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Vitamin K analogue as a new fluorescence probe for quantitative antioxidant assay

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ABSTRACT

A synthesized vitamin K model compound, NQ-6 (2-hexyloxy-1,4-naphthoquinone) showed weak fluorescence around 440 nm in ethanol. Addition of antioxidants such as vitamin E to a NQ-6 solution suppressed the NQ-6 emission quantitatively. A kinetic study on the quenching of the NQ-6 emission by hydrogen-donor type antioxidants (three tocopherol analogues, catechin, and 2,6-di-*tert*-butyl-4-methylphenol) in ethanol was performed. The quenching rate constant obtained from the Stern–Volmer plots for the steady-state fluorescence intensity was consistent with the second-order rate constant of each antioxidant for the free-radical scavenging. The NQ-6 emission is thought to be the delayed fluorescence caused by the thermal population to the excited singlet state from the triplet state. Thus, the fluorescence quenching occurred through a hydrogen atom transfer reaction from an antioxidant to NQ-6 in the excited triplet state (^{3}NQ-6*). The second-order rate constant for the reaction between ^{3}NQ-6* and α-tocopherol was estimated to be 1.2×10^{10} M^{-1} s^{-1} from the quenching parameter and the ^{3}NQ-6* lifetime in ethanol (1.2 μs) measured with the transient absorption. From the high reactivity of ^{3}NQ-6* to the antioxidants and the amphiphilic property of NQ-6 as a vitamin K model, NQ-6 is applicable to the quantitative antioxidant assay as a new fluorescence probe.

1. Introduction

Antioxidant assays in vivo or in vitro have been practiced widely in medical, pharmacological, and biological research, because many natural antioxidants contained in biological systems have been believed to play vital roles for protecting livings against oxidative stresses [1–4]. Some methods for measuring antioxidant activity of materials have already been known and used in research of many fields [5–8]. For example, inhibition of substrate oxidation by antioxidants has been measured by steady-state absorption or spin-trapping EPR method [5,9–11]. There, meta-stable radicals such as DPPH (1,1-diphenyl-2-picrylhydrazyl) and the aryloxyl radical, or more reactive short-lived radicals such as *t*-butyloxyl have often been used as models of the reactive oxygen species [9–12]. Time-resolved laser-photolysis measurements have also been applied using excited state molecules such as benzophenone in the $n\pi^*$ triplet state as a reactant in place of the model radicals [12,13]. However, more sensitive methods which can be applicable in quantitative antioxidant assays are desirable because they could realize measurements for a little bit amount of samples, and even those for spatial distribution of antioxidant actions. Fluorescence detection is one of the best solutions to these needs, and many fluorescence probes for antioxidant assays have been reported [14–16]. But such synthesized probe molecules are rather different from biomolecules in structure, and sometimes have obstacles to use for antioxidant assays in model biological systems because of their solubility and activities. One of the desirable probes is a model of some biologically important molecules, having good bioaffinity or amphiphilic property such as surfactants.

2-Methyl-1,4-napthoquinone derivatives are found in biological systems, and are known as vitamin K (VK) [17]. VKs have some biological functions. For example, they are required for normal blood clotting for animals and human beings, and they play important roles as an electron acceptor in the photoelectron transport system of the photosynthetic reaction center [17–19]. Natural VKs such as vitamin K_1 (Fig. 1a) and K_2 have a lipophlic long alkyl chain at the 3-position and their head, 1,4-naphtoquinone moiety, has some polarity. Thus, these natural VKs are thought to be amphiphilic and can be distributed in the interface region of hydrophobic phase and aqueous phase in biological systems as well as vitamin E (VE). Vitamin K_3 (VK3, 2-methyl-1,4-napthoquinone, Fig. 1b) is a synthesized VK having no alkyl chain and has almost the same biochemical functions as the natural VKs. The photophysics and photochemistry of 1,4-napthoquinone analogs (NQs) including these VKs have been a

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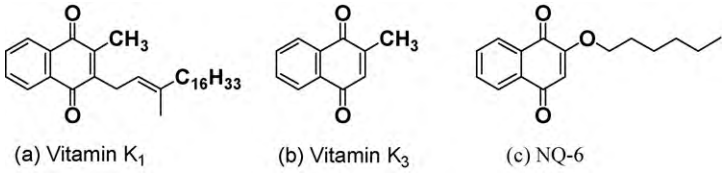


Fig. 1. Molecular structures of vitamin K_1, vitamin K_3, and NQ-6.

subject of many studies [20–27]. Most of NQs have scarce fluorescence (FL), because the quantum yield of the intersystem crossing (ISC) from the lowest excited singlet state (S_1) to the excited triplet state (T_1) is very large (ϕ_{isc} = 0.74 for 1,4-napthoquinone in acetonitrile [24]). In contrast, strong phosphorescence from the $n\pi^*$ triplet state of NQs can be observed in glassy media at low temperature. Previous studies also reported the unique photophysical phenomena such as the dual phosphorescence and the delayed fluorescence due to the property that the S_1 and two excited triplet states (T_1 and T_2) lie nearly from each other in energy [21,22].

Because of the large ISC quantum yield and the high reactivity of the triplet state, photochemical actions by NQs occur mainly in the excited triplet state [24–28]. UV-A irradiation from the sun to biological systems containing VKs has possibility to induce injuries and photodegradations of tissues, such as skins of animals and leaves of plants. In biological and pharmacological studies, NQs are often used as a photosensitizer for generating the reactive oxygen species, such as singlet oxygen, or for generating the radicals or cations of functional biomolecules, such as DNA and thymine [28]. The excited triplet state NQs ($^3NQs^*$) often abstract a hydrogen atom from hydrogen-donors, such as phenols and amines, in some cases at the rate near the diffusion controlled limit [24–29]. Such hydrogen atom transfer reactions to $^3NQs^*$ from hydrogen-donors are also a model of the bioprotection reaction by the antioxidants suppressing photodegradations and photoinjuries. Therefore, NQs may be used as a photochemical tool for antioxidant assays in place of benzophenone. However, the complicated method, such as the time-resolved absorption or the spin-trapping EPR, may be required for the antioxidant study using NQs.

