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Optical Properties of Talaporfin-Serum Albumin Complex

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Optical properties of Talaporfin, a photosensitizer for photodynamic therapy, under the human serum albumin (HSA) existence were investigated using phase-resolved fluorescence. Talaporfin-HSA complex was analyzed in the phosphoric acid buffer solution (PBS) at concentrations ranging from 1.95×10^{-7} M to 1.00×10^{-4} M. The fluorescence spectra of Talaporfin PBS solution show a peak around 662 nm, whereas the peak of Talaporfin-HSA complex is red shifted to 670 nm. The photophysical parameters of Talaporfin-HSA complex is attributed to the electronic transition from the lowest singlet excited state to the ground state at concentrations below 1.25×10^{-5} M. As for the PBS solution of a Talaporfin-HSA complex, its fluorescence intensity is stronger than that of Talaporfin PBS solution. This result could be explained as follows: in the Talaporfin-HSA complex Talaporfin molecules combine to the HSA site II, and hence they feel the less polar circumstance than in the bared PBS solution because of the surrounding amino acid residues of HSA. The fluorescence life time of Talaporfin-HSA complex increases slightly with Talaporfin concentration, and its value of 4.9–5.5 ns is longer than that of the PBS solution.

Keywords: phase-resolved fluorescence; photosensitizer; Talaporfin-human serum albmin complex

INTRODUCTION

Photodynamic diagnosis (PDD) and photodynamic therapy (PDT) have been developed quickly in recent years. In PDD and PDT photosensitizers play an important role and the selection of them becomes one of the key factors. The hematoporphyrin derivative (HpD) was developed by Lipson *et al.* in 1960 from the hydrochloric hematoporphyrin

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treated with acetic acid and sulfuric acid [1], since then the research of localized diagnosis and treatment of a tumor has been advanced enormously [2,3]. However the dosage of HpD causes side reactions such as photodermatosis under the daylight because its lifetime is quite long and remained in the skin for long time after dosage. Moreover, the absorption of light with long wavelengths which reach to the deep part of living tissues is weak. NPe6 (Mono-L-aspartyl chlorin e6, ME2906) was developed as a second generation photosensitizer recently [4], and named Talaporfin by World Health Organization (WHO) in 2003. Talaporfin is approved to use to an early lung cancer (a stadium term or I term lung cancer) clinically by Ministry of Health, Labor and Welfare in Japan now.

The chemical structure of Talaporfin is shown in Figure 1. Talaporfin has a similar skeleton as porphyrin, but an aspartic acid salt is combined to the ethanoic acid in the 15th position through a peptide linkage and a propanoic acid salt is combined in the 17th position of a tetrapyrrole ring [5]. The molecular weight of Talaporfin of a chlorin system is 799.69. Prominent characteristics of Talaporfin are as follows: Firstly an absorption wavelength is longer than HpD, secondly the affinity to a tumor tissue is higher than HpD, and thirdly the staying time of Talaporfin in a normal tissue is also shorter than HpD.

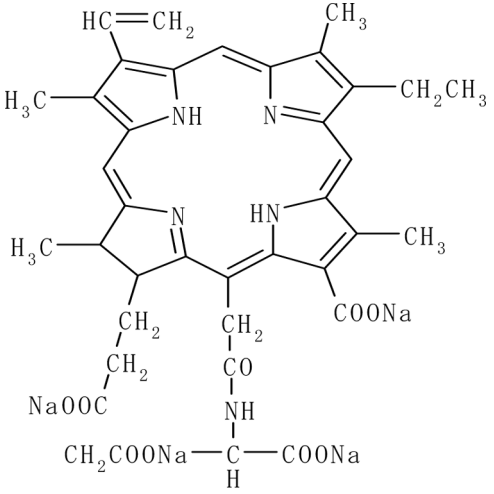

FIGURE 1 The molecular structure of Talaporfin. Talaporfin is a compound with a molecular weight of 799.69, in which the double bond porphyrin ring has been reduced and an aspartic acid is attached to the propionic group at the 17th carbon of the tetrapyrrole ring via a peptide linkage.

The process of PDT using Talaporfin is considered as the active (singlet) oxygen is generated by the intersystem crossing of excited energy of Talaporfin under photoexcitation and hence it causes a tumor tissue necrosis nearby. Some researchers, on the other hand, pointed out a curative effect due to the hemostasis of fine vessels in tumor tissues under Talaporfin PDT, so that the detailed mechanism of tumor recovery by PDT is not clarified as yet.

