Communication

Photosensitized oxidative damage of human serum albumin by water-soluble dichlorophosphorus(V) tetraphenylporphyrin

Dongyan Ouyang and Kazutaka Hirakawa*

Department of Optoelectronics and Nanostructure Science, Graduate School of Science and Technology, Shizuoka University
Johoku 3-5-1, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan

ABSTRACT: Biomolecular photo-damaging activity of a water-soluble cationic porphyrin was examined using human serum albumin (HSA), a water-soluble protein as a target biomolecule model by a fluorometry. Dichlorophosphorus(V) tetraphenylporphyrin (Cl$_2$P(V)TPP), was synthesized and used as a photosensitizer. This porphyrin could bind to HSA and cause the photosensitized oxidation of HSA through the singlet oxygen generation and the oxidative photo-induced electron transfer (ET). Near infrared emission spectroscopy demonstrated the photosensitized singlet oxygen generation by this porphyrin. Decrement of the fluorescence lifetime of Cl$_2$P(V)TPP by HSA supported the ET mechanism. Furthermore, the estimated Gibb's energy indicated that the ET mechanism is possible in the terms of energy. Because oxygen concentration in cancer cell is relatively low, ET mechanism is considered to be advantageous for photosensitizer of photodynamic therapy.

Porphyrins have been used as the drugs for photodynamic therapy (PDT), which is a less invasive treatment of cancer [1,2]. The oxidation of biomolecules is the major mechanism of PDT. It is triggered by singlet oxygen (^{1}O$_2$) generation or photo-induced electron transfer (ET). Biomolecule oxidation by ^{1}O$_2$ is considered as the main mechanism of PDT, however, the low concentration of oxygen in cancer cells restricts the PDT effect [3,4]. Therefore, ET mechanism might become a more important mechanism of PDT in the future. The ET mechanism requires highly oxidative activity (a lower reduction potential) in the photoexcited state of the photosensitizer. Larger excitation energy is advantageous for the lower reduction potential of the photosensitizer in the photoexcited state. Indeed, ultra-violet photosensitizers mainly induce biomolecule photodamage through the ET mechanism, whereas a visible-light photosensitizer is not appropriate for this mechanism [5]. Therefore, an appropriate molecular design to achieve ET mediated biomolecule damage using a visible-light photosensitizer is important. Since high-valent porphyrin complexes demonstrate a lower reduction potential in their photoexcited state than free-base or low-valent metal complexes, these porphyrins are advantageous for the oxidative ET reaction [5-7]. For example, in this research, a synthesized porphyrin, Cl$_2$P(V)TPP (Figure1) was used.

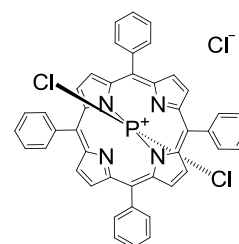*To whom correspondence should be addressed.
E-mail: hirakawa.kazutaka@shizuoka.ac.jp

Figure 1. Structure of Cl$_2$P(V)TPP used in this research.

The protein damage photosensitized by Cl$_2$P(V)TPP was examined by the method of the previous reports [8,9]. The sample solution containing 10 μM porphyrin and 10 μM human serum albumin (HSA), a water-soluble protein, in a sodium phosphate buffer (pH 7.6) was irradiated with a light-emitting diode (LED) (519 nm, 1 mW cm^{-2}, CCS Inc., Kyoto, Japan). The intensity of the LED light source was measured with a photo-power meter (8230E, ADCMT, Tokyo, Japan). Protein damage by porphyrins was evaluated by measurement of the fluorescence intensity from the amino acid residues as previously reported (excitation wavelength: 298 nm, dete ction wavelength: 350 nm). The concentration of damaged HSA, [Damaged HSA], is determined as follows:

$$[\text{Damaged HSA}] = \frac{F_0 - F}{F_0} \times [\text{HSA}]_0, \qquad (1)$$

where F and F_0 are the fluorescence intensities of HSA with or without the treatment of photosensitized reaction by porphyrins, respectively and [HSA]$_0$ is the initial concentration of HSA (10 μM).