In a previous study [29], we prepared 2-hexyloxy-1,4-napthoquinone (NQ-6, Fig. 1c) as a VK model as a photochemical probe in biomembrane systems. We found that NQ-6 has stronger FL than the other NQs, and that the FL is efficiently suppressed by existence of some antioxidants, such as vitamin E (VE). This property of NQ-6 may be applicable to the antioxidant assay using the steady-state FL method. In this report, we have investigated quenching of the NQ-6 FL by some typical antioxidants (Fig. 2) quantitatively, and the origin of the NQ-6 emission and its quenching mechanism are discussed. The rate constants of the FL quenching by the antioxidants are estimated from quantitative analysis based on Stern–Volmer plots, and the values are compared to the rate constants for free-radical scavenging in the literature.

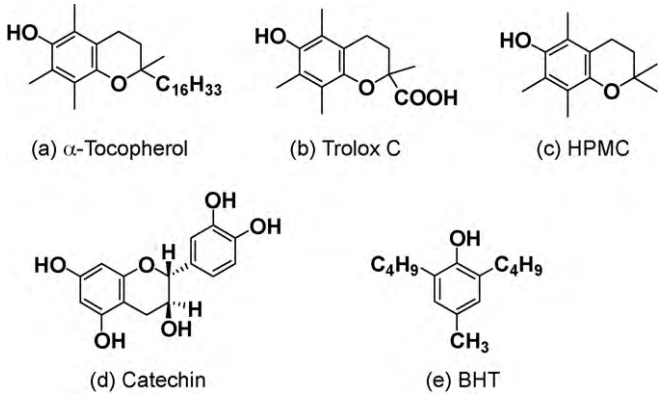


Fig. 2. Molecular structures of the antioxidants used in this study.

2. Experimental

NQ-6 was prepared according to the reported method [29]. 1,4-Napthoquinone (NQ), VK3, 2,6-di-*tert*-butyl-4-methylphenol (BHT), and α-tocopherol were commercially available reagents (Wako Pure Chemicals) and were used as received. Catechin from Sigma–Aldrich, and Trolox C (TC, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Fig. 2b) from Fluka were also used as received. 6-Hydroxy-2,2,5,7,8-pentamethylchroman (HPMC, Fig. 2c) was obtained from Eisai and was used as received. Ethanol (Wako) was dried and purified by distillation. Diethyl ether from Wako was used as received.

Absorption spectra were measured with a Shimadzu UV-1240 spectrophotometer controlled by a PC. Emission spectra were measured at room temperature (R.T.) with a Shimadzu RF-5000 spectrofluorophotometer controlled by a PC. Emission time profiles were measured at R.T. by a single-photon counting time-resolved near-infrared fluorescence spectrophotometer (Hamamatsu C-7990-01) using an Nd-YAG laser (CryLas FTSS355Q, THG: 355 nm, 14 kHz, FWHM 1 ns) as an excitation light source [30]. Emission quantum yields were measured by an absolute photoluminescence (PL) quantum yield measurement system (Hamamatsu C-9920-02). Sample solutions were deoxygenated by pump-freeze-thaw cycles before the emission measurements.

Transient-absorption spectra were measured at R.T. by a nanosecond laser-photolysis system (UNISOKU TSP-1000) using an Nd-YAG laser (Continuum Surelight-I, THG 355 nm, FWHM < 5 ns, 2 Hz) as an excitation light source [31,32]. Time profiles of the transient absorption were obtained by accumulating 8 waves with a digital oscilloscope (Sony-Tektronix TDS3032). Sample solutions were deoxygenated by pump-freeze-thaw cycles before the measurements.

3. Results and discussion

3.1. Absorption and emission spectra of NQ-6

Fig. 3 shows absorption spectra of NQ-6, NQ, and VK3 in ethanol. Almost the same absorption peak wavelength (330 nm) and molar absorption coefficient ($\log \varepsilon$ = 3.51 at 330 nm) were obtained for these NQs. A broad absorption band of each NQ around 330 nm has been assigned to the S_0–S_1 transition having large $n\pi^*$ character [21]. For NQ-6, an increase in absorption at 360–400 nm was observed. A similar band was also observed for 2-hydroxy-1,4-naphthoquinone. This band is thought to be another $^1n\pi^*$ transition induced by the alkyloxy chain in the 2-position of NQ-6, otherwise due to an increase in allowance of the S–T (singlet–triplet) forbidden absorption.

Fig. 4a shows emission spectra of NQ-6 obtained in ethanol at R.T. with RF-5000 and at 77 K with C-9920-02, together with the absorption spectrum at R.T. The excitation wavelength was set at 335 nm for the emission measurement at R.T. and at 355 nm for that

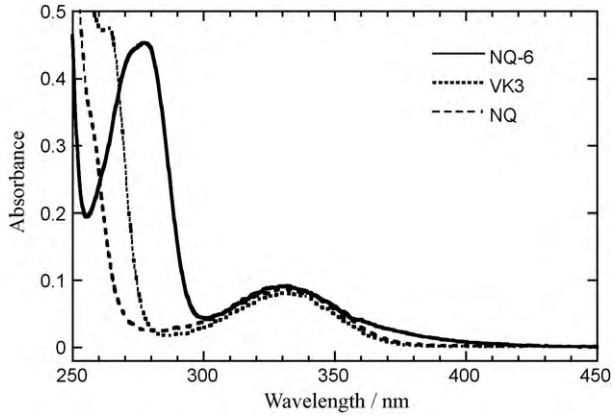


Fig. 3. Absorption spectra of NQ-6, NQ, and VK3 in ethanol. The concentration of NQs was 2.80×10^{-5} M.

at 77 K. An emission band around 440 nm was observed at R.T. and the small corresponding band was also at 77 K. As shown in Fig. 4b, the excitation spectrum for this band almost coincided with the absorption band of NQ-6. From the small energy gap between the absorption and emission bands, this emission band around 440 nm can be assigned to FL due to the $S_1 \rightarrow S_0$ transition ($n\pi^*$). The quantum yield of this NQ-6 emission band in ethanol at R.T. could not be determined, but it should be a little less than the lowest limit of our PL apparatus (~0.004). On the other hand, another emission band around 600 nm was observed in the glass medium at 77 K. This emission band is thought to be the phosphorescence band of NQ-6. The quantum yield of this band was 0.009.

