water and organic solvent), a singlet oxygen trap was prepared using 20 mM DMA. PCe6-1 (Ce6, 1.5 mg/ml) was dissolved in DMSO and added to DMA stock solutions to give a final concentration of 20 mM DMA. After a 10 min equilibration period, the appropriate amount of DMA was added. The samples containing sensitizers and DMA were irradiated with a light intensity of 2.4 J/cm^2 using a 670 nm He–Ne laser source (Institute of Electronics). A collimated laser beam was directed at the sample cuvette through an optical fiber. The decrease in fluorescence intensity of DMA (excitation, 360 nm; emission, 380–550 nm) as a result of the photosensitizer reaction was monitored using a spectrofluorophotometer controlled by a PC [36].

To evaluate the singlet oxygen generation (SOG) of PCe6-1 in distilled water, the singlet oxygen sensor green (SOSG) was introduced at a concentration of 2.0 mM. SOG was induced by irradiation with a light intensity of 2.4 J/cm^2 using a 670 nm He–Ne laser source. After irradiation, SOSG fluorescence was read at an excitation and maximum wavelength of 494 and 534 nm, respectively, to determine the SOG of the samples in distilled water. SOG was evaluated by SOSG fluorescence enhancement compared to background or a control sample [37].

2.4. Cell culture and incubation conditions

CT-26 cells were obtained from the Korean Cell Line Bank (KCLB No. 10247) and cultured in 10 ml of DMEM medium that was supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were cultured at 37 °C with 100% humidity and 5% CO_2, and the medium was replaced every 2–3 days. PCe6-1 was dissolved in serum-free (SF) medium. Water-insoluble free Ce6 was dissolved in DMSO and then diluted in SF medium until the DMSO concentration reached <0.1%. All of the reported concentrations refer to the free Ce6 equivalents. Untreated cells were irradiated or kept in the dark and used as a reference standard.

2.5. Cellular uptake test of PCe6-1

To verify the cellular uptake of PCe6-1, CT-26 cells were exposed to free Ce6 or PCe6-1 (5 μg/ml of Ce6). The cells were incubated for 30 min and 6 h, washed, harvested, and resuspended with DPBS. Fluorescence-activated cell sorting (FACS) was performed using a FACScan flow cytometer (Beckman, San Jose, CA, USA). For each sample, 10,000 cells (gated events) were counted, and free Ce6 fluorescence was detected with logarithmic settings (FL4; Em = 670 nm). The cells were considered to be positive if their fluorescence (FL4) was higher than that of cells from an untreated cell suspension. The statistical analyses were using the CXP Analysis program.

To observe the subcellular localizations of the PCe6-1, CT-26 cells (1 × 10^5 cells/well in a 12-well plate) were treated with PCe6-1 (5 μg/ml of Ce6) for 30 min and 2 h at 37 °C. The cells were then washed twice with PBS, fixed with 4% paraformaldehyde, and visualized using a confocal laser scanning microscope (LSM 510 Meta; Zeiss, Germany). An optimal pinhole size of 120 mm was selected to exclude fluorescent light emitted from out-of-focus planes above and below the focusing plane. An objective with a magnification of 1200 was used for image capture. A laser line with a wavelength of 630 nm was used to excite DAPI, and a He–Ne laser was used to excite Ce6. A long-pass filter (LP 650 nm) was used at the emission end to detect Ce6. Fluorescence images were analyzed using LSM Image Browser software (Zeiss).

2.6. Cytotoxicity of PCe6-1

CT-26 cells (1 × 10^4 cells/well) were seeded onto 96-well plates and allowed to attach for 1 d. After cell attachment, the medium was replaced with 100 ml of SF culture medium containing free Ce6 or PCe6-1 (0.002–2 mg/ml of Ce6) and incubated for 2 h. Next, the cells were washed twice with PBS, and fresh culture medium was added. Free Ce6 and PCe6-1 treated cells were irradiated with a He–Ne laser (670 nm, 2.4 J/cm^2). Irradiated cells were then incubated at 37 °C for 24 h, and cell viability was evaluated using MTT assay. The data represent the percent of viable cells compared to the control.

2.7. In vivo photodynamic therapy and histology

All experiments involving live animals were performed in compliance with the relevant laws and institutional guidelines of the Korea Institute of Science and Technology (KIST), and institutional committees approved the experiments.

CT-26 cells (1 × 10^5) were subcutaneous injected into the left flank of the mice. When the xenograft tumors reached 150–200 mm^3, 200 ml of saline, free Ce6 or

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