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Preparation and sonodynamic activities of water-soluble tetra-α-(3-carboxyphenoxyl) zinc(II) phthalocyanine and its bovine serum albumin conjugate

He-Nan Xu [1], Hai-Jun Chen [1], Bi-Yuan Zheng, Yun-Quan Zheng, Mei-Rong Ke, Jian-Dong Huang *

College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou, China

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ABSTRACT

Sonodynamic therapy (SDT) is a new approach for cancer treatment, involving the synergistic effect of ultrasound and certain chemical compounds termed as sonosensitizers. A water-soluble phthalocyanine, namely tetra-α-(3-carboxyphenoxyl) zinc(II) phthalocyanine ($ZnPcC_4$), has been prepared and characterized. The interactions between $ZnPcC_4$ and bovine serum albumin (BSA) were also investigated by absorption and fluorescence spectroscopy. It was found that there were strong interactions between $ZnPcC_4$ and BSA with a binding constant of $6.83 \times 10^7\ M^{-1}$. A non-covalent BSA conjugate of $ZnPcC_4$ ($ZnPcC_4$–BSA) was prepared. Both $ZnPcC_4$ and $ZnPcC_4$–BSA exhibited efficient sonodynamic activities against HepG2 human hepatocarcinoma cells. Compared with $ZnPcC_4$, conjugate $ZnPcC_4$–BSA showed a higher sonodynamic activity with an IC_{50} value of 7.5 μM. Upon illumination with ultrasound, $ZnPcC_4$–BSA can induce an increase of intracellular reactive oxygen species (ROS) level, resulting in cellular apoptosis. The results suggest that the albumin conjugates of zinc(II) phthalocyanines functionalized with carboxyls can serve as promising sonosensitizers for sonodynamic therapy.

1. Introduction

Sonodynamic therapy (SDT) is a new approach for cancer treatment, which was developed on the basis of photodynamic therapy (PDT) [1–3]. PDT utilizes the combined action of photosensitizer, light, and molecular oxygen to eradicate cancer cells [4,5]. However, clinical application of PDT is limited to the treatment of superficial and small solid tumors due to the poor tissue penetration of excitation light [6]. Similarly to PDT, SDT utilizes the combined action of a sonosensitizer and low-intensity ultrasound to cause cell and tissue damage. Ultrasound has an appropriate tissue attenuation that lets it penetrate and reach deep-seated tissues without losing ability to focus energy into small volume. This unique advantage makes SDT more efficient for noninvasive treatment of deep-seated tumors compared with PDT [7–10]. Accumulating evidences indicate that SDT has a great potential in cancer therapy [11–15].

The overall efficacy of sonodynamic therapy depends greatly on the behavior of the sonosensitizer. To date, the most common sonosensitizers are porphyrin derivatives such as hematoporphyrin (the first generation photosensitizer), portoporphyrin IX, and ATX-70 (a gallium porphyrin complex) [7,16]. Nonetheless, these porphyrin-based sonosensitizers have some shortcomings such as skin photosensitivity and unsatisfactory sonocytotoxicity. Significant efforts have therefore been put in new sonosensitizers which have better sonochemical properties, higher tumor specificity, and less skin photosensitivity.

Phthalocyanines have been proposed as highly promising photosensitizers over the last three decades, owing to their intense absorption in the red visible region, high efficiency to generate reactive oxygen species, low dark toxicity, and low skin photosensitivity [17–19]. However, the use of phthalocyanines as sonosensitizers for SDT remains relatively little studied [20–23]. Herein, we reported the preparation and *in vitro* sonodynamic activities of a tetra-α-(3-carboxyphenoxyl) zinc(II) phthalocyanine ($ZnPcC_4$) and its bovine serum albumin conjugate ($ZnPcC_4$–BSA). Zinc(II) phthalocyanines functionalized with carboxyl(s) have been used as efficient photosensitizers, due to their water solubility and high singlet oxygen yield [24–26]. However, the sonodynamic activity of this type of phthalocyanine has not been investigated. In addition, despite recent advances using serum albumin as the protein carrier for anticancer drugs or photosensitizers to improve their

* Corresponding author. Tel.: +86 591 22866235; fax: +86 591 22866227.
E-mail address: jdhuang@fzu.edu.cn (J.-D. Huang).
[1] These authors contributed equally to this work.