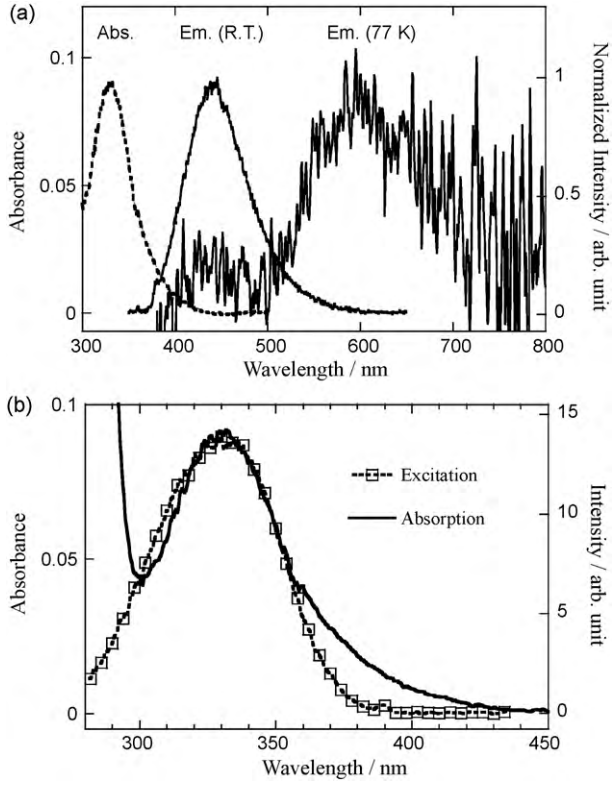
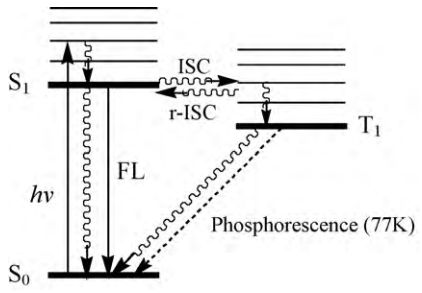


Fig. 4. (a) Emission spectra of NQ-6 (2.77×10^{-5} M) in ethanol at room temperature and at 77 K. The emission intensities were normalized at the peak intensity. The absorption spectrum of NQ-6 is also shown as the broken line. (b) Coincidence between absorption spectrum and excitation spectrum of NQ-6 emission at 430 nm observed in ethanol at R.T.



Scheme 1. Kinetic diagram of photoexcitation and decay processes for NQs.

The photophysical property of NQ-6 should resemble those of NQ and VK3. Actually, the NQ-6 emission spectrum resembled those for NQ and VK3, although the intensity of the emission band around 440 nm for NQ-6 in ethanol at R.T. was more than 20 times as large as those for NQ and VK3. In the literature [21,22], the fluorescence and phosphorescence bands of NQ and VK3 in the gas phase were located around 450 nm (22,000 cm^{-1}) and 540 nm (18,500 cm^{-1}), respectively. There, the fluorescence of these NQs was explained by an E-type delayed fluorescence [21]. The delayed fluorescence is an emission from the S_1 state which is thermally populated from the T_1 state lying nearby S_1 in energy. Scheme 1 shows a possible diagram for the delayed fluorescence of NQs. After the photoexcitation of NQs, the rapid ISC from S_1 to T_1 occurs efficiently. As a result, the direct FL from the S_1 state is always scarce in NQs. However, the thermal population from T_1 to S_1 is partially possible through the reversed ISC (r-ISC) when the S–T energy gap in NQs is relatively small. Then, the emission due to the transition from thermally populated S_1 to the S_0 state can be observed as the delayed fluorescence. In this case, the lifetime of the delayed fluorescence is essentially equal to that of the precursor T_1 state.

The NQ-6 emission around 440 nm observed at R.T. is also likely due to the delayed fluorescence as well as those in NQ and VK3. On the basis of this mechanism, the large emission intensity of NQ-6 can be explained by enlargement of the S_1–T_1 interaction through r-ISC. This situation should be induced by the alkyloxy ($-O-(CH_2)_nCH_3$) group in the 2-position of NQ-6. In fact, NQ-6 analogues, which are NQ-1 (2-methoxy-1,4-naphthoquinone), NQ-10 (2-decyloxy-1,4-naphthoquinone), and NQ-16 (2-hexadecyloxy-1,4-naphthoquinone), show similar property in absorption and emission to that of NQ-6.

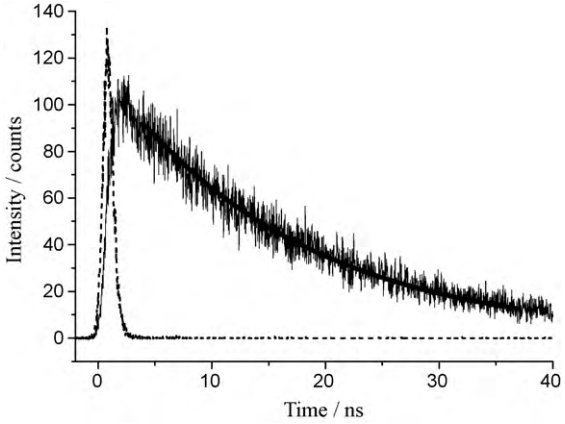


Fig. 5. Time profile of NQ-6 emission in ethanol at 425 nm measured by single-photon counting ([NQ-6] = 3.02×10^{-5} M). (Dotted line shows the pulse shape of the excitation laser at 355 nm.)