Since serum albumin is a major component of a plasma protein, it forms a complex with substances such as photosensitizers used in PDT, biomolecules and dosed drugs during blood flow in the vein [6]. Therefore, it is important to know what kind of affinity exists between a serum albumin and these substances. In the case of photosensitizers used in PDT, it is also required to clarify their photophysical properties under the serum albumin existence. Following our former report on phase-resolved fluorescence study of NPe6 (Talaporfin) [7], we investigated fundamental optical properties of Talaporfin itself and of Talaporfin-serum albumin complex in this paper.

MATERIALS AND METHODS

It is reported that Talaporfin forms a complex with serum albumin with a molar ratio of 1:1 [8]. In order to investigate optical properties of Talaporfin under the serum albumin existence, the phosphoric acid buffer solution (PBS, $pH = 7.4$) of Talaporfin and a serum albumin was prepared. The serum albumin used is a human serum albumin (HSA), and the specification is fraction V and 96–99%. The concentration of Talaporfin and HSA was changed in the range from 1.95×10^{-7} M to 1.00×10^{-4} M, keeping the molar ratio at 1:1. As a reference we also prepared Talaporfin ethanol solution in the same concentration range. Excitation and fluorescence spectra of the PBS solution prepared were measured using a fluorescence spectrophotometer (FLUOLOROG-3: SPEX-JOBIN-YVON, New Jersey, USA). Perylene (standard-grade, Merck, Darmstadt, Germany) was dissolved in ethanol, which was used as a standard sample of fluorescence efficiency measurement. We obtained a quantum efficiency of 0.89 in the perylene-ethanol solution under the excitation at 411 nm. The optical absorption spectra of Talaporfin and perylene solutions were measured using a UV-VIS spectrophotometer (UV-2500: SHIMADZU, Kyoto, Japan), and each molar absorption coefficient was determined.

The fluorescence life time τ of Talaporfin-PBS solution was determined in the frequency domain [9] using a fluorescence measurement system (FLUOLOROG-3-TAU3: SPEX- JOBIN-YVON, New Jersey, USA). The fluorescence life time can be approximated by a single

exponential function in the range from 1.95×10^{-7} M to 1.00×10^{-4} M. The fluorescence quantum efficiency η was determined by means of a relative measurement technique using a perylene as a standard fluorescent substance, that is, from Eq. (1).

$$\eta_x = \eta_{st}(FA_x/FA_{st})(A_{st}/A_x)(I_{ex,st}/I_{ex,x})(n_x^2/n_{st}^2) \quad (1)$$

where FA is an integration of corrected fluorescence spectrum against wavelength, A and I are an absorbance and a light intensity at the excitation wavelength, respectively, and n is an averaged refractive index of the solvent in the fluorescence spectrum (wavelength) range. The suffix x means a sample, st a standard, and ex an excitation. It should be noted that the excitation light intensity I was corrected over the wavelengths range, whereas the refractive index n was not corrected because of little effect on the fluorescence efficiency. Using the fluorescence efficiency η thus obtained, the rate constants of the radiative (k_F) and the nonradiative deactivation process (k_{NR}) were determined by Eqs. (2) and (3), respectively.

$$k_F = \eta/\tau \quad (2)$$

$$k_{NR} = (1 - \eta)/\tau \quad (3)$$

Then the fluorescence life time τ is given by Eq. (4)

$$\tau = 1/(k_F + k_{NR}) \quad (4)$$

RESULTS AND DISCUSSION

Fluorescent Characteristics of a Talaporfin-HSA Complex–Steady State

The absorption spectra of PBS solutions of Talaporfin only and Talaporfin-HSA complex are shown in Figure 2. The concentration of Talaporfin is 2.50×10^{-5} M, and the molar ratio of Talaporfin and HSA is 1:1. PBS solution of Talaporfin (pH = 7.4) showed the optical absorption peaks at 400, 502, 600, and 654 nm, and the corresponding peaks of the Talaporfin-HSA complex were observed at 406, 508, 606 and 660 nm. The absorption band of the tetrapyrrol ring changed structurally by binding with an HSA molecule and shifted towards longer wavelength (bathochromic shift) [4]. This tendency is understandable as the π–π^* transition was induced through the interaction between polar solvent and substituent group of Talaporfin.

