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Photodynamic Therapy With Hyperbranched Poly(Ether-Ester) Chlorin(e6) Nanoparticles On Human Tongue Carcinoma Cal-27 Cells

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Abstract

Background—Hyperbranched polymers represent a new class of drug delivery vehicle that can be used to prepare nanoparticles with uniform size distribution.

Methods—In this study we prepared covalent conjugates between the photosensitizer chlorin(e6) and hyperbranched poly(ether-ester), HPEE. HPEE-ce6 nanoparticles were synthesized by carbodiimide-mediated reaction between HPEE and ce6, and characterized by ultraviolet-visible absorption spectroscopy (UV-Vis), and transmission electron microscopy (TEM). The uptake and phototoxicity of HPEE-ce6 nanoparticles towards human oral tongue cancer CAL-27 cells was detected by confocal laser scanning microscopy (CLSM) and MTT assay respectively.

Results—The absorption peak of HPEE-ce6 nanoparticles was red-shifted 12-nm compared with ce6, and TEM showed uniform nanoparticles with a diameter of 50-nm. HPEE-ce6 nanoparticles were taken up by CAL-27 cells after 4 hour incubation and localized in the cytoplasm. The MTT assay showed a significantly ($P < 0.05$) higher phototoxicity compared to free ce6 after 12 J/cm² of 660-nm laser illumination.

Conclusions—This is the first time to our knowledge that hyperbranched polymers have been used in PDT drug delivery.

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Keywords

Photodynamic therapy (PDT); Chlorin e6; hyperbranched poly(ether-ester) (HPEE); drug delivery system (DDS); nanoparticles; transmission electron microscopy

INTRODUCTION

Photodynamic therapy (PDT) is an emerging approach to treat cancer and other diseases involving unwanted tissue [1]. PDT has received approval for treating esophageal, lung, head and neck, skin, bladder and other cancers [2]. PDT uses a photosensitizer (PS) molecule that absorbs laser (or other light) and forms a long-lived excited state that can transfer energy to ground state oxygen producing reactive oxygen species (ROS), such as singlet oxygen and hydroxyl radical that can exert a cytotoxic effect [3]. Singlet oxygen is generally considered to be the predominant active ROS during PDT [4]. PDT shows selectivity because (a) the PS is selectively accumulated in the tumor, and (b) the lesion site is specifically illuminated therefore sparing surrounding normal tissue from damage.

One limitation of many porphyrin PS that are currently used for PDT is their spectral absorption below 650 nm; this wavelength of light can only penetrate millimeters of soft tissue. The optimum wavelengths for increased transmission depth in human tissue are in the far-red and infrared regions (wavelength of 650 ~ 900 nm), and moreover the ideal PS molecule should have a large absorption peak while porphyrins have very small peaks. Chlorin e6 has a relatively large absorption peak at 660-nm and this makes it a good candidate for PDT for cancer.

To date, most of the available PS are hydrophobic and insoluble in water; this fact necessitates some formulation be developed for drug delivery. In addition, the selectivity PS accumulation in tumors for clinical applications is sub-optimal. Recently, workers have reported a variety of nanoparticle delivery platforms for PS in an attempt to resolve these deficiencies [5–7]. PS can be associated with nanoparticles in different ways (such as adsorption, covalent bonding, non-covalent bonding, encapsulation, etc.). Many nanoparticles are hydrophilic and compatible with aqueous environments, and can passively accumulate in tumors through the enhanced permeability and retention (EPR) effect due to the leaky vasculature and poor lymphatic drainage that are characteristic of tumors [8]. Furthermore the nanoparticles can also be actively targeted to tumors by attaching monoclonal antibodies [9], proteins [10], peptides [11] or other molecules [12] that serve as tumor specific ligands.