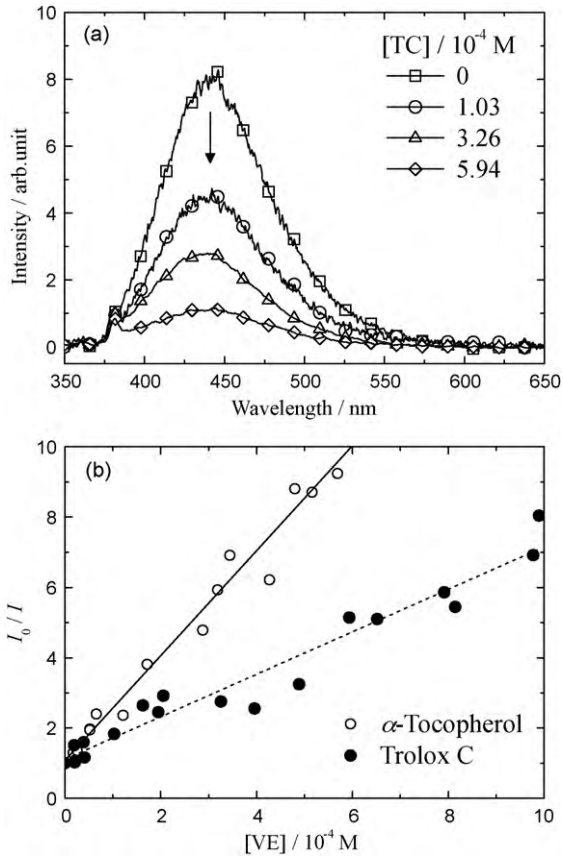


Fig. 6. (a) Change of NQ-6 emission spectrum in ethanol with addition of TC. [NQ-6] = 2.79×10^{-5} M and [TC] = 0, 1.03, 3.26, and 5.94×10^{-4} M. (b) Stern–Volmer plots on quenching of the NQ-6 emission at 410 nm by α-tocopherol (open circles) and TC (closed circles).

Fig. 5 shows a time profile of the NQ-6 emission at 440 nm measured in ethanol by single-photon counting. A fast decay was observed within 50 ns, and the decay data could be fitted to a single exponential curve whose lifetime $\tau_f = 16.5$ ns. This short lifetime suggests that this emission decay comes from the direct FL of NQ-6 due to the $S_1 \rightarrow S_0$ transition. There may be another weak and slow component, whose contribution is less than 1%. The decay component due to the delayed FL could not be measured with our time-resolved FL spectrophotometer, because the intrinsic intensity of the delayed FL was much smaller than that of the direct FL.

3.2. Quenching of NQ-6 emission by antioxidants

The steady-state FL intensity of NQ-6 decreased significantly by addition of antioxidants, such as VE. Fig. 6a shows variation of NQ-6 emission spectrum in ethanol at R.T. with addition of TC. The excitation wavelength was set at 335 nm where TC has no absorption. The FL intensity decreased gradually with increase of the TC concentration ([TC]). The result indicates that TC quenched the NQ-6 FL state (NQ^*) quantitatively (reaction (1)). Similar result was obtained by using each of α-tocopherol, HPMC, catechin, and BHT, as an additive. FL of VK3 was also quenched by addition of these antioxidants, but the FL intensity of VK3 was too small for us to analyze the obtained data quantitatively.

$$NQ^* + TC \rightarrow (\text{quenching products}) \quad (1)$$

In general, when quenching of an emission state by a quencher (Q) progresses through a bimolecular process, the relation represented by Eq. (2) based on Stern–Volmer (S–V) analysis should hold.

Table 1
Kinetic values ($\tau_0 k_Q$ and k_Q) of the antioxidants in ethanol estimated in this study. Reported k_s values of the antioxidants for free-radical scavenging are also listed [11,33–36].

Antioxidant	$\tau_0 k_Q / 10^3$ M^{-1}	$k_Q / 10^9$ M^{-1} s^{-1}	$k_s / 10^2$ M^{-1} s^{-1}
α-Tocopherol	14.9	12	51.2
Trolox C (TC)	6.04	5.0	22.3
HPMC	11.8	9.8	40.2
Catechin	1.41	1.2	0.826
BHT	1.17	0.97	0.350

$$\frac{I_{FL}^0}{I_{FL}} = 1 + \tau_0 k_Q [Q] \quad (2)$$

Here, I_{FL} and I_{FL}^0 are apparent FL intensities with and without a quencher Q, and τ_0, k_Q, and [Q] are the lifetime of the emission state in the absence of Q, the quenching rate constant for Q, and the concentration of Q, respectively. In the analysis, reduction of the quencher concentration during a few minutes of FL measurements by some processes such as photolysis or side-reactions is thought to be negligible. Fig. 6b shows S–V plots for quenching of the NQ-6 emission by VE analogues (α-tocopherol and TC). A linear relationship was obtained for each VE, suggesting that the FL quenching by VE progressed quantitatively. The $\tau_0 k_Q$ values estimated from slopes of the S–V plots were 1.49×10^4 and 6.04×10^3 M^{-1} for α-tocopherol and TC, respectively. Linear relationships on S–V plots were also obtained for other antioxidants (HPMC, catechin, and BHT), and the estimated $\tau_0 k_Q$ values are listed in Table 1. The standard errors in the $\tau_0 k_Q$ values were 2.5–5%. The order (α-tocopherol ≥ HPMC > TC ≫ catechin > BHT) in the $\tau_0 k_Q$ value is analogous to that in the rate constant for the aryloxyl-radical scavenging in the literature [11,33–36]. The k_Q values in the NQ-6 quenching may be related to the antioxidant activity. In diethyl ether solutions, the $\tau_0 k_Q$ values (3.65×10^3 M^{-1} for α-tocopherol and 5.87×10^2 M^{-1} for BHT) could also be estimated in the same way.

Assuming that the NQ-6 emission consisted only of the direct FL, the k_Q value for α-tocopherol was estimated to be 8.8×10^{11} M^{-1} s^{-1} by using $\tau_0 = 17$ ns as the lifetime of the emission state. This k_Q value is too large for a rate constant of an intermolecular reaction in solutions, because it is to a great extent over the diffusion-controlled limit ($k_{dif} = 0.92 \times 10^{10}$ M^{-1} s^{-1} for ethanol [37]). Such a large value is possible only for limited reactions, such as the reactions in geometrically restricted donor–acceptor systems. In the present system, each concentration of NQ-6 and antioxidants was very low (10^{-5}–10^{-4} M), and association or complex formation between NQ-6 and antioxidants was thought to be negligible because the shapes of the absorption and emission spectra of NQ-6 were not affected by addition of the antioxidants. Furthermore, the decay rate of the NQ-6 FL measured by single-photon counting was constant with addition of the antioxidants. Therefore, the antioxidants did not quench S_1 directly, but quenched another state related to the NQ-6 emission. Thus, we should use another value as τ_0 in this NQ-6 FL quenching system.

