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Dendritic nanoconjugates of photosensitizer for targeted photodynamic therapy

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ABSTRACT

Application of photodynamic therapy for treating cancers has been restrained by suboptimal delivery of photosensitizers to cancer cells. Nanoparticle (NP)-based delivery has become an important strategy to improve tumor delivery of photosensitizers; however, the success is still limited. One problem for many NPs is poor penetration into tumors, and thus the photokilling is not complete. We aimed to use chemical conjugation method to engineer small NPs for superior cancer cell uptake and tumor penetration. Thus, Chlorin e6 (Ce6) was covalently conjugated to PAMAM dendrimer (generation 7.0) that was also modified by tumor-targeting RGD peptide. With multiple Ce6 molecules in a single nanoconjugate molecule, the resultant targeted nanoconjugates showed uniform and monodispersed size distribution with a diameter of 28 nm. The singlet oxygen generation efficiency and fluorescence intensity of the nanoconjugates in aqueous media were significantly higher than free Ce6. Targeted nanoconjugates demonstrated approximately 16-fold enhancement in receptor-specific cellular delivery of Ce6 into integrin-expressing A375 cells compared to free Ce6 and thus were able to cause massive cell killing at low nanomolar concentrations under photo-irradiation. In contrast, they did not cause significant toxicity up to 2 μM in dark. Due to their small size, the targeted nanoconjugates could penetrate deeply into tumor spheroids and produced strong photo-toxicity in this 3-D tumor model. As a result of their great cellular delivery, small size, and lack of dark cytotoxicity, the nanoconjugates may provide an effective tool for targeted photodynamic therapy of solid tumors.

1. Introduction

Photodynamic therapy (PDT) is a clinically approved and minimally invasive therapeutic procedure, which is based on the combined use of a photosensitizer (PS), oxygen, and light radiation. PSs are usually pharmacologically inactive in the dark and are activated by irradiation at a specific wavelength to produce reactive oxygen species (ROS), which are capable of rendering cancer cell death [1]. Clinical use of many PSs has been hampered by their intrinsic limitations including poor solubility, aggregation in aqueous solution, and poor tumor selectivity [2]. To overcome these limitations, various nanoparticles (NPs), including liposomes, inorganic NPs and polymeric NPs, have been developed to increase water solubility and enhance tumor delivery of PSs [3–8]. Among these NPs, various polymer-PS conjugates, such as Pluronic F127/Ce6 and glycol chitosan/pheophorbide A conjugates, have been reported [9,10]. Chemical conjugation of hydrophobic photosensitizer to hydrophilic polymers allows self-assembly into NPs. Importantly, chemical conjugates demonstrate higher solubility and increased stability in vitro and in vivo than free PSs [3–7]. Further, chemical conjugation method can avoid the release of PSs from NPs before reaching to the target tissues and thus reduces side effects caused by accumulation of PSs in non-target tissues [9,11,12].

Poor tumor penetration is still a limitation for NP-based delivery of PSs. For complete eradication of solid tumors, anticancer drugs including PSs should penetrate deeply in the solid tumors and reach all cancer cells in a therapeutic concentration [13]. Abraxane is a FDA-approved albumin-bound paclitaxel NP for treatment of solid tumors. Their large size (about 130 nm) allows them to preferentially accumulate in solid tumors by the Enhanced Permeability and Retention (EPR) effect [13–15]. However, although it causes lower side effects than free paclitaxel, this NP only provides modest survival benefits, likely due to poor penetration of Abraxane into deep tumor tissues [13,16]. It has

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been reported that NPs smaller than 50 nm may penetrate into deep tissue far from tumor vasculature [13,17,18]. However, most of the NPs that are used for delivery of PSs are too large for effective tumor penetration. For example, liposomes and albumin NPs that are prepared via complexation are typically larger than 100 nm [7,19]. Those prepared by chemical conjugation, though smaller than liposomes, are still larger than 50 nm [9,10].

This study aimed to use chemical conjugation method to construct NPs that are cancer cell-selective and are smaller than 50 nm. Thus, the resultant NPs can deliver PSs deeply into tumor tissues and effectively into cancer cells. For this purpose, we chose poly (amido amine) generation 7.0 (PAMAM G7) dendrimer with a diameter of 8 nm as a core molecule to prepare ultra-small NPs. By modification with sufficient PEG on the surface, PAMAM dendrimers showed reduced cytotoxicity. In addition, to achieve tumor selectivity, cRGD, a ligand to integrin $\alpha_v\beta_3$ that is overexpressed in many cancer cells [20], were functionalized to the surface of PEGylated PAMAM dendrimers. Photosensitizer Ce6 were covalently conjugated onto the RGD functionalized PAMAM and the resultant nanoconjugates (RGD-P-Ce6) were characterized in terms of size, zeta potential, fluorescence and singlet oxygen generation efficiency. We then examined cellular delivery and phototoxicity of the nanoconjugates in integrin $\alpha_v\beta_3$-expressing A375 cells, and further studied their penetration and phototoxicity in spheroids of A375 cells, a 3-D model of solid tumors.

