Fluorometry of singlet oxygen generated via a photosensitized reaction using folic acid and methotrexate

Kazutaka Hirakawa*

*Department of Basic Engineering (Chemistry), Faculty of Engineering, Shizuoka University,
Johoku 3-5-1, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan*

*Corresponding author:

Department of Basic Engineering (Chemistry), Faculty of Engineering, Shizuoka University,
Johoku 3-5-1, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan

Phone: +81-53-478-1287

Fax: +81-53-478-1287

E-mail: tkhirak@ipc.shizuoka.ac.jp

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Graphical abstract

The singlet oxygen quantum yield of photosensitizers can be determined by fluorometry based on the formation of strongly fluorescent pteridine via the oxidative decomposition of folic acid and methotrexate.

Abstract A method for the fluorometry of singlet oxygen ($^1\text{O}_2$) using less fluorescent folic acid and its analogue, methotrexate (MTX), was examined. Folic acid and MTX were decomposed into a strongly fluorescent pteridine compound via a photosensitized reaction by $^1\text{O}_2$ -generating photosensitizers in a deuterium oxide solution. The fluorescence intensity increased in proportion to the irradiation time or the number of photons absorbed by the photosensitizer. This method using the fluorescence enhancement of these folic acid analogues can be applied to determine the quantum yield of $^1\text{O}_2$ generated through a photosensitized reaction in deuterium oxide. The background fluorescence of MTX is quite smaller than that of folic acid, indicating that MTX can be used for the more sensitive detection of $^1\text{O}_2$.

Introduction

Singlet molecular oxygen ($^1\text{O}_2$ ($a^1\Delta_g$), denoted throughout as $^1\text{O}_2$) is the lowest excited electronic state of molecular oxygen and a reactive oxygen species that can mediate the oxidative degradation of many molecules [1-4]. Singlet oxygen is commonly generated by the irradiation of a molecule, a photosensitizer, that is either endogenous or specifically added to the system. In this process, energy transfer to ground-state molecular oxygen from an excited electronic state of the photosensitizer, generally the lowest excited triplet state, results in the production of $^1\text{O}_2$. One of the most important topics in photosensitized $^1\text{O}_2$ generation is photodynamic therapy (PDT), which is a promising treatment of cancer and some non-malignant conditions [5-7]. Furthermore, the modification of biomacromolecules through $^1\text{O}_2$ generation is the likely initial event involved in the induction of mutagenic and lethal effects [8-12]. The detection of $^1\text{O}_2$ and determination of the quantum yield of $^1\text{O}_2$ generation (Φ_Δ) by a photosensitizer are necessary to evaluate the PDT activity or phototoxic risk of photosensitizers. The measurement of the near-infrared emission through a deactivation process of $^1\text{O}_2$ is sensitive and one of the most important methods to detect $^1\text{O}_2$ [13-15], but this method requires the use of costly apparatuses. Another sensitive method for $^1\text{O}_2$ detection is fluorometry using a molecular probe, which is the less fluorescent precursor of the fluorescent molecule [16-19]. In general, the sensitive fluorometry of $^1\text{O}_2$ also requires costly fluorescence probes. We previously reported a method for the fluorometry of hydrogen peroxide (H_2O_2) using folic acid (Fig. 1), which is an inexpensive and nontoxic compound [20]. Hydrogen peroxide with copper ion or ultraviolet irradiation causes the oxidation of folic acid, leading to the formation of pterine-6-carboxylic acid (PCA) [20-24]. The fluorescence intensity of PCA is strong, although folic acid itself weakly fluoresces [20,24,25]. This fluorescence enhancement mechanism can be applied as a

fluorescence probe to detect reactive oxygen species. Since folic acid itself shows weak background fluorescence, improvement of this molecule to suppress the background fluorescence is necessary for the more sensitive detection of reactive oxygen species. In this paper, the fluorometry of $^1\text{O}_2$ (photosensitized $^1\text{O}_2$ generation) using folic acid and its commercially available less fluorescent analogue, *N*-[4-[[[(2,4-diamino-6-pteridiny)l)methyl]methylamino]benzoyl]-L-glutamic acid (methotrexate; MTX, Fig. 1) [26], was investigated. The determination method of Φ_Δ using these molecules is also reported.