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passive targeting properties [27–29], to the best of our knowledge, the application of serum albumin for delivery of sonosensitizer has no precedents in the literature.

2. Experimental

2.1. General

All the reactions were performed under an atmosphere of nitrogen. *N*,*N*-dimethylformamide (DMF) was dried over molecular sieves and further distilled under reduced pressure before use. Potassium carbonate was activated by muffle at 300 °C under normal pressure. Chromatographic purifications were performed on silica gel columns (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd., China) with the indicated eluents. Bovine serum albumin was purchased from Sigma–Aldrich Co. All other solvents and reagents were of reagent grade and used as received.

^{1}H NMR spectra were recorded on a Bruker AVANCEIII 400 spectrometer (400 MHz). Chemical shifts were relative to internal $SiMe_4$ (δ = 0 ppm). Mass spectra were recorded on a Finnigan LCQ Deca xpMAX mass spectrometer. IR spectra were recorded on a Perkin-Elmer SP2000 FT-IR spectrometer, using KBr disks. Elemental analyses were performed by Element Vario EL III equipment. Electronic absorption spectra were measured on a Shimadzu UV-2450 UV–vis spectrophotometer. Fluorescence spectra were taken on an Edinburgh FL900/FS900 spectrofluorometer. The fluorescence quantum yields (Φ_F) were determined by the equation: $\Phi_{F(sample)} = (n^2_{sample}/n^2_{ref})\cdot(F_{sample}/F_{ref})\cdot(A_{ref}/A_{sample})\,\Phi_{F(ref)}$, where *F*, *A*, and *n* are the measured fluorescence (area under the emission peak), the absorbance at the excitation position, and the refractive index of the solvent, respectively. Unsubstituted zinc(II) phthalocyanine (ZnPc) in DMF [$\Phi_{F(ref)}$ = 0.28] was used as the reference [30]. The singlet oxygen quantum yields (Φ_Δ) was measured in DMF by a steady-state method using 1,3-diphenylisobenzofuran (DPBF) as the scavenger and ZnPc [$\Phi_{\Delta(ref)}$ = 0.56] as reference [31,32].

2.2. Synthesis of $ZnPcC_4$

2.2.1. 3-(3-carboxyphenoxyl)phthalonitrile (α-C)

A mixture of 3-nitrophthalonitrile (0.87 g, 5 mmol), 3-hydroxybenzoic acid (0.69 g, 5 mmol), and anhydrous K_2CO_3 (2.07 g, 15 mmol) in dry DMSO (20 mL) was stirred at room temperature for 24 h under nitrogen atmosphere. The reaction mixture was filtered by sand core funnel and the filtrate was poured into ice water (200 mL). Subsequently, HCl aqueous solution (2 M) was added to the filtrate until pH = 1–3 to give white precipitate, which was collected by filtration, washed with water until pH = 7 and dried in vacuum. The crude product was purified by recrystallization with DMF/water to afford white solid (1.11 g, 84%). R_f = 0.53 (EtOH). IR (KBr, cm^{-1}): 3078.4 (Ar–H); 1585.6, 1466.7, 1450.9 (C=C, Ar); 1303.7, 1278.7, 1208.9 (Ar–O–Ar); 2232.9 (C≡N); 1688.8 (C=O). MS (ESI): m/z 263.1 $[M-H]^-$. ^{1}H NMR (DMSO-d_6, ppm): δ 13.16 (br., 1H), 7.81–7.88 (m, 3H), 7.62–7.67 (m, 2H), 7.52 (t, *J* = 0.6 Hz, 1H), 7.32 (d, *J* = 4.2 Hz, 1H). Anal. Calcd for $C_{15}H_8N_2O_3$: C, 68.18; H, 3.05; N, 10.60. Found: C, 67.99; H, 3.27; N, 10.86.