The ^{1}O$_2$ formation was directly measured by near-infrared luminescence from ^{1}O$_2$ at around 1,270 nm, which corresponds to the ^{1}O$_2$ ($^1\Delta_g$)-^{3}O$_2$($^3\Sigma_g^-$) transition, as previous reports [10]. The quantum yield of ^{1}O$_2$ generation (Φ_Δ) was estimated from the comparison of the ^{1}O$_2$ emission intensities by porphyrins (5 μM) in a 2.0 cm^3 solution of sodium phosphate buffer (pH 7.6) with that of methylene blue (Φ_Δ= 0.52 in H$_2$O) [11].

To confirm the binding interaction between HSA and Cl$_2$P(V)TPP, the absorption spectrum of 5 μM Cl$_2$P(V)TPP with or without HSA in a sodium phosphate buffer (pH 7.6) was measured. In the presence of HSA, the hypochromic effect and red-shift were observed in the UV–vis absorption spectra of Cl$_2$P(V)TPP, indicating the static interaction between Cl$_2$P(V)TPP and HSA. In general, binding ratio between porphyrin and HSA depends on the concentrations of molecules and association constant. Job's plot of the absorption
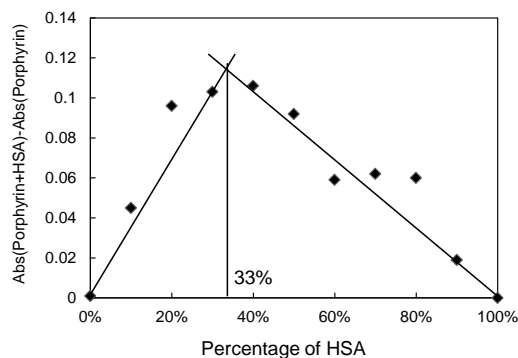
Figure 2. Job′s plot of the Soret band peak of Cl$_2$P(V)TPP with HSA.

change showed the intersection points at *ca.* 33% supporting the 2 : 1 complex formation between Cl$_2$P(V)TPP and HSA (Figure 2). The apparent association constant (K_{ap}) between these poprhyrins and HSA was evaluated under an assumption of the following equation:

$$K_{ap} = \frac{[\text{Porphyrin} - \text{HSA}]}{[\text{Porphyrin}][\text{HSA}]_b} \qquad (2)$$

where [Porphyrin] is the concentration of the non-binding photosensitizer, [HSA]$_b$ is the concentration of the binding sites of HSA without a binding porphyrin (twice of the actual concentration of free HSA), and [Porphyrin-HSA] is the concentration of the HSA-binding photosensitizer. The estimated value of K_{ap} is 1.1×10^6 M^{-1}. This value shows that the binding ratio becomes more than 90% when the concentration of HSA is 20 μM. The affinity between Cl$_2$P(V)TPP and HSA is remarkably large, possibly due to the hydrophobic character of Cl$_2$P(V)TPP.
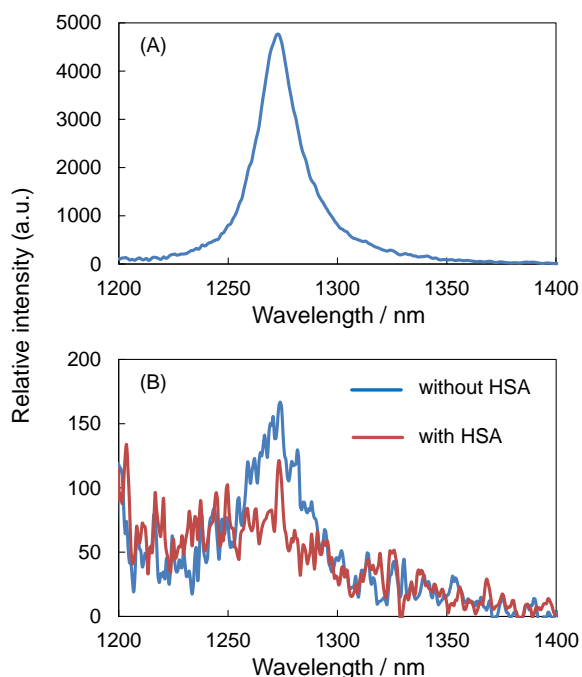
Figure 3. Near-infrared luminescence spectra of ^{1}O$_2$ generated by photosensitized reaction of Cl$_2$P(V)TPP in ethanol (A) and sodium phosphate buffer (pH 7.6) (B). Concentration of HSA is 10 μM.

Table 1. Basic parameters of Cl$_2$P(V)TPP

E_{ox} / V[a]	Φ_Δ[b]	ΔG /eV
1.76	0.64	-0.76

[a]: Oxidation potential in acetonitrile vs saturated calomel electrode (SCE) . [b]: in 10 mM sodium phosphate buffer (pH 7.6).

