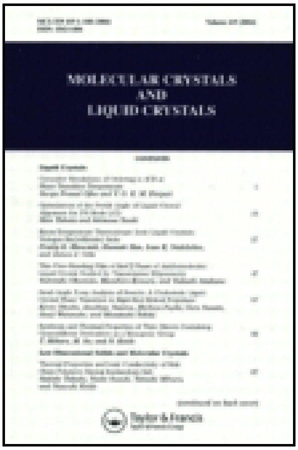

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Singlet Oxygen Generation and Photocytotoxicity against Tumor Cell by Two-Photon Absorption

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We have investigated singlet oxygen generation and photocytotoxicity against tumor cells by two-photon excitation using a two-photon absorbing photosensitizer consisting of a butadiyne-bridged porphyrin dimer terminated with two water-soluble porphyrin monomers. The compound exhibited high efficiency for singlet oxygen generation under not only one-photon but also two-photon irradiating conditions. The successful destruction of Hela cancer cells via two-photon absorption (2PA) is demonstrated.

Keywords: photodynamic therapy; porphyrin; singlet oxygen; two-photon absorption

INTRODUCTION

Photodynamic therapy (PDT) is a gentle treatment modality for cancers based on the localization of photosensitizers such as hematoporphyrin derivatives (HpD) in the cancer cell, followed by

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photoactivation [1,2]. The photoirradiated HpD is promoted to the excited singlet state and decays to the excited triplet state via intersystem crossing (ISC). The excitation energy is transferred to ground state oxygen ($^3O_2$) to generate the singlet oxygen ($^1O_2$), which attacks the cancer. The largest problem in current PDT is the limitation of the penetration depth of light (at 630 nm) to restrict the treatment to cancers on surface. Use of near-infrared light (NIR) is necessary for the treatment of deeper cancer because the penetration of the NIR light into biological tissues is much better than that of visible light.

2PA is a nonlinear optical process, in which two photons are absorbed simultaneously even at nonresonant wavelength in the NIR region to promote the molecule to the higher excited state having the combined energy of two photons. Further, the quadratic dependence of 2PA on the laser intensity produces a high spatial selectivity by using a focused laser beam. Therefore, PDT using 2PA is expected to innovate a treatment method for the deeper cancer with a three-dimensional site selectivity.

Recently, we reported that the conjugated porphyrin **1** (Fig. 1) self-assembled through zinc-imidazolyl coordination exhibited a very large two-photon absorption cross section value ($\sigma^{(2)}$) of 7,600 GM (1 GM = 10^{-50} cm^4 s molecule^{-1} photon^{-1}), which was the largest among the reported values measured by femtosecond pulses [3,4]. Its water-soluble array **2** exhibited the photocytotoxicity for Hela cell under one-photon irradiation [5]. Further, we reported another

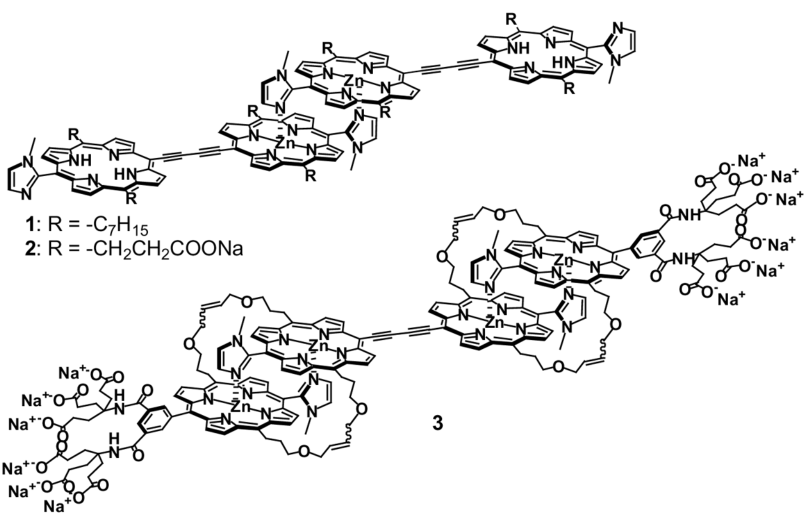

FIGURE 1 Structures of supramolecular porphyrin arrays **1**, **2**, and **3**.

water-soluble porphyrin array **3**, which was composed of one bisacetylene-linked bisporphyrin as a 2PA part and two monomeric porphyrins having in total twelve carboxylate groups as water-solubilizing parts [6]. Array **3** exhibited large effective 2PA cross section values, ${}^{eff}\sigma(2)$, of 33,000 GM at 890 nm and 28,000 GM at 780 nm with 5 ns pulses, although these contain a contribution of excited state absorption, and generated singlet oxygen efficiently under two-photon excitation conditions at 890 nm. In this article, we report a photocytotoxicity of **3** against Hela cancer cells as well as singlet oxygen generation under two-photon excitation conditions using femtosecond pulses at 780 nm, which is a fundamental wavelength of Ti:Sapphire laser and corresponds to another peak maximum in the 2PA spectrum.