NQ-6 emission is thought to consist of the primary direct FL and the delayed FL. Thus, the NQ-6 emission is possible to be quenched both in S_1 and in T_1. Although the S_1 lifetime is too short (17 ns), the lifetime of the delayed FL, which is thought to be equal to the T_1 lifetime, might be large (~1 μs) enough to be quenched by the antioxidants in solutions. Assuming that the NQ-6 FL quenching by antioxidants progresses through the quenching reaction of the excited triplet state of NQ-6 (^{3}NQ-6*), the lifetime of the delayed FL or that of ^{3}NQ-6* should be used as τ_0 in estimation of k_Q from $\tau_0 k_Q$. In this case, from the fact that the NQ-6 emission was approxi-

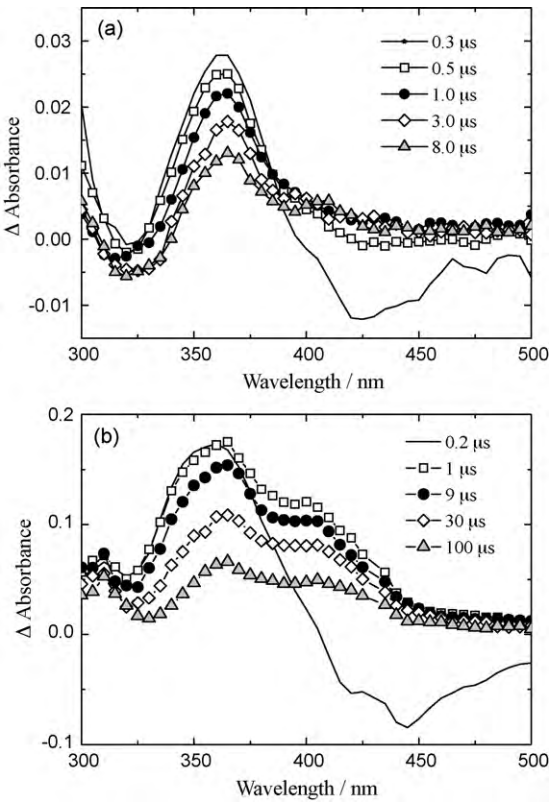


Fig. 7. (a) Time evolution of transient-absorption spectrum in the photolysis of NQ-6 (3.22×10^{-4} M) in ethanol. (b) Time evolution of transient-absorption spectrum in the photolysis of NQ-6 (3.10×10^{-4} M) with α-tocopherol (1.60×10^{-3} M) in ethanol.

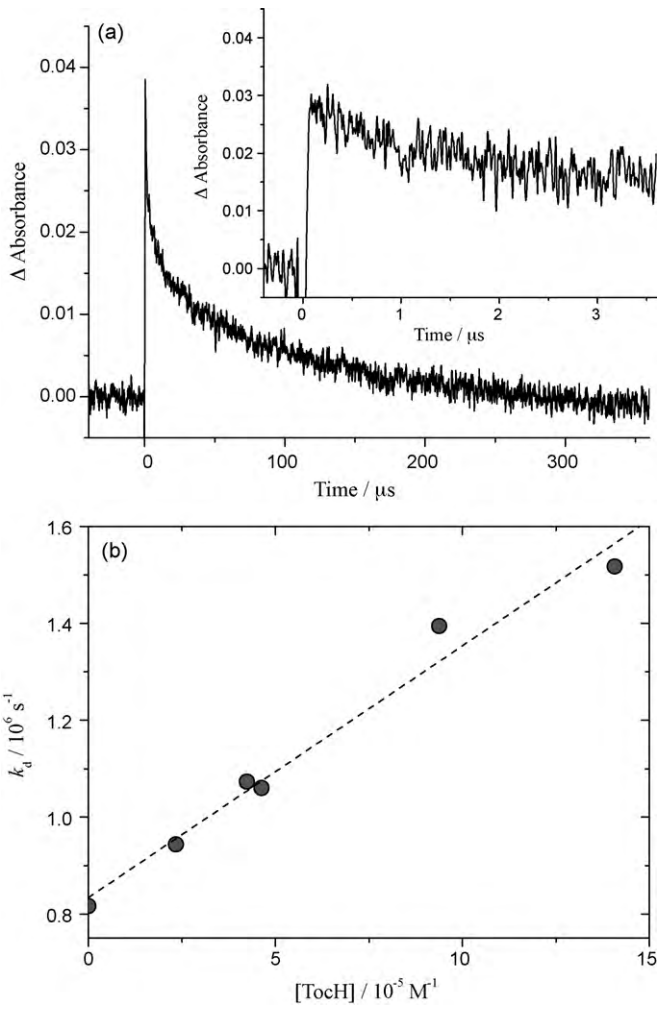


Fig. 8. (a) Time profile of transient absorption at 360 nm in the photolysis of NQ-6 (3.22×10^{-4} M) in ethanol. (b) Plots of the apparent rate constant of the fast decay component (k_d) vs. concentration of α-tocopherol ([TocH]).