Hyperbranched poly(ether ester) (HPEE) belongs to a new class of macromolecule with a highly branched topological structure [13]. Its exterior possesses many hydroxyl and carboxyl functional groups available for functionalization, including the covalent attachment of drug molecules. In addition, HPEE has good biocompatibility and has a biodegradable nature; therefore it could be an ideal PDT drug carrier. In this study we report the synthesis and characterization of nanoparticles consisting of HPEE attached to chlorin e6 and the interaction and PDT effects with CAL 27 human oral cancer cells.

MATERIALS AND METHODS

HPEE

The preparation of the hyperbranched polymer HPEE has been described in detail [13]. Briefly glycidyl methacrylate was co-polymerized with oligo(ethylene glycol) in the presence of potassium hydride in dimethyl sulfoxide solvent at 80°C for 24 hour under argon

atmosphere. Purification was achieved by repeated precipitation in methanol/diethyl ether mixtures. Characterization was by ¹H and ¹³C NMR (400 MHz). Molecular weight and MW distribution was done by gel permeation chromatography connected to a DAWN multi-angle laser light scattering instrument (Wyatt Technology). The HPEE preparation used in the present study had a number average MW of 9200 and a polydispersity value (weight average MW divided by number average MW) of 2.84. The degree of branching was 0.47.

Synthesis of HPEE-ce6 conjugate

The HPEE-ce6 conjugate was prepared by the reaction of HPEE and ce6 in DMSO solution. DMSO solution was dried over Ca(OH)₂ at 40°C for 24 h and then distilled under reduced pressure at 80°C. Calculated amounts of HPEE (500 mg), ce6 (200 mg), dicyclohexylcarbodiimide (412 mg), N-hydroxysuccinimide (230 mg) and 4-dimethylaminopyridine (214 mg) were dissolved in DMSO. All reactions were carried out in a dark room. The mixture was stirred at 50°C for 72 h. Then, ether was added and the resulting precipitate was dialyzed against distilled water for 24 h to remove solvents and reagents. The remaining insoluble material including free ce6 and other impurities was filtered by centrifugation and discarded. The supernatant was collected and rotary evaporated, and further dried under vacuum at 50°C to constant weight. Finally, the HPEE-ce6 conjugate was obtained. Based on ¹H NMR and UV-Vis techniques, the ce6 content in the HPEE-ce6 conjugate was calculated to be 4.8% by weight. Based on the HPEE MW of 9200 this is equivalent to approximately 0.9 ce6 molecules per

Characterization of HPEE-ce6

UV-visible (UV/Vis) spectra were obtained on an Evolution 300 UV-Vis spectrophotometer (Thermo-Fisher) measured over a wavelength range of 200–800 nm. TEM morphology analysis was carried out in the Analysis and Test Center, Shanghai Jiaotong University using an electron microscope (model JEOL JEM-2010) fitted with a CCD camera (model Gatan 832). The samples were fixed with 3% phosphotungstic acid (50:1 mixture), and placed on copper mesh (Quantifoil Holey carbon films, R1.2/1.3), and dried.

Cell Culture Reagents

DMEM medium (high glucose), fetal calf serum, non-essential amino acids, streptomycin, 0.25% trypsin –0.02% EDTA, MTT, 24 and 96-well plates and other biological reagents and materials came from the Shanghai Ninth People's Hospital laboratory of oral cancer. Cell culture coverslips (13 mm) were purchased from Shanghai Biochemical Technology Co., Ltd. Yuen-chi China

Cell culture

The CAL-27 cell line was provided by the Shanghai Jiao Tong University School and Ninth People's Hospital biological laboratories. Cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum. Cells were cultured at 37°C, 5% CO₂, 90% relative humidity in an incubator (SANYO MCO-17A, Japan SL Shel LAB), and replacing culture medium every 2 to 3 days. Logarithmic growth phase cells were seeded in 24-well or 96-well plates, cultured for 24 hours until the cells attached for confocal laser scanning fluorescence microscopy or cell toxicity tests.