The Φ_Δ in sodium phosphate buffer and the redox potential of Cl$_2$P(V)TPP were also listed in Table 1. The photosensitized ^{1}O$_2$ generation by Cl$_2$P(V)TPP was confirmed by the detection of near-infrared emission around 1270 nm (Figure 3). When adding HSA, the relative intensity at about 1270 nm decreased, suggesting the physical or chemical quenching of ^{1}O$_2$ by HSA (Figure 3B).

The relatively large value of Φ_Δ indicate that the ^{1}O$_2$ mechanism is important for photosensitized biomolecule damage by Cl$_2$P(V)TPP in the presence of a sufficient concentration of molecular oxygen. Redox potential for one electron oxidation of Cl$_2$P(V)TPP is relatively high. Thus, it is expected that photoexcited Cl$_2$P(V)TPP shows the large oxidative activity in its photoexcited state through ET mechanism.

In the presence of Cl$_2$P(V)TPP, the fluorescence intensity of HSA around 350 nm, assigned to the tryptophan residue, was decreased by photo-irradiation. The fluorescence decrement of HSA can be explained by tryptophan oxidation through the photosensitized reaction [8,9]. Protein damage by Cl$_2$P(V)TPP was evaluated by measurement of the fluorescence intensity from tryptophan residue as previously reported [8]. The quantum yield of tryptophan degradation (Φ_{DT}) photosensitized by Cl$_2$P(V)TPP for 60 min irradiation was estimated from the decrease of the tryptophan fluorescence (Table 2).

The solvent effect on HSA photosensitized damage by porphyrins has been also examined. HSA damage was enhanced in D$_2$O, in which the lifetime of ^{1}O$_2$ is markedly elongated (about 2–4 μs in H$_2$O to 70 μs in D$_2$O) [10,12]. These findings suggest that ^{1}O$_2$ contributes to HSA oxidation.

To determine the contribution of ^{1}O$_2$ generation and ET mechanism, a scavenger effect on HSA damage was investigated. HSA photodamage by Cl$_2$P(V)TPP was partially inhibited by sodium azide (NaN$_3$) (Figure 4), a physical quencher of ^{1}O$_2$ [13], supporting the contribution of ^{1}O$_2$ mechanism to HSA damage. However, HSA damage was not completely inhibited by an excess amount of NaN$_3$ (10 mM), suggesting that the ET mechanism is partly responsible for HSA photodamage, as is the ^{1}O$_2$ mechanism. The quenching rate coefficient of ^{1}O$_2$ by NaN$_3$ (k_q) is almost diffusion control rate coefficient (k_{dif}), which is calculated as follows:

$$k_q \approx k_{dif.} = \frac{8000\,RT}{3\eta} \qquad (3)$$

where R is the gas constant, T is the absolute temperature, and η is the viscosity of water (8.91×10^{-4} kg m^{-1} s^{-1}). The quenching efficiency of ^{1}O$_2$ by sodium azide (Ef_q) can be calculated from the following equation using the lifetime of ^{1}O$_2$ (τ_Δ = 3.5 μs [8]):

$$Ef_q = \frac{k_q[\text{NaN}_3]}{k_q[\text{NaN}_3] + 1/\tau_\Delta} \qquad (4)$$
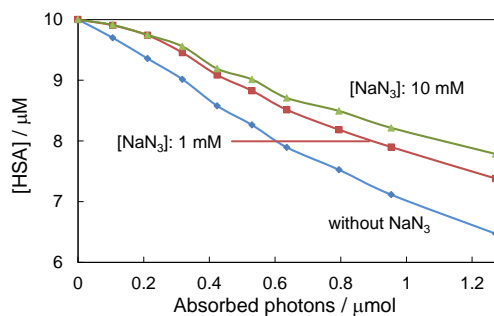
Figure 4. Effect of different concentrations of NaN$_3$ on HSA damage photosensitized by porphyrins.

where [NaN$_3$] is the concentration of NaN$_3$. In the presence of 10 mM NaN$_3$, the Ef_q becomes 0.996. The estimated contribution of the HSA damage through the ET mechanism and $^1O_2$ mechanism for 60 min irradiation were shown in Table 2. The relatively high percentage of electron transfer effect may be attributed to the stronger binding ability of Cl$_2$P(V)TPP for HSA.