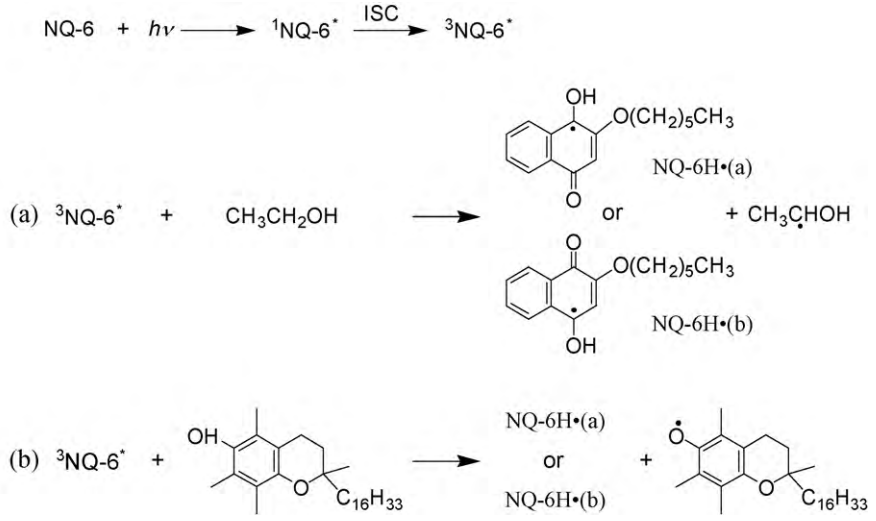
mately quenched in [VE] > 1.2×10^{-3} M, contribution of the primary FL in the total emission intensity would be negligible in the analysis. We tried the time-resolved measurement for the delayed FL lifetime but failed because of the relatively small intensity. Then, the transient-absorption measurements for the lifetime of ^{3}NQ-6* (τ_T) were performed.

3.3. Transient absorption in photolysis of NQ-6

Fig. 7a shows time-evolution of transient-absorption spectrum obtained for a photolysis of NQ-6 in ethanol. There are a transient-absorption band around 360 nm and a transient bleaching around 320 nm which decayed with time. Another negative band around 430 nm which disappeared rapidly within 300 ns after the laser pulse is due to the NQ-6 emission. The peak of the absorption shifted slightly from 360 nm to 370 nm in a few microseconds. The result suggests the existence of two transient species whose absorption bands are overlapped in 350–420 nm. According to the literature on photolysis of NQ and VK3 [26,27,38], one is thought to be ^{3}NQ-6*, and another is thought to be the semiquinone radical of NQ-6 (NQ-6H$^\bullet$). Fig. 8a shows the time profile of the transient absorption at 360 nm. The decay curve of the absorption band around 360 nm consists of two components; one is a fast decay within 4 μs and another is a slow component still observed at more than 100 μs after the photoexcitation. The fast decay component is thought to be due to ^{3}NQ-6*, and the slow decay component is thought to come from NQ-6H$^\bullet$.

The photoreaction processes for NQ-6 in ethanol are represented in Scheme 2. ^{3}NQ-6* produced through ISC from S_1 of NQ-6 (^{1}NQ-6*) abstracts a hydrogen atom from ethanol, and then NQ-6H$^\bullet$ and the ethanol radical are produced. This photoreaction scheme was confirmed by the time-resolved EPR (TR-EPR) [29], and the formation of two kinds of NQ-6H$^\bullet$ (NQ-6H$^\bullet$(a) and NQ-6H$^\bullet$(b) in Scheme 2a) was shown. These NQ-6H$^\bullet$ could not be distinguished from each other in the transient-absorption spectrum. A least square fits of the fast decay component data at 360 nm to a single exponential curve gave τ_T = 1.21 μs as the lifetime of ^{3}NQ-6* in ethanol. The standard error in the τ_T value was ~10%. This τ_T value is smaller than that reported for NQ or VK3 in acetonitrile (~1.7 μs). The reason is that ^{3}NQ-6* can reacts with solvent ethanol and the reactivity of ^{3}NQ-6* is larger than NQ and VK3 in the T_1 state as described in the TR-EPR reports [25,29]. On the other hand, the slow component due to NQ-6H$^\bullet$ whose apparent lifetime is τ_R = 101 μs should be characterized by a second-order decay. The NQ-6H$^\bullet$ decay is thought to be mainly caused by a bimolecular self-quenching.

Coexistence of antioxidants decreased the ^{3}NQ-6* lifetime through fast quenching reactions. Fig. 7b shows time-evolution of transient-absorption spectrum observed on a photolysis of NQ-6 in the presence of α-tocopherol in ethanol. The spectrum has the transient bleaching around 330 nm and the transient-absorption band around 360 nm due to ^{3}NQ-6* and NQ-6H$^\bullet$. In addition, a new band due to the tocopheroxyl radical (Toc$^\bullet$) could be observed in 380–440 nm. This result indicates that a rapid reaction between ^{3}NQ-6* and α-tocopherol occurred as the hydrogen atom transfer reaction and it produced NQ-6H$^\bullet$ and Toc$^\bullet$ (Scheme 2b). This result agrees with the TR-EPR result [29]. The Toc$^\bullet$ band around 420 nm



Scheme 2. Photoreaction schemes for NQ-6 in ethanol with and without α-tocopherol.

decayed slowly in 100 μs [32]. The time profile of absorbance at 360 nm consists of two components. One is the fast decay within a few microseconds due to ^{3}NQ-6*. Another slow component should be characterized by the NQ-6H• bimolecular quenching. The apparent lifetime of the slow component was constant (101 μs) and independent of the α-tocopherol concentration ($0–5 \times 10^{-4}$ M), suggesting that the reaction between NQ-6H• and α-tocopherol is rather slow ($\ll 10^6\ M^{-1}\ s^{-1}$). In contrast, the rate constant of the fast decay component due to ^{3}NQ-6* increased with increase of the α-tocopherol concentration (Fig. 8b).

The rate constant (k_Q^T) of quenching ^{3}NQ-6* by an antioxidant Q can be obtained from a plot of the apparent decay rate (k_d) of ^{3}NQ-6* versus concentration of Q ([Q]), according to Eq. (3). The transient-absorption experiments on the NQ-6 photolysis were tried in some concentrations of α-tocopherol. However, the measurements for the ^{3}NQ-6* time profile were very difficult in large α-tocopherol concentration because of interference by overlap of large absorption of NQ-6H• and Toc•. From the k_d values estimated by least squares fits of decay data for the 360 nm absorption to a single exponential curve, the k_Q^T value of α-tocopherol in ethanol was estimated to be $5.2 \times 10^9\ M^{-1}\ s^{-1}$ (Fig. 8b). This value agreed with that expected from the TR-EPR result ($\geq 10^9\ M^{-1}\ s^{-1}$) [29]. However, this k_Q^T value might have rather large error because of the difficulty in measuring the ^{3}NQ-6* time profiles. Although we could estimate the k_Q^T value from the transient-absorption measurements, such a method is difficult, complicated, and sometimes inaccurate because of interference of the species other than the object in antioxidant assay.