Laser

The light source was a 660-nm diode laser, (Model TSM-1 Made in China) used in continuous wave mode and adjustable power with a maximum power output of 2W.

Confocal laser scanning microscopy (CLSM) observation of cells

After 48-hour culture CAL-27 cells were detached with 0.25% trypsin, and a cell suspension, of 2×10^5 cells/mL seeded in 24-well plate (1 mL/well) and allowed to attach for 24 hours. Medium was aspirated and replaced with 1 mL fresh serum-free DMEM medium containing 100 μ M HPEE-ce6 nano-PS. After 4 hours incubation DAPI (500 ng/mL) was added for 10 minutes, the PS was aspirated, washed 3 times with 1.5 mL PBS and fixed with 4% paraformaldehyde. A confocal laser scanning microscope (Zeiss LSM10) was used to observe cell morphology and cell fluorescence using 40X objective. For DAPI excitation was 364-nm and a band-pass filter of 435–485-nm and for ce6 the excitation was 543-nm with a band-pass filter of 558–719-nm.

Photodynamic Therapy

Different experimental groups consisted of HPEE-ce6, free ce6 and control groups. A cell suspension of 200 μ L of 4×10^4 cells/mL was inoculated into a 96-well plate. All manipulations were carried out in dark conditions. A concentration gradient was constructed with five wells each containing ce6 equivalent concentrations of 1, 5, 10, 15, 25, 50, 100 and 150 μ M and incubated for 4 hours. Then cells were washed with PBS, and each well replaced with 200 μ L growth medium. Light delivery consisted of 660-nm semiconductor laser irradiation, with a power density of 100 mW/cm² and an energy density of 12 J/cm², with a spot arranged evenly over the wells of the 96-well plate. The exposure time was 600 seconds plus a second dark control plate that received no light.

After PDT the plates were returned to incubator for 24 hours and then was added 20 μ L well of 5 mg/mL of MTT solution (prepared with PBS) and incubated for 4 hours. Wells were then aspirated and 200 μ L of DMSO added with shaking for 5–10 minutes to dissolve the purple crystals. Plates were read at 490 nm in a microplate reader (SpectraMaxM3, Molecular Devices) to record the optical density and corrected for blank absorbance (wells without cells). Cell survival rate was calculated from following formula: Cell viability = OD value of experimental group/control group OD value $\times$ 100%. Each experiment was repeated three times and averaged.

Statistical analysis

The SAS 6.12 software package was used to compare values of MTT cytotoxicity and perform t test. When $P < 0.05$ the difference was statistically significant.

RESULTS

HPEE and chlorin(e6) covalently bound to form nano-PS

The use of hybrid polymerization HPEE [13], allows structures to be prepared as shown in Figure 1-A. Due to the hyperbranched structure with a large number of ether bonds and ester bonds, it has both good biocompatibility and biodegradability. HPEE has many free hydroxyl groups that can be esterified with the carboxyl groups of chlorin(e6) (Fig. 1-B), producing HPEE-ce6 nanoparticles (Figure 1-C).

TEM observation of HPEE-ce6 nanoparticles

The HPEE-ce6 nanoparticles can be visually observed by transmission electron microscopy of the particle size and morphology. Figure 2 shows that HPEE-ce6 nanoparticles were round or oval shaped and uniformly dispersed, with a particle size of approximately 50 nm.

UV-visible absorption spectra PS in PBS

The absorption spectra of HPEE-ce6 and ce6 in PBS are shown in Figure 3. Most of the peaks are similar for the two species although the Q-band of ce6 is around 657 nm while the HPEE-ce6 is at around 669 nm (red-shift of 12 nm). Both peaks are relatively strong and the absorption intensity is basically the same.