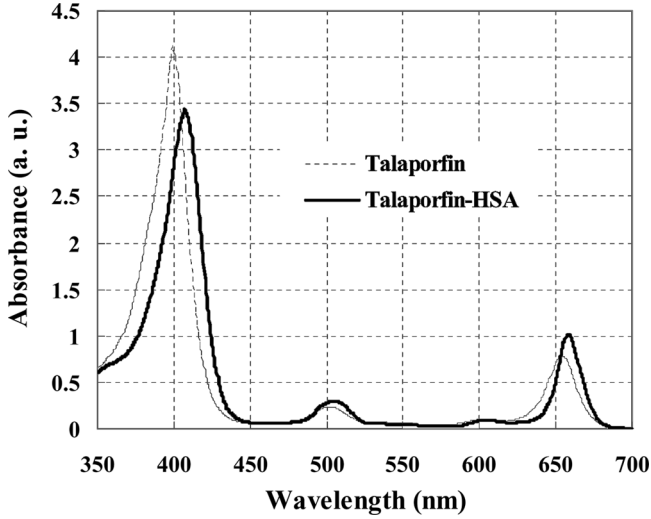

FIGURE 2 Absorption spectra of the Talaporfin-HSA complex (solid line) and the Talaporfin only (dashed line) in PBS solution. The concentration of Talaporfin is 2.50×10^{-5} M.

Figure 3 shows the fluorescence excitation spectra and the fluorescence spectra of PBS solutions of Talaporfin only and Talaporfin- HSA complex. The excitation spectra were detected at 670 nm, and the fluorescence spectra were excited by 500 nm, respectively. The excitation spectra (solid and dashed lines in Fig. 3) is exactly the same as the absorption spectra (Fig. 2). The similar bathochromic shifts (red shifts) caused by Talaporfin-HSA and polar solvent were observed in the concentration range from 1.95×10^{-7} M to 1.00×10^{-4} M.

The fluorescence spectra of Talaporfin PBS solution shows a peak around 662 nm, whereas that of Talaporfin-HSA complex is red shifted to 670 nm. This red shift rarely depends on Talaporfin concentration in the range from 1.95×10^{-7} M to 1.00×10^{-4} M. Figure 4 indicates the change of fluorescence intensity of the Talaporfin-HSA complex as a function of Talaporfin concentration. The open squares represent the experimental results of a Talaporfin-HSA complex, and the solid line is fitted to these results in the linear fashion. The solid squares and asterisks show the results of the Talaporfin in PBS solution and of the Talaporfin in ethanol solution for comparison, respectively. These results suggest that the fluorescence intensity of a Talaporfin-HSA complex is linearly dependent on the Talaporfin concentration (the slope = 1 in the full-log plot of Fig. 4) up to 1.25×10^{-5} M above

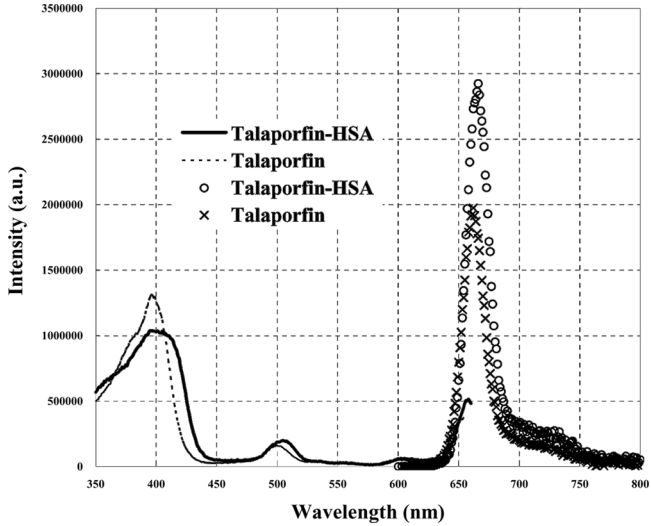

FIGURE 3 Fluorescence excitation spectra of the Talaporfin-HSA complex (solid line) and the Talaporfin only (dashed line) in PBS solution, and fluorescence spectra of the Talaporfin-HSA complex (open circle) and the Talaporfin PBS solution (cross). The concentration of Talaporfin is 2.50×10^{-5} M. The excitation spectra was detected at 670 nm and the fluorescence spectra was excited at 500 nm.

which the concentration quenching of fluorescence occurs due to, say, dimmer formation of Talaporfin molecules [7]. Therefore the fluorescence of Talaporfin-HSA complex could be assigned to the electronic transition from the lowest singlet excited state to the ground state in the same manner as isolated Talaporfin molecules.