Materials and methods

Materials

MTX, Nile blue (NB), tetrakis-(*N*-methyl-4-pyridyl)porphyrin (TMPyP), Azure A (AZA), and Azure B (AZB) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Folic acid, methylene blue (MB), acetonitrile, and sodium azide were from Wako Pure Chemical Industries (Osaka, Japan). New methylene blue (NMB) was from Tokyo Kasei (Tokyo, Japan). Deuterium oxide (D_2O) and rose bengal (RB) were from Across Organics (Morris Plains, NJ, USA).

Measurements of the absorption and fluorescence spectra

The absorption spectra of folic acid, MTX, and photosensitizers were measured with a UV-vis spectrophotometer (UV-1650PC, Shimadzu, Kyoto, Japan). The fluorescence spectra were measured with a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan). The fluorescence intensity of the sample solution at single wavelength was measured with a fluorescence photometer (650-60, Hitachi).

Photosensitized reaction

A sample solution containing 20 μM folic acid or MTX and an indicated concentration of the photosensitizer in D_2O was irradiated with a light-emitting diode (LED) (green: ISL-150X150-GG, $\lambda_{\text{max}}=519\text{ nm}$ (1.2 mW cm^{-2}) and red: ISL-150X150-RR, $\lambda_{\text{max}}=659\text{ nm}$ (0.9 mW cm^{-2}), CCS Inc., Kyoto, Japan). The intensity of the LED light source was measured with a light meter (LM-331, AS ONE, Osaka, Japan). The determination of the photon fluence of these light sources is described in the Results and discussion section.

Calculation of the molecular orbital energies of folic acid and MTX

The molecular orbital (MO) energy of folic acid and MTX was estimated from the *ab initio* MO calculation at the Hartree-Fock/6-31G* level. The structures of these molecules were also optimized by this calculation. The order of the energy levels of MO in the ground state was calculated by this method to estimate the electron transfer direction in the photoexcited state. This calculation was performed utilizing Spartan'06 (Wavefunction Inc., CA, USA).

Results and discussion

Strategy for the fluorometry of $^1\text{O}_2$ using folic acid and MTX

The calculated energy level of the MO showed that the aminobenzoyl moieties of folic acid and MTX are the electron-donating groups, suggesting that the fluorescence from their pteridine moieties can be quenched through intra-molecular electron transfer from their aminobenzoyl moieties [20,24,26]. It has been reported that the $\text{C}^9\text{-N}^{10}$ bonds connecting the pteridine and aminobenzoyl moieties of folic acid [20-24,27-29] and its analogues [26,29-32] are unstable against oxidative stress agents. Singlet oxygen-mediated cleavage of

this C⁹-N¹⁰ bond or oxidation of the electron-donating moiety should recover the fluorescence intensity of their pteridine moieties (Fig. 1).

Photochemical properties of folic acid and MTX and their photosensitized decomposition

The absorption and fluorescence spectra of folic acid and MTX are presented in Fig. 2. In general, pteridine compounds absorb the ultraviolet A region and show fluorescence in the visible-light region. Folic acid and MTX scarcely absorb visible light, indicating that visible light irradiation itself does not decompose these molecules. Folic acid showed weak fluorescence in D₂O, whereas the photosensitized folic acid by MB, a ¹O₂-generator [33], exhibited strong fluorescence due to the formation of strongly fluorescent 6-formylpterin [29] through the oxidation of folic acid by ¹O₂. Similarly, the photosensitized MTX in the presence of MB in D₂O showed strong fluorescence in the visible light region, although the fluorescence of MTX itself could not be detected. This fluorescence can be assigned to the π^* - π transition of the pteridine compound [26]. The fluorescence enhancement of photosensitized MTX can be explained by the oxidative decomposition of MTX by ¹O₂.

The photosensitized fluorescence enhancement of folic acid was observed in H₂O as well as D₂O (Fig. 3). This fluorescence enhancement of folic acid in H₂O is not due to ¹O₂ (described in latter). On the other hand, the fluorescence enhancement of MTX photosensitized by MB was hardly observed in H₂O. This result can be explained by the short lifetime of ¹O₂ (τ_A) in H₂O (~3 μ s) compared with that in D₂O (~68 μ s) [34]. Fig. 4 shows the effect of sodium azide, a strong physical quencher of ¹O₂, on the fluorescence enhancement of folic acid and MTX. The fluorescence enhancement of folic acid and MTX in D₂O was suppressed by sodium azide, supporting the ¹O₂-mediated decomposition of folic acid and MTX. However, sodium azide did not completely suppress their fluorescence