2.2.2. 1,8(11),15(18),22(25)-tetrakis-(3-carboxyphenoxyl) zinc(II) phthalocyanine ($ZnPcC_4$)

A mixture of 3-(3-carboxyphenoxyl)phthalonitrile (0.26 g, 1.0 mmol) and K_2CO_3 (0.14 g, 1.0 mmol) in n-pentanol (20 mL) was stirred at 90 °C under nitrogen atmosphere for 15 min, and then zinc acetate (0.11 g, 0.6 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.4 mL, 2.6 mmol) were added. The mixture was heated to reflux at 130 °C for 10 h. The solvent was then removed in vacuum and the residue was treated with water and acidified with HCl aqueous solution (1 M) to induce precipitation. The precipitate was collected by filtration, washed with water until pH 7, and dried in vacuum. The crude product was subjected to column chromatography using DMF/ethyl acetate (1:3, v/v) and then DMF as eluents. The last green fraction was collected, and dried in vacuum to give dark green solid (0.10 g, 37%). R_f = 0.67 (MeOH). IR (KBr, cm^{-1}): 3273.8 (O–H); 1702.1 (C=O); 3065.5 (Ar–H), 1577.3, 1481.1, 1440.2 (C=C, Ar); 1247.2 (Ar–O–Ar); 1129.7 (C–N). MS (ESI): m/z 1121.2 $[M+H]^+$. ^{1}H NMR (DMSO-d_6, ppm): δ 9.10–9.28 (m, 2H), 8.68–8.75 (m, 1H), 8.55 (t, *J* = 8.2 Hz, 1H), 8.20 (t, *J* = 7.6 Hz, 1H), 8.07 (t, *J* = 7.6 Hz, 2H), 7.84–7.92 (m, 4H), 7.63–7.73 (m, 17H). Anal. Calcd for $C_{60}H_{32}N_8O_{12}Zn$: C, 64.21; H, 2.87; N, 9.98. Found: C, 64.03; H, 3.01; N, 9.82.

2.3. Preparation of phthalocyanine-BSA conjugate

The non-covalent BSA conjugate of $ZnPcC_4$ was prepared according to the literature procedure [33]. The conjugate was obtained by stirring a mixture of $ZnPcC_4$ and BSA (molar ratio: $ZnPcC_4$/BSA = 2/1) in a phosphate buffered saline (PBS) at ambient temperature overnight, followed by gel chromatography on a G-100 Sephadex column using deionized water as eluent. The conjugate $ZnPcC_4$–BSA collected as the first blue fraction was lyophilized to remove water. The protein content in the conjugate was calculated from the absorbance at 280 nm in a diluted PBS solution (pH = 7.4) with reference to the corresponding molar absorptivity of BSA ($\varepsilon = 4.85 \times 10^4\ M^{-1}\ cm^{-1}$). The phthalocyanine concentration was calculated from the Q band absorbance in a diluted DMF solution with reference to the corresponding molar absorptivity ($\varepsilon = 2.19 \times 10^5\ M^{-1}\ cm^{-1}$).

2.4. In vitro studies

For *in vitro* studies, $ZnPcC_4$ was first dissolved in DMF (1.0 mM) and the solution was diluted to 80 μM with 0.5% (wt.) aqueous solution of Cremophor EL (Sigma, 0.5 g in 100 mL of water). $ZnPcC_4$–BSA conjugate was dissolved in PBS with a concentration of 80 μM. BSA was also dissolved in PBS with a concentration of 80 μM. These solutions were clarified with 0.45 μm filter, and then diluted with the cellular culture medium (as described below) to appropriate concentrations.

Human hepatocellular carcinoma HepG2 cells were obtained from the cell bank of the Chinese Academy of Science, Shanghai, China. The cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, streptomycin (50 μg mL^{-1}), and penicillin (50 units mL^{-1}). The cells were incubated at 37 °C in a humidified CO_2 (5%) incubator, and the medium was refreshed every 1–2 days. Cells in the exponential phase of growth were used in the following experiments.

2.4.1. Sonodynamic activity assay

HepG2 cells were exposed to ultrasound after an incubation of 45 min with $ZnPcC_4$–BSA, $ZnPcC_4$, and BSA, respectively. The experimental set-up for ultrasound exposure is showed in Fig. 1. The transducer with a diameter of 45 mm was submerged in the stainless steel container filled with cold degassed water. Polystyrene tube containing 0.5 mL of cell suspension (2×10^5 cells mL^{-1} in RPMI 1640 medium) was fixed vertically on the focal area of the transducer. The distance between the bottom of the polystyrene tube and the transducer was 1 cm. The spatial average ultrasonic intensity was 2.0 W cm^{-2} with a frequency of 1.0 MHz in continuous waves and the ultrasonic time was set at 3 min. The ultrasound system (Therapy Ultrasound 4150) was manufactured by the CARCI Company. For all experiments, the cold degassed water was used as the ultrasonic coupling medium, thereby reducing