EXPERIMENTAL

The array **3** was synthesized by nine steps in total and identified by MALDI-TOF mass and analytical gel permeation chromatography [6]. 2PA-PDT experiments with Hela cells were performed as follows. A stock solution of Hela cells in MEM (minimal essential medium) was prepared. From this solution, a 2 mL MEM aliquot containing ca. 100 Hela cells was withdrawn and placed in a glass chamber on a glass slide. The chamber was incubated at 37°C overnight. A 1 mL aliquot of 1 μM **3** in H_2O was added and the mixture was incubated for two hours. MEM and the glass chamber were removed from the glass slide. The excitation source was a mode-locked Ti:Sapphire laser (Coherent Mira 900), which provides 100 fs pulses with average power of 2 mW. The cells were irradiated at 780 nm for 5 min covering a spot diameter of 1 μm with an objective lens resulting to average power of 600 mJ/cell. The progress of photosensitization was monitored using a CCD camera.

RESULTS AND DISCUSSION

Singlet Oxygen Generations

The predominant photocytotoxic species produced during PDT have been identified as singlet oxygen (Type-II reaction) [1,2]. The malignant tumor is killed by the attack of singlet oxygen generated as a result of the energy transfer from the photosensitized agent in the relatively long-lived excited triplet state to the ground state oxygen. Therefore, the efficient generation of singlet oxygen in water is required for the PDT agent. Singlet oxygen is known to emit phosphorescence from ${}^1\Delta_g$ to ${}^3\sum_g$ at 1270 nm. Although, its emission intensity is rather weak even under one-photon excitation condition due to its nature of forbidden transition, the emission from singlet oxygen at 1270 nm upon one-photon irradiation was measured by time-resolved experiment. The samples in water were irradiated with 5 ns Nd:YAG-OPO (optical parametric oscillator) pulses, and the phosphorescence was detected with InP/InGaAsP (Hamamatsu Photonics R5509-43) as a detector operated at $-80^{\circ}C$ through an interference filter. The excitation wavelength was selected as its absorbance equal to 0.8 for all samples ($550 \sim 600$ nm) and power was adjusted to 30 mW. Figure 2 shows time-resolved emission profiles of (a) **3**, (b) tetra(4-sulfophenyl)porphyrin (TPPS), and (c) TPPS with NaN_3, which is a typical quencher for singlet oxygen. In cases of **3** and TPPS, The rise components were observed after the excitation indicating the formation of singlet oxygen by energy transfer from photosensitizer [7,8]. Since the emission was quenched by the addition of NaN_3, the signal can be assigned to come from singlet oxygen (Fig. 2 (c)). The compound **3** produced almost the same or even higher emission intensity as TPPS indicating that **3** can generate singlet oxygen sufficient for PDT upon photoirradiation.

The singlet oxygen generation by two-photon excitation was measured by using 9,10-anthracenedipropionic acid sodium salt (ADPA) that scavenges the generated singlet oxygen by rapid Diels-Alder

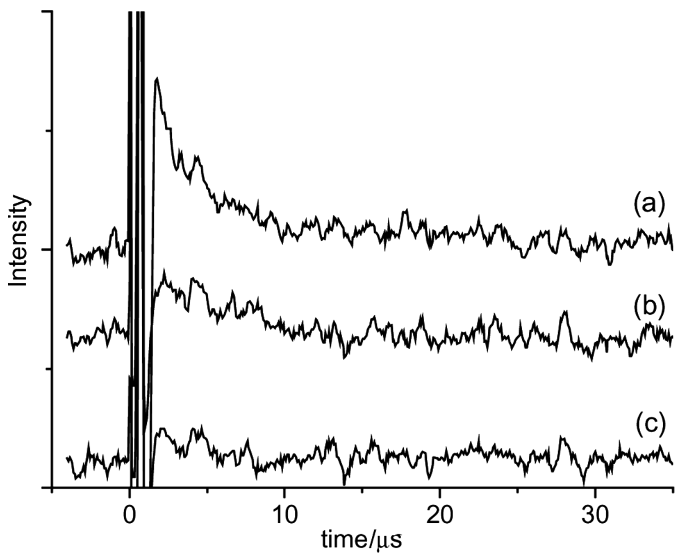

FIGURE 2 Time-resolved emission profiles of singlet oxygen with (a) **3**, (b) tetra(4-sulfophenyl)porphyrin (TPPS), and (c) TPPS with NaN_3.

