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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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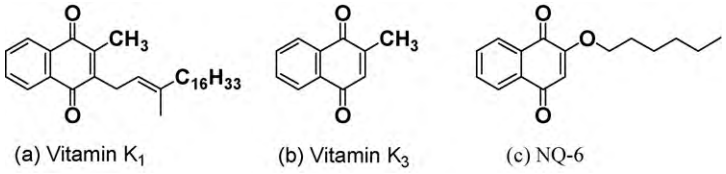

Fig. 1. Molecular structures of vitamin K_1, vitamin K_3, and NQ-6.

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.

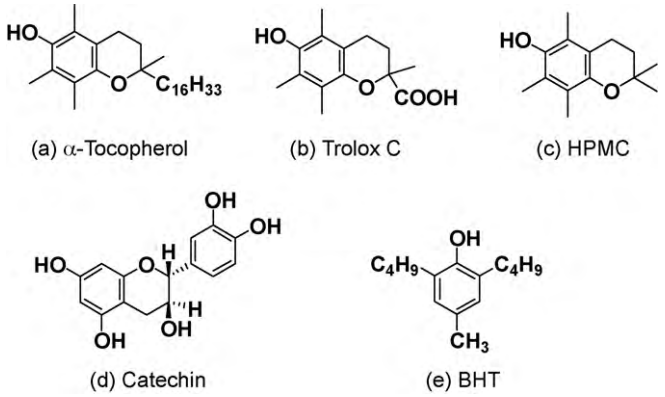

Fig. 2. Molecular structures of the antioxidants used in this study.

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