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Visible light-induced biocidal activities and mechanistic study of neutral porphyrin derivatives against *S. aureus* and *E. coli*

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ABSTRACT

Positive charged porphyrins have long been regarded as effective biocidal agents, however neutral porphyrins have rarely been studied in their ability photoinactivating microbials, and the structure-activity relationship such as correlation of electronic effect and biocidal activity of porphyrins still remains unclear. Herein, four neutral porphyrins with various electronic effects were selected to undergo light-induced biocidal processes. It turned out that the TPPOH and TPPNH$_2$ with electron-donating groups $-NH_2$ and $-OH$, respectively, exhibited much more powerful light-induced biocidal activities against *E. coli* and *S. aureus* than TPP and TPPNO$_2$ with electron-withdrawing group $-NO_2$. This phenomenon suggested that neutral porphyrins may be treated as a new class of biocidal agents and functional groups with various electronic effects on porphyrins can dramatically affect porphyrins' light-induced biocidal activities. Mechanistic studies demonstrate that despite a better light-induced antibacterial ability of TPPOH, its singlet oxygen generation efficacy is a little lower than that of TPPNH$_2$, together with charge characteristics and lipophilicity, it is clear that (1) the oxidative species singlet oxygen and ROS played the key role in the photo-activated antimicrobial processes of porphyrins, and (2) higher singlet oxygen or ROS yields of TPPOH and TPPNH$_2$ may originate from their structural characteristics, namely electron-donating groups $-OH$ or $-NH_2$, and (3) a synergistic effect of all other factors including the electrostatic and hydrophobic effects must involve in the process and cooperatively determine their biocidal activities.

1. Introduction

Every year millions of people died of bacterial infections. A bacterial infection caused great threat to the public health, also caused large social cost to society. Based on the data from acute care hospitals according to the U.S. centers for disease control and prevention, about 722,000 people infected in hospitals in 2011, and about 75,000 hospital patients died during their hospitalizations [1]. Despite antibiotics saving lives of many people, a realistic problem also should not allow to be ignored that a variety of bacteria have developed resistance to antibiotics owing to the abuse of antibiotics. Even more alarming is the appearance of multi- or pan-resistant Gram-negative strains with extended spectrum β-lactamase and the New Delhi metallo-β-lactamase resistance [2]. More recently, multidrug-resistant *Acinetobacter baumann* and *Pseudomonas aeruginosa* have become a major cause in controlling infectious diseases [3]. Against this threat, it is necessary to develop new antimicrobial strategies. For years, photodynamic inactivation (PDI) of pathogens as an alternative strategy to deactivation of bacteria has attracted tremendous and intensive research interest due to its significant advantages in biocidal efficacy, no bacterial resistance developed, controllable, recyclable and green over traditional biocides [4–6]. PDI is dependent on light, oxygen, photosensitizer (PS) and with the following mechanism: upon irradiation of light, PS absorbs light and leads to its excitation and energy transfer, then ultimately produces strong oxidizing reactive oxygen species to inactivate pathogens [7]. Gram-positive and Gram-negative bacteria exhibit fundamentally different susceptibility to PS due to their distinct physiology [8].

A variety of PS has been developed over the past decades [9–11]. Among those promising PS, porphyrins, which have been found to be endogenous in cells, have emerged as a major type of photodynamic agents because porphyrins are able to be activated by visible light to perform cytotoxicity to certain bacterial species [12–15]. So far, a few photodynamic agents with porphyrin backbone have been approved for

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treating cancers and other diseases [16]. Porphyrin has several significant characteristics: (1) it owns the characteristics of both type I and type II photo processes [17] meaning that porphyrin can produce both reactive oxygen species (ROS) by electron transfer of the triplet state of PS followed by reacting with oxygen (type I), and high quantum yield of singlet oxygen ($^1O_2$) by energy transfer of the triplet state of PS to ground state oxygen ($^3O_2$) (type II) [18–20], (2) porphyrin is highly photostable, and is not prone to photobleaching, (3) the photophysical and biocidal properties of porphyrin is tunable by introducing side chains or metal ions into its structure [12]. These properties make porphyrin an attractive antimicrobial agent. Hence, intensive research efforts have been made to enhance porphyrin's biocidal ability. Up to date, positive charged porphyrins have long been regarded as effective biocidal agents, however neutral porphyrins have rarely been studied in their light-induced biocidal abilities, and the structure-activity relationship, such as correlation of electronic effect and biocidal activity of porphyrins, still remains unclear [21]. Additional reports observed that lipophilicity may also play an important role in cytotoxicity of porphyrins [22, 23].