$$k_d = \frac{1}{\tau_T} + k_Q^T[Q] \quad (3)$$

By using the value $\tau_T = 1.2$ μs from the transient absorption as the emission lifetime τ_0 in Eq. (2), the quenching rate constant (k_Q) for α-tocopherol was determined to be $1.2 \times 10^{10}\ M^{-1}\ s^{-1}$. This k_Q value is larger than the k_Q^T value estimated above, and also larger than the value reported for the quenching of triplet benzophenone by α-tocopherol ($5 \times 10^9\ M^{-1}\ s^{-1}$ in benzene [12]). The τ_T value estimated in the transient-absorption experiment might be smaller than the real value for the delayed FL quenching. Otherwise, the above k_Q^T value estimated from the transient absorption of ^{3}NQ-6* might be inaccurate because of interference of large absorption of NQ-6H• and Toc•, or the excitation laser scattering. The k_Q value of α-tocopherol is a little larger than the diffusion controlled limit described above. The practical rate constant of the diffusion controlled limit in ethanol is thought to be slightly larger than $0.9 \times 10^{10}\ M^{-1}\ s^{-1}$ according to the rate constants reported for $^1O_2$ quenching by carotenoids [39]. From the results, it is sure that the NQ-6 FL quenching occurs through the ^{3}NQ-6* quenching reaction by antioxidants.

In the same way, the k_Q values for the other antioxidants in ethanol were determined from $\tau_0 k_Q$ values, and summarized in Table 1. The k_Q values should have ~10% of uncertainties due to that of the τ_T value. The k_Q value of each antioxidant is reasonable as the rate constant for the quenching of the triplet state carbonyl compounds. In diethyl ether solutions, the k_Q values could be determined as $1.1 \times 10^{10}\ M^{-1}\ s^{-1}$ for α-tocopherol and $1.7 \times 10^9\ M^{-1}\ s^{-1}$ for BHT by using the value $\tau_T = 0.34$ μs estimated from the transient absorption. Although $\tau_0 k_Q$ value of each antioxidant in diethyl ether was much smaller than that in ethanol, the k_Q value in diethyl ether was close to that in ethanol. This fact also confirms that the NQ-6 FL quenching occurs through the ^{3}NQ-6* quenching reaction by antioxidants.

The results demonstrate that the k_Q values estimated from the steady-state quenching measurements on the NQ-6 delayed FL are reliable as well as those from the time-resolved measurements, even though the steady-state experiment is fairly easy and simple compared with the transient absorption and TR-EPR.

3.4. Comparison in activities of NQ-6 FL quenching and free-radical scavenging by antioxidants

The k_Q values of α-tocopherol, HPMC, TC, catechin, and BHT determined in this study are listed in Table 1, together with the second-order rate constants of these compounds reported for the aryloxyl-radical-scavenging reaction (k_s). The k_Q and k_s values are largely different from each other in individual antioxidant, but show a similar trend on the structure–activity relationship. The difference between k_Q and k_s comes from, of course, the difference in reactivity between ^{3}NQ-6* and the aryloxyl radical. While the ratio of the k_Q value for α-tocopherol to that for TC is close to the corresponding ratio of k_s, the ratio of the k_Q value for catechin to that for BHT is smaller than the corresponding ratio of k_s. The difference in k_Q between antioxidants having relatively low activity might be small. However, the higher reactivity of ^{3}NQ-6* makes it possible to measure the antioxidant parameter (k_Q) in lower antioxidant concentration in contrast to the k_s measurements, where solubility sometimes causes experimental difficulties. On the other hand,

measurements for some antioxidants having larger k_Q values than α-tocopherol may be difficult, because the k_Q values would be close to the diffusion control limit. However, concerning α-tocopherol is known as the most effective antioxidant for the radical scavenging, this FL quenching method can be used for the antioxidant assay for most of the other antioxidants, which have smaller k_Q than α-tocopherol.

As described above, the measurements of NQ-6 FL quenching by antioxidants would give a quantitative parameter for the antioxidant activity against the reactive oxygen species, as well as the time-resolved measurements using photochemical probes such as the other carbonyl compounds. The steady-state FL measurement is always more sensitive than absorption measurements, and is much easier than EPR, transient-absorption, and time-resolved fluorescence measurements. So, the FL detection can be applied to investigation for smaller amount of sample, for spatial distribution, and for opaque systems such as oil/water emulsions and cell dispersions. Furthermore, amphiphilic compounds such as NQ-6 is thought to exist in the interface region of the oil and water phases in micelle or reversed micelle systems, and also in biomembrane systems. This property is useful in the investigation of biological model systems. We have also succeed to prepare some NQ-6 analogues, such as NQ-1 (2-methoxy-1,4-naphthoquinone), NQ-10 (2-decyloxy-1,4-naphthoquinone), and NQ-16 (2-hexadecyloxy-1,4-naphthoquinone). These NQ-6 analogues modeled on natural VKs show similar FL property to NQ-6. On the other hand, their solubility and location in biomembranes or micelle systems are somewhat different from each other, because of their alkyl side-chain length. We can choose an appropriate compound from the NQ-6 derivatives for the purpose or the target system of our study. From these properties, even though the FL intensity of NQ-6 analogue is much smaller than those of the other FL probes, NQ-6 analogues give an easy and reliable method for antioxidant assays by simple steady-state FL measurements applicable to a wide variety of systems.

4. Conclusion

A VK analogue NQ-6 showed weak FL around 440 nm in ethanol. This NQ-6 FL was quantitatively quenched by antioxidants such as VE. The quenching rate parameter ($\tau_0 k_Q$) for three tocopherol analogues, catechin, and BHT was estimated from the Stern–Volmer plot of the steady-state FL intensity. Because the NQ-6 emission is thought to come from the delayed FL caused by the thermal population to S_1 from T_1 through r-ISC, the FL quenching occurs through a hydrogen atom transfer reaction from each antioxidant to ^{3}NQ-6*. The second-order rate constant (k_Q) for the reaction between ^{3}NQ-6* and α-tocopherol was estimated to be $1.2 \times 10^{10}\,M^{-1}s^{-1}$ from the $\tau_0 k_Q$ value and the ^{3}NQ-6* lifetime in ethanol. The k_Q values estimated from the NQ-6 FL quenching measurements are consistent with the second-order rate constant of each antioxidant for the free-radical scavenging, suggesting that the k_Q values can be used as a reliable parameter for antioxidant assay. Because NQ-6 has VK-like amphiphilic property and the reaction between ^{3}NQ-6* and antioxidants can be detected with the sensitive steady-state FL, NQ-6 has a large possibility as a new FL probe applicable to antioxidant assays in a wide variety of systems.