enhancements, suggesting the partial contribution of another mechanism, such as photo-induced electron transfer, to the decomposition of folic acid and MTX. The fluorescence intensity of the folic acid solution photosensitized by MB in H₂O was not decreased by the addition of sodium azide, indicating that folic acid is photo-oxidized by MB through a mechanism other than ¹O₂ formation in H₂O. Therefore, the ¹O₂-mediated decomposition of folic acid and MTX and the resulting fluorescence enhancement can be evaluated in D₂O. The contribution of ¹O₂ to the fluorescence enhancement (ΔI_{SO}) is expressed as follows:

$$\Delta I_{SO} = \Delta I_{obs} - \Delta I_{azide} \quad (1),$$

where ΔI_{obs} represents the observed fluorescence enhancement in D₂O and ΔI_{azide} the fluorescence enhancement in the presence of sodium azide. On the other hand, superoxide and H₂O₂ themselves do not decompose folic acid and MTX in the absence of a metal ion (data not shown), which indicates that the effects of these reactive oxygen species on the fluorescence enhancement can be negligible.

Time course of the fluorescence enhancement of MTX photosensitized by MB

The photosensitized decomposition of folic acid and MTX in the presence of MB proceeded linearly with the irradiation time. The value of the ΔI_{SO} was estimated from the photosensitized reaction of MTX by MB and plotted within 60 min (Fig. 5). The decomposition rate of MTX increased in proportion to the concentration of MB up to ~2 μ M under this experimental condition. Due to a filter effect, the fluorescence intensity of the decomposed MTX reached a plateau when a high concentration (> 5 μ M) of MB was used

(data not shown). The inset shows the relationship between the slope of the fluorescence enhancement of MTX and the absorbed photon ratio (PR) at the maximum wavelength of the light source (659 nm), which depends on the concentration of MB and is expressed as follows:

$$PR = 1 - 10^{-Abs} \quad (2),$$

where Abs represents the absorbance of the photosensitizer at the maximum wavelength of the light source. Similar results were obtained in the case of folic acid. These results indicate that the quantitative fluorometry of 1O_2 is possible when the value of PR is within ~ 0.6 (absorbance: ~ 0.4).

Relationship between the fluorescence enhancement of folic acid analogues and the Φ_Δ of the photosensitizer

Folic acid and MTX were irradiated in the presence of several photosensitizers, NB (Φ_Δ in D_2O : 0.005) [35], AZB (0.20) [35], AZA (0.43) [35], MB (0.52) [33], TMPyP (0.57) [1], NMB (0.68) [35], and RB (0.76) [1], using appropriate LED light sources. The value of the PR of these photosensitizers at the maximum wavelength of the light source was about 0.2 $\sim$ 0.5 (absorbance: 0.1 $\sim$ 0.3). The number of absorbed photon (PN , $\mu mol\ min^{-1}$) by these photosensitizers was calculated from the overlap of their absorption spectra and the emission spectra of the light source (Fig. 6). The values of PN can be calculated by the following equation:

$$PN = \int (1 - 10^{-Abs(\nu)}) E(\nu) S d\nu \quad (3),$$

where $Abs(\nu)$ represents the absorbance of the photosensitizer, $E(\nu)$, the photon fluence of the light source at the indicated light frequency ($\mu\text{mol cm}^{-2} \text{ min}^{-1} \text{ s}$), ν , the light frequency (s^{-1}), and S , the irradiated area (1 cm^2 , under this experimental condition). The fluorescence intensity increased proportionally to the irradiation time with each photosensitizer used in the experiment, as in Fig. 5 (data not shown). In the case of NB, a clear fluorescence enhancement of folic acid was not observed due to the background fluorescence of folic acid itself. The value of ΔI_{SO} divided by PN almost linearly increased with the Φ_{Δ} of the photosensitizers (Fig. 7). These findings showed that this method using folic acid and MTX can be applied to determine the Φ_{Δ} of a photosensitized reaction. MTX is superior to folic acid for the determination of the Φ_{Δ} of photosensitizers because the background fluorescence of MTX is negligible.