Confocal laser scanning microscope (CLSM) observation of CAL-27 cells incubated with HPEE-ce6

We used a confocal laser-scanning microscope to observe the CAL-27 cell uptake and the intracellular localization of the HPEE-ce6. CAL-27 cells were incubated for 4 hours with 100 μ M HPEE-ce6 and for 10 min with x μ M DAPI. Figure 4A shows the gray scale image, 4B is the image of red ce6 fluorescence and 4C is the blue nuclear fluorescence from DAPI. The overlap (Fig 4D) shows that ce6 is located outside the nucleus inside the cytoplasm and is present in all the cells. Uptake of HPEE-ce6 by CAL-27 is likely to occur by endocytosis that will result in the fluorescence being present in endocytic vesicles in the cytoplasm.

Comparison of HPEE-ce6-PDT and ce6-PDT on CAL-27 cells

In order to compare the PDT killing activity of HPEE-ce6 nano-PS with the PDT activity of free ce6-PDT it was necessary to vary the concentration of ce6 equivalent and use a single fluence of light. Figure 5 shows the cell survival obtained after a 4-hour incubation and subsequent exposure to 12 J/cm² of 660-nm laser light delivered at an irradiance of 100 mW/cm². The results indicate that the CAL-27 cell survival after PDT with HPEE-ce6 was significantly lower than the survival obtained with the free ce6 (about 3–4 times less) demonstrating a more powerful photodynamic destruction. The difference was statistically significant ($p < 0.05$) at all ce6 concentrations from 5 μ M upwards.

DISCUSSION

Hyperbranched polymers are a relatively new class of drug-delivery vehicle [14]. The term was coined by Kim and Webster from Du Pont Research in 1992 [15]. Hyperbranched polymers have a very large number of branches compared to linear or lightly branched polymers, but do not have the maximum number of branches that is only found in dendrimers. Hyperbranched polymers are characterized by a degree of branching (DB), which represents the percentage of dendritic and terminal monomers among the total monomers in the polymer:

$$DB = D + T / (D + T + L).$$

DB is usually about 0.45–0.7 for hyperbranched polymers while for dendrimers it is 1. They can be prepared in several ways, but most commonly from AB₂ monomers or from combining A₂ and B₃ monomers. When prepared from AB₂ monomers, gelation does not occur. The polymer molecules have a single “A terminus” (the focus) and many “B termini” (the chain ends). Statistical analysis show that batch polymerization of AB₂ monomers gives a DB of ~0.5. Higher degrees of branching can be obtained using special reaction conditions (slow addition of monomer, addition of a core).

In HPEE the functional groups at the end of the branches are hydroxyl groups and since chlorin e6 has free carboxyl groups, the hydroxyl and carboxyl groups can be joined by an esterification reaction, and by controlling the reaction conditions, we can easily obtain chlorin e6 and HPEE covalent cross-linking into nanoparticles (Figure 1). Figure 2 shows

the particles have an average size of about 50 nm, and a small degree of dispersity or in other words a good uniformity of size distribution.

The optimum transmission of human tissue is located in the red and near-infrared spectral region (wavelength of 620 ~ 900 nm), therefore, the preferred absorption peak wavelength of the PS should be located in the same wavelength region. Chlorin e6 has a strong absorption band at 660-nm. Even though the molecule has three carboxyl groups, chlorin(e6) is not water soluble at pH=7.4. It can be induced to dissolve by initially solubilizing ce6 in basic solution (pH>10) and then reducing the pH with buffer until 7.4 is approached, but the aggregation of the ce6 becomes a serious problem after spending some time at pH=7.4. Therefore innovative methods are being sought to solubilize ce6 in water and at the same time to reduce its tendency to aggregate in order to produce a pharmaceutically acceptable formulation. One such drug-delivery method has been reported to be the non-covalent complex formed between ce6 and polyvinylpyrrolidone (PVP) with a weight-average molecular weight of 12,000 known as Fotolon [16]. The efficacy of Fotolon as a PS to mediate photodestruction of cancer cells is significantly higher than that of the free ce6 [17].