2. Materials and methods

2.1. Preparation of RGD-P-Ce6

PAMAM G7 (Sigma–Aldrich, St. Louis, MO) was conjugated with Mal-PEG_{5K}-NHS (Nanocs Inc., New York, NY) at a 1:30 M ratio of PAMAM to PEG in PBS (pH 7.4) for 30 min at room temperature, and this was followed by adding cRGDyC peptide (Peptides International, Inc., Louisville, KY) to react with the maleimide group on PEG at the ratio of RGD to PAMAM as 40:1 to obtain RGD-PAMAM (RGD-P). Ce6 (MedKoo Biosciences Inc., Chapel Hill, NC) was dissolved in DMSO, followed by the addition of 10 M equivalents of EDC and Sulfo-NHS (Thermo Fisher, Rockford, IL). After 3 h, activated Ce6 (Ce6-NHS) was added into the above RGD-PAMAM solution with a 15:1 M ratio of Ce6 to PAMAM. The reaction was gently stirred for 12 h in dark at room temperature. For the purification, crude nanoconjugates were chromatographed using Sephadex LH-20 (GE Healthcare, Pittsburgh, PA) to remove unreacted Ce6, and mobile phase was mixture of methanol and distilled water (v/v = 1:2). After that, the resulting product was purified by gel filtration using Sephadex G100 (GE Healthcare) and PBS was used as mobile phase. In addition, monofunctional PEG_{5K}-NHS (Nanocs Inc.) was used for preparation of non-targeting nanoconjugates PEG-P-Ce6 with a similar method (Sfig. 1).

2.2. Characterization of RGD-P-Ce6

The Ce6 content in the RGD-P-Ce6 nanoconjugates was measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific). Free Ce6 dissolved in DMSO was used as standard solution and the amount of Ce6 of RGD-P-Ce6 (diluted with methanol) was measured as the absorption at the 405 nm peak. The fluorescence of Ce6 and RGD-P-Ce6 was detected by NanoDrop 3300 (Thermo Scientific).

To evaluate the singlet oxygen generation (SOG) by light irradiation of the nanoconjugates, the singlet oxygen sensor green (SOSG, Life Technologies, Carlsbad, CA) was mixed with RGD-P-Ce6 or free Ce6 in PBS with all concentrations at 1 μM. SOG was induced by irradiation at a light intensity of 3.5 mW/cm^2 using a 660 nm laser module diode (LaserLands, Wuhan, China) equipped with a concave lens (Edmund Optics Inc., NJ). After irradiation, SOSG fluorescence was measured at an excitation and emission of 488 nm and 525 nm using FLUOstar Omega plate reader (BMG Labtech, Germany). PBS with SOSG was irradiated and served as a negative control.

The hydrodynamic sizes of the nanoconjugates were measured by dynamic light scattering using a Zetasizer Nano (Malvern Instruments, Malvern, UK). The zeta potentials were also measured using this instrument. The final Ce6 concentration of the nanoconjugates was adjusted to 20 μM in DI water in these measurements. The morphology of the RGD-P-Ce6 nanoconjugates was observed using a cryo-Transmission Electron Microscope (TEM, Oberkochen, Germany). In this experiment, the nanoconjugates were diluted and dropped on 200 mesh carbon coated copper grids and were allowed to attach for 2 min. Uranyl acetate aqueous solution (4%) was dropped on the grid to counterstain the nanoconjugates.

2.3. Cell culture and intracellular uptake

A375 cells (a non-pigmented melanoma cell line) and NIH3T3 cells (mouse fibroblasts) were cultured in DMEM medium supplemented with 10%FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2.

To compare the intracellular uptake, A375 cells and NIH/3T3 cells were treated with free Ce6, PEG-P-Ce6 and RGD-P-Ce6 (150 nM equivalent Ce6) for 12 h. Then the cells were trypsinized and analyzed by flow cytometry using BD LSR II, with a 405 nm laser and a 670/20 nm emission filter set for Ce6 fluorescence. To investigate the endocytosis pathway [21], A375 cells were pretreated with various inhibitors (dynasore 30 μM, chlorpromazine 12.5 μM, filipin 5 μg/ml, methyl-β-cyclodextrin 1.3 mg/ml, amiloride 100 μM and wortmannin 100 nM) for 30 min and followed by incubation with RGD-P-Ce6 (100 nM of Ce6) in the presence of the inhibitors for 4 h. After washing with PBS, the cells were trypsinzed and the Ce6 fluorescence intensity was measured using flow cytometry. To determine whether the cellular uptake of RGD-P-Ce6 is energy dependent, A375 cells were incubated with RGD-P-Ce6 (100 nM) for 4 h at 4 °C and the cellular uptake was compared to that at 37 °C.

2.4. Intracellular trafficking of RGD-P-Ce6

Live cell confocal microscopy was performed to examine subcellular distribution of the targeted nanoconjugates. After cultured in glass-bottom dishes overnight, A375 cells were treated with RGD-P-Ce6 (400 nM of Ce6) for 6 h. The cells were then treated with lysosomal marker Lysotracker Green DND-26 (75 nM), ER tracker green (1 μM) or transferrin-Alexa 488 (25 μg/ml) for 1 h. After washing twice with PBS, images were captured using an Olympus FV1200 confocal microscope.

2.5. Intracellular singlet oxygen detection after photo-irradiation

After incubated with RGD-P-Ce6 (75 nM) for 12 h, A375 cells were further incubated with 10 μM CM-H_2DCFDA (Life Technologies) for 60 min and were irradiated with a 660 nm laser (3.5 mW/cm^2) for 0, 10, 20 or 30 min. After that, the cells were harvested and the fluorescence of the cells was detected by flow cytometry (FITC channel) to measure the intracellular ROS level [22]. In addition, the intracellular ROS was also detected by observing DCF using confocal microscopy.