Protocol for the determination of Φ_{Δ}

This method can be applied to the determination of the Φ_{Δ} of visible light photosensitizers. Because folic acid and MTX absorb ultraviolet light, resulting in their decomposition, $^1\text{O}_2$ generation by ultraviolet photosensitizers cannot be measured using these folic acid analogues. To detect $^1\text{O}_2$ generated via a photosensitized reaction, a sample solution containing $20 \mu\text{M}$ folic acid or MTX and photosensitizers are mixed in D_2O . The appropriate absorbance of the photosensitizer at the maximum wavelength of the light source is about $0.04 \sim 0.4$. The concentration of the photosensitizers should be adjusted to this condition. A visible-light-emitting LED is recommended as the light source because of its defined emission spectrum and reduced heat formation. The sensitivity of this assay is proportional to the irradiation time, and several minutes ($10 \sim 30 \text{ min}$) may be sufficient for a general photosensitizer. The PN value can be estimated from the measurement of the absorption

spectrum of the photosensitizer and the emission spectrum of the light source using Equation (3) or, in practice, with the following equation:

$$PN = \sum \left\{ (1 - 10^{-Abs(\nu)}) E(\nu) S \Delta \nu \right\} \quad (4),$$

where $\Delta \nu$ represents the sampling interval of the light frequency. The value of Φ_{Δ} is calculated by the following equation:

$$\Phi_{\Delta} = \Phi_{\Delta}^r \frac{\Delta I_{SO}^s}{PN_s} \frac{PN_r}{\Delta I_{SO}^r} \quad (5),$$

where Φ_{Δ}^r represents the 1O_2 quantum yield of a reference photosensitizer, ΔI_{SO}^s , the fluorescence enhancement of the sample solution, ΔI_{SO}^r , the fluorescence enhancement of the reference solution, PN_s , the absorbed photon number by the sample photosensitizer, and PN_r , the absorbed photon number by the reference photosensitizer.

In addition, the quantitative determination of 1O_2 is possible using this method. The amount of substance of 1O_2 (n_{SO}) generated by photosensitized reaction can be determined by the following equation:

$$n_{SO} = PN \Phi_{\Delta} T = \Phi_{\Delta}^r PN_r \frac{\Delta I_{SO}^s}{\Delta I_{SO}^r} T \quad (6),$$

where T represents the irradiation time. In the steady state during photo-irradiation, the concentration of 1O_2 ($[^1O_2]_{st}$) can be expressed as follows:

$$[^1O_2]_{st} = \frac{PN \Phi_{\Delta} \tau_{\Delta}}{V} = \frac{PN_r \Phi_{\Delta}^r \tau_{\Delta}}{V} \frac{\Delta I_{SO}^s}{\Delta I_{SO}^r} \quad (7),$$

where V represents the volume of the sample.

Reactivity of folic acid and MTX with 1O_2

In general, 1O_2 is easily quenched by various molecules through a physical mechanism into ground state 3O_2 rather than by a chemical reaction [2,29]. It has been reported that folic acid is oxidized by 1O_2 into 6-formylpterin [29]. In the interaction between 1O_2 and folic acid, the rate constants for the physical quenching (k_q) and the chemical reaction (k_r) are $2.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $2.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively [29]. In the present study, the decomposition yield (Φ_{dc}) of MTX by 1O_2 was roughly estimated from the analysis with high-performance liquid chromatography (HPLC) [36] to determine the value of k_r . The obtained value of Φ_{dc} was 4.0×10^{-3} , and k_r can be expressed as follows:

$$\Phi_{dc} = \frac{k_r [\text{MTX}]}{\frac{1}{\tau_\Delta} + (k_q + k_r) [\text{MTX}]} \quad (8),$$

where $[\text{MTX}]$ represents the MTX concentration (20 μM under this experimental condition). For this calculation, the value of k_q ($4.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) [29] by *N*-methylpterin, which has only one different substituent (R1 of *N*-methylpterin = OH in Fig. 1) from MTX, is used, since the k_q is considered to be scarcely affected by the slight difference of the substituent. The value of k_r can be roughly calculated using the above mentioned values, and the resulting value of k_r is $3.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This reaction rate is slightly slower than that of reported sensitive fluorescence probes, such as dimethylantracene derivatives [17], and suggests that several minutes is required for the 1O_2 assay using these folic acid analogues.