In recent years the number of reports of nanoparticles of various kinds being used as delivery vehicles for PS in PDT has increased at a remarkable rate [18, 19]. Classification systems for different kinds of nanoparticles have been devised [6]; nanoparticles can be passive carriers of the PS or can participate in the actual photochemical reaction e.g. quantum dots; they can either be actively targeted to cancer cells by attachment of ligands or passively targeted to tumors by the EPR effect; they can either be or non-biodegradable etc. In our case the HPEE-ce6 nanoparticles would be biodegradable passively targeted carriers of PS. There have been several reports that PS-loaded nanoparticles (particularly those with covalent bonds between the nanoparticle and the PS) are taken up into cells by the process of endocytosis [20]. Our results suggested that CAL-27 cells also took up the HPEE-ce6 nano-PS by an endocytosis mechanism. It should be noted that the cellular uptake of nanoparticle bound PS is one-way in character, in other words it is not easy for the PS to diffuse out of the cells or expelled by exocytosis. The only way for the PS to leave the cells once it has been taken up, is for the biodegradable nanoparticle to be degraded by hydrolytic enzymes present in lysosomes thus releasing the free ce6 that could then diffuse out of the cell.

The greater effectiveness of the HPEE-ce6 nanoparticles in PDT cell killing compared to the free ce6 could have several possible explanations. In our opinion the most likely explanation is that the uptake of ce6 calculated on a number of molecules per cell basis was higher when delivered by the nanoparticle vehicle than when delivered as free ce6 solution. Several laboratories have suggested that this greater PS uptake was the explanation of their observations that different nanoparticle-based PS were more effective than the equivalent free PS in solution [21–23]. A second possible explanation is that the disaggregating effect of covalently binding ce6 to a hydrophilic polymer played a role in enhancing the PDT activity of each ce6 molecule. It is well known that the undesirable process of PS aggregation is highly effective in reducing the quantum yield of reactive oxygen species produced during PDT by self quenching between PS molecules [24]. It been reported that a nanoparticle-bound PS is less aggregated and more fluorescent and photoactive than its free equivalent [21]. The third possibility is that delivery of PS in a nanoparticle-bound form could lead to its localization in a more sensitive sub-cellular site and this consideration was suggested as a possible explanation of the increased effectiveness of nanoparticle-bound methylene blue [25].

It has also been reported that nanoparticle-bound PS are effective as PS in vivo. systems such as chick embryos [26], subcutaneous mouse tumors [27], intraperitoneal ovarian cancer in rats [28], and human lung cancer xenografts in mice [29] have all been employed to demonstrate enhanced efficacy of nanoparticle delivered PS.

In summary, HPEE-ce6 nanoparticles are capable of simple preparation and demonstrate improved in vitro PDT activity over free ce6. This is the first time to our knowledge that hyperbranched polymers have been used in PDT drug delivery. They may have particular application for treatment of superficial oral cancer or precancerous lesions,

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Figure 1. Preparation of HPEE-ce6 nanoparticles
Chemical structures of (A) HPEE; (B) ce6; (C) HPEE-ce6

Figure 2. TEM images of HPEE-ce6 nanoparticles
Scale bar is 200-nm.

Figure 3.
The UV/Vis absorption spectra of free ce6 and HPEE-ce6 nanoparticles.

Figure 4. CLSM images of CAL-27 cells incubated with HPPE-ce6 nano-particles for 4 h
(A) Grayscale image of CAL-27 cells. (B) Red fluorescence from HPPE-ce6. (C) Blue nuclear fluorescence from DAPI. (D) Overlaid images from B and C. Scale bar is 100 μm .

Figure 5. Cell survival of CAL-27 treated with PDT mediated by HPEE-ce6 nanoparticles and with free ce6

Cells were incubated with PS for 4 hours, exposed to 12 J/cm^2 of 660-nm laser light delivered at 100 mW/cm^2 . After a further 24 hour incubation a 4-hour MTT-assay was performed.