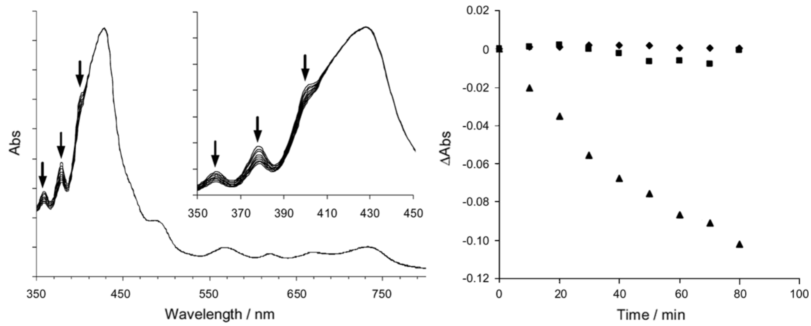

FIGURE 3 (a) Change in the absorption spectra of ADPA with **3** upon two-photon irradiation at 780 nm; (b) change in the absorbance at 379 nm of ADPA with **3** (triangle), TPPS (square), and nothing (diamond).

reaction [9]. Solutions of around 10^{-5} M of ADPA, with TPPS, and with **3** in D_2O were irradiated with 5 ns pulses at 780 with average power of 30 mW. Since ADPA exhibits characteristic absorption peaks of anthracene at 399, 378, 359 and 342 nm and loses those by photo-bleaching, absorption change was observed at these wavelengths (Fig. 3(a)). No change was observed in the Q-bands of **3** indicating that the sensitizer itself is affected neither during two-photon excitation nor singlet oxygen generation. Almost no decrease in the anthracene absorbance was observed for the solutions without any sensitizer and even in the presence of TPPS, which has very weak 2PA at this wavelength. The time profile of the absorption peak at 379 nm was illustrated in Figures 3(b). The experiment under one-photon excitation conditions without a lens at 780 nm (30 mW) showed no decrease in the anthracene absorption, indicating no contribution from one-photon excitation in the two-photon singlet oxygen measurements for **3**.

2PA-PDT Experiments with Hela Cells

The PDT experiments under two-photon irradiation were performed using Hela cells. Hela cells incubated with **3** on a glass slide were irradiated for 5 min with a mode-locked Ti:Sapphire laser at 780 nm with an average power of 2 mW and a spot diameter of 1 μm (FWHM = 100 fs). This irradiation condition gives average power of 600 mJ/cell. In Figure 4(a), the central Hela cell was selectively targeted at the position marked by a white arrow while the lower left cell was not irradiated. As shown in Figure 4(b), degradation of the cell membrane of the central cell after two-photon irradiation

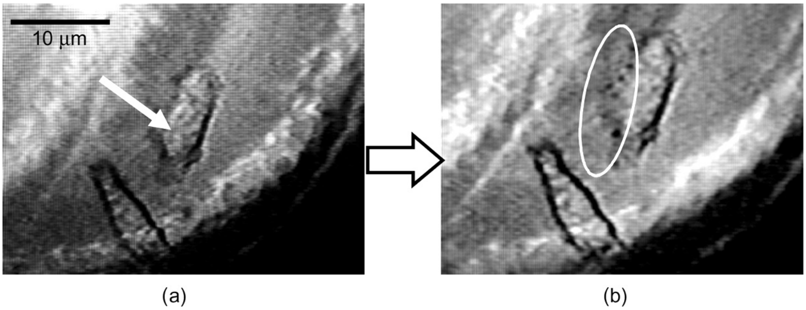

FIGURE 4 Pictures of Hela cells incubated with **3** before (a) and after (b) two-photon excitation at 780 nm. The irradiated position is marked by a white arrow. The degradation of the cell membrane was observed as indicated by an oval.

could be observed. The non-targeted lower left cell remained intact. As references, we performed similar control experiments without any photosensitizer and with hematoporphyrin (HP) on Hela cells. Both experiments resulted in no cell death under two-photon irradiation conditions. These experiments conclude the photodynamic activity of **3** toward Hela cancer via two-photon excitation.

CONCLUSIONS

We have demonstrated singlet oxygen generation and photocytotoxicity against tumor cells under two-photon irradiation using a water soluble two-photon absorbing photosensitizer. Singlet oxygen was successfully generated on laser irradiation of a long wavelength such as 780 nm as evidenced by ADPA photobleaching after two-photon excitation. Further, we have successfully demonstrated the photocytotoxicity of **3** via two-photon excitation on Hela cancer cells.

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