Some substances emit stronger fluorescence when their environment changes from strong polar environment to weak environment. This tendency explains well the result from Figure 4 that Talaporfin in the weak polar ethanol solution shows stronger fluorescence than Talaporfin in the polar PBS solution. As for the PBS solution of a Talaporfin-HSA complex, its fluorescence intensity is stronger than that of Talaporfin PBS solution. This result could be explained as follows: in the Talaporfin-HSA complex Talaporfin molecules combine to the HSA site II, and hence they feel the less polar circumstance than in the bared PBS solution because of the surrounding amino acid residues of HSA. This situation is the same as Talaporfins were dissolved in the weak polar solvent, and their polarization is reduced, which in turn causes fluorescence intensity enhancement [10].

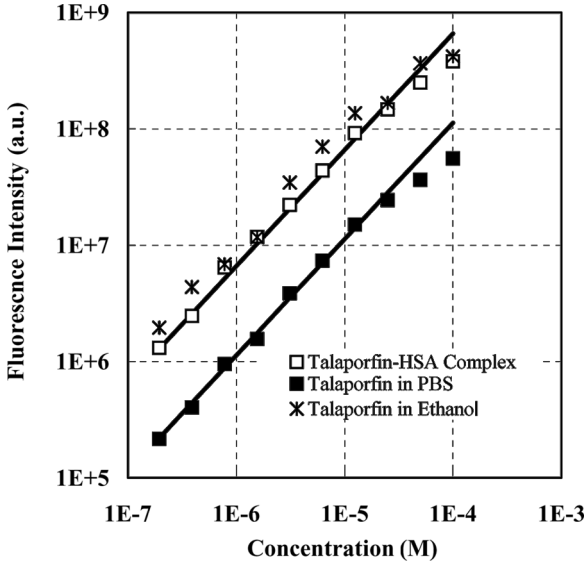

FIGURE 4 The fluorescence intensity as a function of Talaporfin concentration. The intensity was determined by calculating the area of emission spectra. Open squares indicate the experimental results of Talaporfin-HSA, solid squares of Talaorfin PBS solutions, asterisks of Talaporfin ethanol solutions, respectively, and the solid line represents a linear dependence of the intensity on Talaporfin concentration.

Fluorescent Characteristics of a Talaporfin-HSA Complex – Phase Resolution Measurement

Figure 5 shows the Talaporfin concentration dependence of fluorescence life time. The open square indicates a result in a Talaporfin-HSA complex. The results in Talaporfin PBS and in Talaporfin ethanol solutions are indicated with the solid square and asterisk, respectively. The fluorescence life time of Talaporfin-HSA complex increases slightly with Talaporfin concentration in the range investigated, and its value 4.9–5.5 ns is longer than that of the PBS and ethanol solutions. Here it is noted that the life time of Talaporfin PBS solution is almost constant as 4.0 ns in the from 1.95×10^{-7} M to 1.00×10^{-4} M.

Quantum efficiency is also shown in Figure 5 as a function of Talaporfin concentration. The symbols of open triangle, solid triangle and cross indicate results in a Talaporfin-HSA complex, Talaporfin PBS solution and Talaporfin ethanol solution, respectively. The quantum efficiency of Talaporfin-HSA complex increases with an increase of Talaporfin concentration, reaches a maximum (ca. 0.2) around

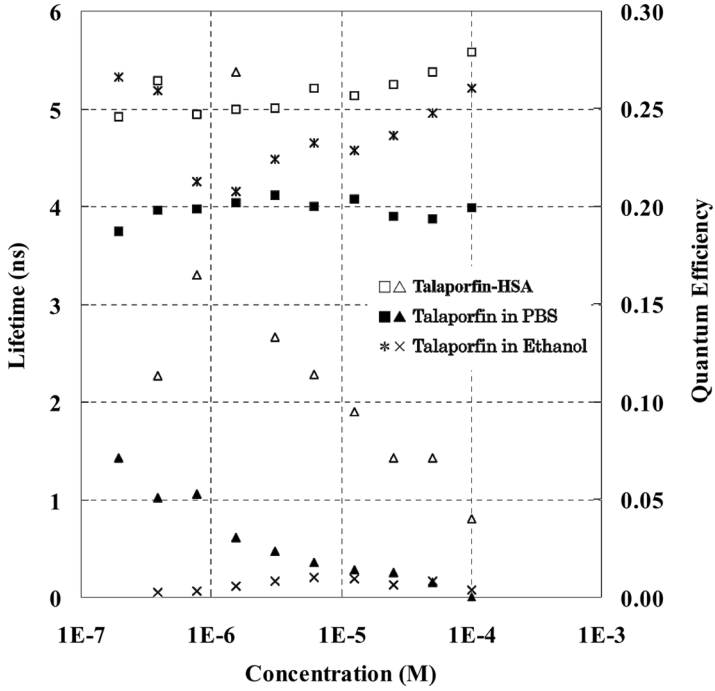

FIGURE 5 Quantum efficiency (open triangle, solid triangle and cross) and decay time (open square, solid square and asterisk) of fluorescence as a function of Talaporfin concentration.