The large value of K_{ap} for Cl$_2$P(V)TPP with HSA suggests the deep penetration to HSA. This characteristic may be advantageous for the ET mechanism, whereas the association with HSA should inhibit the energy transfer to molecular oxygen to generate $^1O_2$. The Gibbs energy (-ΔG) of the ET from tryptophan to the photoexcited Cl$_2$P(V)TPP was roughly estimated using the following equation:

$$-\Delta G = E(S_1) - e\,(E^+ - E^-), \qquad (5)$$

where $E(S_1)$ is the S$_1$ energy of Cl$_2$P(V)TPP estimated from the fluorescence maximum, e is the charge of electron, E^+ is the oxidation potential of the tryptophan (0.65 V vs SCE) [14] and E^- is the reduction potential of Cl$_2$P(V)TPP. These calculated value of -ΔG demonstrate that the oxidation of tryptophan by Cl$_2$P(V)TPP through the ET is possible from the energetically point of view (Table 1).

Table 2. Ratio and Quantum Yield of the Protein Damaging Mechanisms

R_{ET}	R_Δ	Φ_{DT}	Φ_{DET}	$\Phi_{D\Delta}$
63%	37%	1.1×10^{-3}	6.7×10^{-4}	3.9×10^{-4}

R_{ET}: Contribution of the ET mechanism

R_Δ: Contribution of the $^1O_2$ mechanism

Φ_{DET}: The quantum yield of HSA damage through the ET mechanism, $\Phi_{DET} = \Phi_{DT} \times R_{ET}$

$\Phi_{D\Delta}$: The quantum yield of HSA damage through the $^1O_2$ mechanism, $\Phi_{D\Delta} = \Phi_{DT} \times R_\Delta$

In the absence of HSA, two fluorescence lifetime (τ_f) species were observed, suggesting the two kinds of electric states of the S$_1$ state of Cl$_2$P(V)TPP (Table. 3). This result can be explained by that two conformations of Cl$_2$P(V)TPP are possible in a sodium phosphate buffer. Indeed, the similar results were reported in the case of other P(V)porphyrins [15]. In the presence of HSA, the time profile of the fluorescence decay of Cl$_2$P(V)TPP could be analyzed by the triple exponential function. Therefore, we could speculate the possibility of three kinds of species for the S$_1$ state of Cl$_2$P(V)TPP under an interaction with HSA. The observed values of the two species (9.0 ns and 4.4 ns) are rather larger than those without HSA, suggesting that

the S$_1$ state of Cl$_2$P(V)TPP is stabilized by the restriction of the vibrational quenching through an interaction with HSA. On the other hand, the shorter lifetime species (1.4 ns) supports the quenching of S$_1$ state of Cl$_2$P(V)TPP through the ET from tryptophan residue. Previous reports indicated the ET from the tryptophan residue to the S$_1$ state of other kinds of P(V)porphyrins [8,9,15]. The reaction mechanism is presented in the Scheme 1.

Table 3. Fluorescence Lifetime of Cl$_2$P(V)TPP and Relative Amplitude

without HSA	with HSA
5.7 ns (79%),	9.0 ns (23%),
3.6 ns (21%)	4.4 ns (66%),
	1.4 ns (11%)

The sample solution contained 5 μM Cl$_2$P(V)TPP with or without 10 μM HSA in a sodium phosphate buffer (pH 7.6). The excitation wavelength is 394 nm.

In summary, this study determined the contribution of the ET and the $^1O_2$ mediated mechanisms of protein damage by the photosensitized reaction of Cl$_2$P(V)TPP. It is speculated that the binding ability may play an important role in the protein damage by photosensitized reaction beside the redox potential and Φ_Δ of photosensitizers.
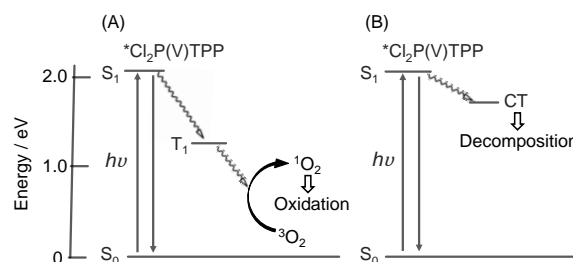
Scheme 1. Mechanisms of the photosensitized HSA damage through $^1O_2$ generation (A) and ET (B).

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