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References

[1] B. Halliwell, Annu. Rev. Nutr. 16 (1996) 33–50.
[2] B. Halliwell, J.M.C. Gutteridge, C.E. Cross, J. Lab. Clin. Med. 119 (1992) 598–620.
[3] E. Niki, Free Radic. Res. 33 (2000) 693–704.
[4] I.S. Young, J.V. Woodside, J. Clin. Pathol. 54 (2001) 176–186.
[5] D. Huang, O.U. Boxin, R.L. Prior, J. Agric. Food Chem. 53 (2005) 1841–1856.
[6] J.-K. Moon, T. Shibamoto, J. Agric. Food Chem. 57 (2009) 1655–1666.
[7] G. Cao, H.M. Alessio, R.G. Cutler, Free Radic. Biol. Med. 14 (1993) 303–311.
[8] B. Ou, M. Hampsch-Woodill, R.L. Prior, J. Agric. Food Chem. 49 (2001) 4619–4626.
[9] P. Stocker, J.-F. Lesgards, N. Vidal, F. Chalier, M. Prost, Biochim. Biophys. Acta 1621 (2003) 1–8.
[10] S. Rohn, L.W. Kroh, Mol. Nutr. Food Res. 49 (2005) 898–907.
[11] K. Mukai, A. Tokunaga, S. Itoh, Y. Kanesaki, K. Ohara, S. Nagaoka, K. Abe, J. Phys. Chem. B 111 (2007) 652–662.
[12] C. Evans, J.C. Scaiano, K.U. Ingold, J. Am. Chem. Soc. 114 (1992) 4589–4593.
[13] M.V. Encinas, E.A. Lissi, A.F. Olea, Photochem. Photobiol. 42 (1985) 347–352.
[14] A. Gomes, E. Fernandes, J.L.F.C. Lima, J. Fluoresc. 16 (2006) 119–139.
[15] P. Wardman, Free Radic. Biol. Med. 43 (2007) 995–1022.
[16] A. Gomes, E. Fernandes, J.L.F.C. Lima, J. Biochem. Biophys. Methods 65 (2005) 45–80.
[17] L. Stryer, Biochemistry, Freeman, New York, 2007.
[18] C.M. Jackson, Y. Nemerson, Annu. Rev. Biochem. 49 (1980) 765–811.
[19] S.L. Booth, Annu. Rev. Nutr. 29 (2009) 89–110.
[20] M.-A. Hangarter, A. Hörmann, Y. Kamdzhilov, J. Wirz, Photochem. Photobiol. Sci. 2 (2003) 524–535.
[21] T. Itoh, H. Baba, Bull. Chem. Soc. Jpn. 52 (1979) 3213–3216.
[22] T. Itoh, J. Chem. Phys. 87 (1987) 4361–4367.
[23] M.T. Craw, M.C. Depew, J.K.S. Wan, J. Magn. Reson. 65 (1985) 339–343.
[24] I. Amada, M. Yamaji, M. Sase, H. Shizuka, J. Chem. Soc. [Faraday Trans.] 91 (1995) 2751–2759.
[25] K. Ohara, Y. Hashimoto, C. Hamada, S. Nagaoka, J. Photochem. Photobiol. A 200 (2008) 239–245.
[26] Z. Zhang, S. Hao, H. Zhu, W. Wang, J. Photochem. Photobiol. B 92 (2008) 77–82.
[27] H. Görner, Photochem. Photobiol. Sci. 3 (2004) 71–78.
[28] J.R. Wagner, J.-E. van Lier, L.J. Johnston, Photochem. Photobiol. 52 (1990) 333–343.
[29] Y. Nishioku, K. Ohara, K. Mukai, S. Nagaoka, J. Phys. Chem. B 105 (2001) 5032–5038.
[30] K. Ohara, T. Origuchi, K. Kawanishi, S. Nagaoka, Bull. Chem. Soc. Jpn. 81 (2008) 345–347.
[31] H. Yamada, Y. Yamashita, M. Kikuchi, H. Watanabe, T. Okujima, H. Uno, T. Ogawa, K. Ohara, N. Ono, Chem. Eur. J. 11 (2005) 6212–6220.
[32] K. Ohara, A. Shimizu, Y. Wada, S. Nagaoka, J. Photochem. Photobiol. A 210 (2010) 173–180.
[33] A. Mitarai, A. Ouchi, K. Mukai, A. Tokunaga, K. Abe, J. Agric. Food Chem. 56 (2008) 84–91.
[34] K. Mukai, W. Oka, K. Watanabe, Y. Egawa, S. Nagaoka, J. Terao, J. Phys. Chem. A 101 (1997) 3746–3753.
[35] K. Mukai, Y. Kanesaki, Y. Egawa, S. Nagaoka, in: F. Shahidi, C.-T. Ho (Eds.), Phytochemicals and Phytopharmaceuticals, AOCS Press, Champaign, 2000, pp. 222–238.
[36] K. Mukai, S. Yokoyama, K. Fukuda, Y. Uemoto, Bull. Chem. Soc. Jpn. 60 (1987) 2163–2167.
[37] S.L. Murov, Handbook of Photochemistry, Macel Dekker, New York, 1973.
[38] J.F. Chen, X.W. Ge, G.S. Chu, Z.C. Zhang, M.W. Zhang, S.D. Yao, N.Y. Lin, Radiat. Phys. Chem. 55 (1999) 35–39.
[39] K. Fukuzawa, Y. Inokami, A. Tokumura, J. Terao, A. Suzuki, Lipids 33 (1998) 751–756.