1.00×10^{-6} M, then decreases down to 0.05 at 1.00×10^{-4} M, but these values are quite larger than the case of the Talaporfin PBS solution as expected from the results of the fluorescence life time and fluorescence intensity. Excess energy which does not return to the ground state directly is transferred to the excitation triplet state through the intersystem crossing, and then excites the triplet excitation state of oxygen atoms nearby. On the other hand the quantum efficiency of Talaporfin ethanol solution is constant (ca. 0.01) in the concentration range from 1.95×10^{-7} M to 1.00×10^{-4} M. Although the fluorescence intensity is as large as that of Talaporfin-HSA, the fluorescence quantum efficiency is small because of large absorbance.

The rate constants of a radiative process k_F and a nonradiative process k_{NF} were determined by Eqs. (2) and (3) using the results of fluorescence efficiency and a life time measurements. The results are shown in Figure 6 as a function of Talaporfin concentration. Symbols of open square, solid square and asterisk indicate k_{NR} of the

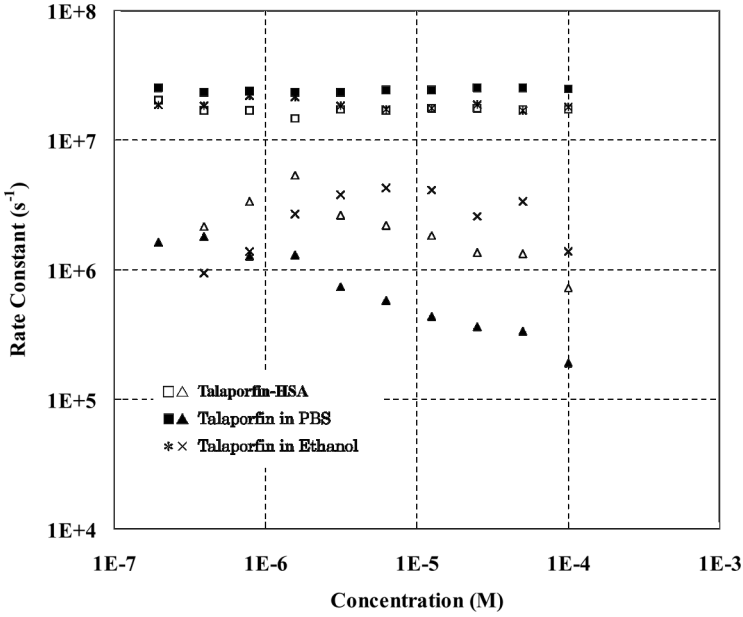

FIGURE 6 Rate constants of the radiative (open triangle, solid triangle and cross) and the nonradiative processes (open square, solid square and asterisk) as a function of Talaporfin concentration.

Talaporfin-HSA complex, Talaporfin PBS solution and Talaporfin ethanol solution, respectively. Similarly symbols of open triangle, solid triangle and cross indicate k_F of a Talaporfin-HSA complex, Talaporfin PBS solution and Talaporfin ethanol solution, respectively. The rate constants of radiative processes have a tendency to decrease with an increase of Talaporfin concentration, but, on the contrary, the rate constants of nonradiative process is almost independent of Talaporfin concentration in the concentration range investigated. It is clear from the comparison with the results in Talaporfin PBS solution that the rate constant of a nonradiative process decreases and that of a radiative process increases by forming a complex with HSA.

CONCLUSION

Optical properties of photosensitizer Talaporfin were clarified by the spectrum measurement and phase resolution fluorescence measurement. Fluorescence of a Talaporfin-HSA complex in PBS solution shows a similar behavior to the isolated Talaporfin in PBS solution

in the concentration range from 1.95×10^{-7} to 1.25×10^{-5} M, and it was attributed to the electronic transition from the lowest singlet excited state to the ground state of Talaporfin molecule. When Talaporfin forms a complex with HSA the peak of the fluorescence spectra locates at 670 nm, which corresponds to the red shift by ca. 8 nm compared in the Talaporfin PBS solution. This is also in good agreement with a result when Talaporfin combines with tissues in the living body. It was suggested that in Talaporfin-HSA complex PBS solution Talaporfin molecule behaves as if isolated in the weak polar solvent. This was confirmed from the increase in fluorescence intensity of Talaporfin-HSA complex, which is similar behavior to the Talaporfin ethanol solution.

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