Although the oxidation mechanism of MTX is complicated and the oxidized products could not be identified in this study, the formation of strongly fluorescent pteridine compounds, such as 2,4-dimethyl-6-hydroxymethylpteridine (fluorescence quantum yield in D₂O, $\Phi_f=0.41$), 2,4-dimethyl-6-formylpteridine, 2,4-dimethyl-6-pteridinecarboxylic acid, aminopterin ($\Phi_f=0.024$), and/or *N*¹⁰-methylpteroic acid ($\Phi_f=0.021$), is speculated. These compounds were reported as decomposed products of MTX through ultraviolet irradiation [26,30-32] or a product resulting from its catabolic oxidation [37]. Although the Φ_f of some compounds was not determined and samples could not be obtained, these pteridine compounds should be strongly fluorescent compared with MTX. The fluorescence spectrum of *N*¹⁰-methylpteroic acid, a hydrolysate of MTX, was quite similar to that of the decomposed product of MTX by ¹O₂, although direct evidence of its formation has not been obtained.

Conclusions

Singlet oxygen generated via photosensitized reaction decomposes less-fluorescent folic acid and MTX, leading to the formation of a strongly fluorescent pteridine compound in D₂O. This method can be applied to determine the Φ_Δ of photosensitizers absorbing visible light. One of the most important applications of the photosensitized reaction is PDT, in which a water-soluble photosensitizer absorbing visible light is necessary. The determination of Φ_Δ is required to evaluate the activity of the PDT photosensitizer. In addition, the value of Φ_Δ is important for the risk evaluation of phototoxic materials and various other studies about the photochemical oxidation process. In many cases, the determination of Φ_Δ of the water-soluble photosensitizer was carried out in D₂O, because the detection of ¹O₂ is easier due to its longer lifetime than that of H₂O. The present method can be simply performed for these purposes without an expensive apparatus and reagent.

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References and notes

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36. The decomposition of MTX was evaluated with an HPLC. The apparatus and the method were similar to those in a previous report [20]. A mobile phase containing 0.020 M monobasic potassium phosphate and acetonitrile (800:100, v/v) was prepared. The concentrations of the detected MTX were estimated from the absorbance at 370 nm.
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Figure captions

Fig. 1 Scheme of the formation of strongly fluorescent pteridines through $^1\text{O}_2$ -mediated decomposition of folic acid and MTX

Fig. 2 Absorption and fluorescence spectra of folic acid and MTX in D_2O . The photo-irradiated sample contained 20 μM folic acid or MTX and 2 μM MB. Where indicated, the sample was irradiated for 30 min with a red-color LED. The excitation wavelengths for the fluorescence measurements were 350 and 370 nm for folic acid and MTX, respectively. The ϵ indicates the molar absorption coefficient

Fig. 3 Fluorescence spectra of folic acid and MTX in H_2O . The sample solution contained 20 μM folic acid (A) or MTX (B) with or without 2 μM MB. Where indicated, the sample was irradiated for 30 min with a red-color LED. The excitation wavelengths for the fluorescence measurements were 350 and 370 nm for folic acid and MTX, respectively

Fig. 4 Effect of sodium azide on the fluorescence enhancement of photosensitized folic acid and MTX by MB. The sample solution contained 20 μM folic acid or MTX, 2 μM MB, and the indicated concentration of sodium azide in D_2O or H_2O . The sample was irradiated for 30 min with a red-color LED. The excitation wavelengths for the fluorescence measurements were 350 and 370 nm for folic acid and MTX, respectively

Fig. 5 Time course of the fluorescence enhancement of MTX photosensitized by MB. A sample solution containing 20 μM MTX and the indicated concentration of MB was irradiated with a red-color LED. The excitation wavelength for the fluorescence measurement was 370

nm. The inset indicates the dependence of the slope of the fluorescence enhancement on the absorbed photon ratio (PR) by MB. The determination of the PR is described in the Results and discussion section

Fig. 6 Emission spectra of the LED light source and the absorption spectra of the photosensitizers used in this experiment

Fig. 7 Relationship between the rate of the fluorescence enhancement and the quantum yield of photosensitized 1O_2 generation. $\Delta I/PN$, the determination of which is described in the Results and discussion section, indicates the rate of the fluorescence enhancement of folic acid and MTX per number of photons absorbed by the photosensitizer

Folic acid or MTX: Less fluorescent

Folic acid: R1 = OH, R2 = H

MTX: R1 = NH₂, R2 = CH₃

Pteridine:
Strongly fluorescent

Folic acid: R3 = CHO
MTX: R3 = unidentified

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7