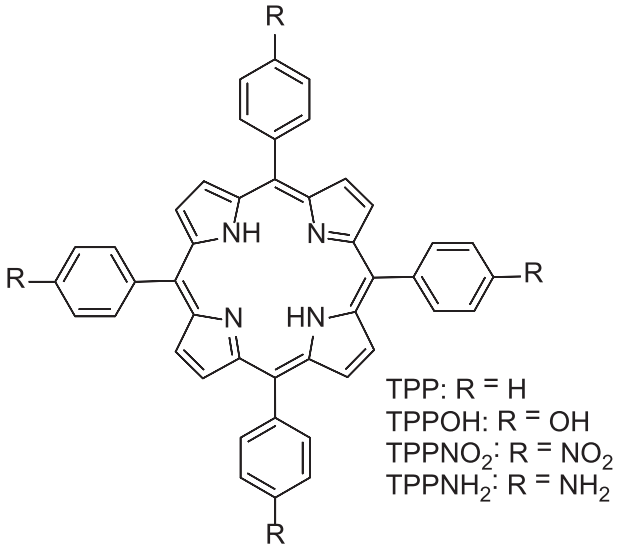
Herein, four neutral porphyrins with various electronic effects were selected to undergo light-induced biocidal processes. It showed that TPPOH (4,4′,4″,4‴-(porphyrin-5,10,15,20-tetrayl)tetraphenol) and $TPPNH_2$ (4,4′,4″,4‴-(porphyrin-5,10,15,20-tetrayl)tetraaniline) with electron-donating groups $-NH_2$ and $-OH$, respectively, exhibited much more powerful light-induced biocidal activities against *E. coli* and *S. aureus* than TPP (5,10,15,20-Tetraphenylporphyrin) and $TPPNO_2$ (5,10,15,20-tetrakis(4-nitrophenyl)porphyrin) with electron-withdrawing group $-NO_2$. This phenomenon indicated that neutral porphyrins may function as a new class of biocidal agents and functional groups with various electronic effects on porphyrins can dramatically affect porphyrins' light-induced biocidal activities. In addition, in order to illuminate the biocidal mechanism of these porphyrins further, their ability of singlet oxygen generation, bacterial membrane perturbations, morphological damages, photostability and the lipophilicity were studied. To the best of our knowledge, there is no such comparison study made, nor study about the potential of these neutral porphyrins as biocide reported so far.

2. Experimental

2.1. Material and Methods

All porphyrin derivatives (Scheme 1), intermediates, 5(6)-Carboxyfluorescein (hereafter referred to as fluorescein), and culture media were purchased from Sigma Aldrich. 1,2-Dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DOPG), *E. coli* total lipid, and cholesterol were purchased from Avanti Polar Lipids. Superfine Sephadex G-25 was purchased from GE Healthcare Bio-Science. All of the solvents were HPLC grade and purchased from Honeywell and used without further purification. *E. coli* strain ATCC25922 and *S. aureus* strain ATCC25923 were supplied by the Institute of Microbiology of Chinese Academy of Sciences. Ultrapure water was used throughout the study (Milli-Q, 18.2 MΩ/cm resistivity).

Lipophilicity was obtained according to the shake-flask method by determining the distribution coefficient ($logD_{7.4}$) of the porphyrin derivatives in n-octanol and PBS buffer (pH 7.4) as aqueous phase [24]. Care was taken to avoid cross-contamination between the phases. The partition coefficient was calculated as the average log ratio of the radioactivity in the organic fraction and the PBS fraction. The photostability of $TPPNH_2$ and TPPOH under visible light irradiation was tested by comparing the absorbance of the porphyrins after light irradiation. The singlet oxygen generation was tested by comparing the absorbance of DPBF (1,3-Diphenylisobenzofuran) at 412 nm with and without porphyrins after light irradiation. The details of other experimental methods, including preparation of fluorescein-loaded vesicles and vesicle leakage assays [25, 26], and observation of cell morphology



Scheme 1. Structure of selected porphyrins: TPP, TPPOH, $TPPNO_2$, and $TPPNH_2$.

[27] are performed based on corresponding references.

2.2. Biocidal Experiments

Gram-negative bacterium *E. coli* and Gram-positive bacterium *S. aureus* were selected for this study. Bacterial samples were transferred from the frozen state onto agar plates 1.5% agar + standard Luria broth (LB) and incubated at 37 °C for 24 h, then stored at 4 °C for use in two weeks. A single colony from the slants was incubated in 5 mL of LB for 18 h with shaking at 30 °C. The bacterial culture was then centrifuged at 4000 r/min for 5 min and the pellet was suspended in 0.9% NaCl solution. This washing procedure was repeated in triplicate. The cell pellet was resuspended in 0.9% NaCl solution to OD_{600}~1.0. The final concentration of bacteria was ~10^9 colony forming units (CFU)/mL. Porphyrin stock solutions were prepared by dissolving a porphyrin compound in dimethyl sulfoxide (DMSO) to form 1 mg/mL solution for biocidal evaluation.

To exam the biocidal effect of porphyrins on Gram-negative and Gram-positive bacteria, *E. coli* and *S. aureus* were incubated with various concentrations of porphyrins (1.0, 3.0, and 9.0 μg/mL in 0.9% NaCl for *E. coli* and 0.01, 0.03, 0.09, and 0.3 μg/mL in 0.9% NaCl for *S. aureus*) at room temperature for 60 or 120 min in the dark or exposed to visible light (11 mw/cm^2, Mejiro Genossen MVL-210 fiber light, wavelength: 400–800 nm). Then the biocidal solutions were taken out and diluted CFU of bacteria in the aforementioned samples were cultured on LB agar plates and incubated for 18–24 h at 30 °C. The ability of porphyrins to inactivate bacteria cells was determined by the plate counting method and was calculated as survival fractions (N/N_0), where N is the number of CFU of the bacteria solution after exposed to porphyrins and N_0 is the CFU of a control (bacteria alone in the dark or exposed to visible light).

2.3. Stability of TPPOH and $TPPNH_2$ Under Light Irradiation

Stock solutions (1 mg/mL) of TPPOH and $TPPNH_2$ in DMSO were prepared, and 500 μL of each porphyrin stock solution was added in 3 mL H_2O. Then the porphyrin solutions were irradiated under visible light (11 mw/cm^2). Absorption spectra of porphyrin solutions were recorded every 20 min (a PerkinElmer Lambda 850 UV/Vis spectrometer) from 700 nm to 250 nm. The specific absorption peaks were observed. And their stability under light irradiation was evaluated by the change of the absorbance peak intensity.