

Effects of Pin-up Oxygen on [60]Fullerene for Enhanced Antioxidant Activity

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Abstract The introduction of pin-up oxygen on C_{60} , such as in the oxidized fullerenes $C_{60}O$ and $C_{60}O_n$, induced noticeable increase in the antioxidant activity as compared to pristine C_{60} . The water-soluble inclusion complexes of fullerenes $C_{60}O$ and $C_{60}O_n$ reacted with linoleic acid peroxyl radical 1.7 and 2.4 times faster, respectively.

Keywords Fullerene C_{60} · Oxidized fullerene $C_{60}O$ · Antioxidant · γ -Cyclodextrin · PVP

Introduction

Fullerenes and its derivatives are well known as a new class of antioxidants and they have attracted considerable attention in biologic applications due to their high reactivity toward radicals [1], especially reactive oxygen species (ROS) such as superoxide [2], hydroxyl radical [3], peroxy radicals [4], and nitric oxide [5]. These harmful radicals attack lipids, proteins, DNA, and other biologic tissues and organs. It has been found that water-soluble fullerenes can be used as potential antioxidants and neuroprotective drugs against degenerative diseases related to oxidative stress [6–11]. Thus, water-soluble fullerenes, including host–guest inclusion complexes, are promising candidates for practical use as antioxidants.

However, such a radical scavenging ability has not been well investigated systematically for functionalized fullerenes, and the development of more efficient and easily accessible fullerene antioxidant derivatives has become an urgent requirement.

In this article, we first report that the introduction of pin-up oxygen on C_{60} , such as that in the oxidized fullerene (fullerene epoxide) $C_{60}O_n$, induces significant increase in the antioxidant activity as compared to pristine C_{60} . The relative radical scavenging rate constant k_{TRS} was kinetically determined using a β -carotene bleaching assay in the presence of water-soluble polyvinylpyrrolidone (PVP)-entrapped [12] and γ -cyclodextrin (CD)-capped [13] C_{60} and $C_{60}O_n$ ($n = 1$ and 0–4) [14] inclusion complexes (Fig. 1).

Experimental

Materials and Apparatuses

Fullerene C_{60} and oxidized fullerene $C_{60}O_n$ were purchased from Frontier Carbon Corporation. Polyvinylpyrrolidone (PVP K 30) was purchased from Wako Pure Chemical Industries, Ltd. Other reagents and organic solvents as well as pure water were all commercially available and used as received. UV-visible spectra were measured on a JASCO V-550 equipped with a thermal controller. LCMS analysis was performed on a SHIMADZU LCMS-2010EV. Ball mill grinding for the preparation of γ -cyclodextrin inclusion complexes was carried out using a FRITSCH pulverisette 6. DFT calculation of molecular orbital energy levels were performed using Spartan '04 software at B3LYP/6-31G* level of theory.

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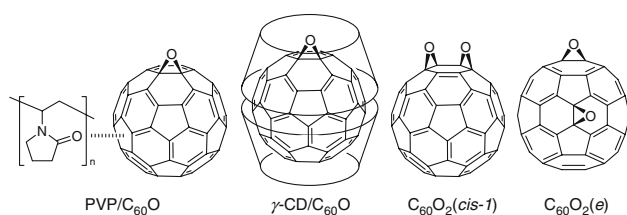


Fig. 1 Plausible structure of water-soluble complexes of [60]fullerene monoepoxide $C_{60}O$ and structure of major isomers of $C_{60}O_2$ (*cis-1* and *e*)

Synthesis of PVP/ C_{60} and its Oxidized Derivatives

A toluene solution (10 mL) of fullerene C_{60} (8 mg) was added to an ethanol solution (5 mL) of PVP (1 g) and stirred for 12 h at room temperature under air. After evaporation of the solvent, drying of the residue under vacuum at room temperature for 18 h gave PVP/ C_{60} quantitatively (1 g) as a brown solid.

Synthesis of γ -CD/ C_{60} and $C_{60}O$

Fullerene C_{60} (10 mg) and γ -cyclodextrin (70 mg) in an agate vessel (50 mL) together with a mixing ball made of zirconia ($0.3 \text{ g} \times 30$) were vigorously mixed by using ball mill at a rate of 650 rpm for 30 min. The milling was repeated by addition of ethanol (5 mL) for 30 min. After drying the ethanol, pure water (5 mL) was added and mixed again for 30 min. The mixture was centrifuged and the obtained solution was filtered through a membrane filter (0.45 and 0.1 μm) to give a clear purple solution. The concentration of solution and the yield were estimated to be 1.40 mM and 31.7%, respectively, by the use of the molar absorption coefficient $\varepsilon = 5.06 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ determined at λ_{max} 329 nm for the cyclohexane solution according to the previously reported method [13b]. The concentration and the yield for $C_{60}O$ were 682 μM and 25.1%, respectively ($\varepsilon = 3.25 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at λ_{max} 322 nm in cyclohexane).

β -Caroten Bleaching Method

Chloroform solutions of 11 μL of β -carotene (1.0 mg/mL), 4.4 μL of linoleic acid (0.1 g/mL) and 22 μL of Tween 40 (0.2 g/mL) were mixed in a quartz cell equipped with a screw-on cap, and then the solvent was removed in vacuo. An aliquot of the emulsion was immediately diluted with 2.4 mL of phosphate buffer solution (0.018 M, pH 7.0), and 0.1 mL of antioxidant (7.5–75 nmol, equivalent to C_{60}) in deionized water was added to the diluted mixture. The solution was mixed well and heated at 50°C under air in a

quartz cell on a UV spectrometer in order to monitor the decrease in the absorbance of β -carotene at 460 nm.

Results and Discussion

The water-soluble fullerene inclusion complexes were synthesized by modified literature method [12]. The formation of γ -CD/ $C_{60}O$ has been confirmed only by a mass spectrum [15]. Thus, we confirmed its formation (obtained as a brownish water solution including an excess of free γ -CD) and determined the concentration of solution using a UV-vis spectrometer by comparison of the peak absorbance around 360 nm in water to that of pristine $C_{60}O$ in cyclohexane (Fig. 2a). On the other hand, PVP/ $C_{60}O$ and $C_{60}O_n$ have not been reported so far and this is the first report (Fig. 2b).

The β -carotene bleaching assay is one of the common methods used in the field of food chemistry for evaluating antioxidant activity. The method is based on the discoloration of the yellowish color of a β -carotene solution due to the breaking of π -conjugation by the addition of lipid peroxy radical (LOO^\bullet) generated from the autoxidation of

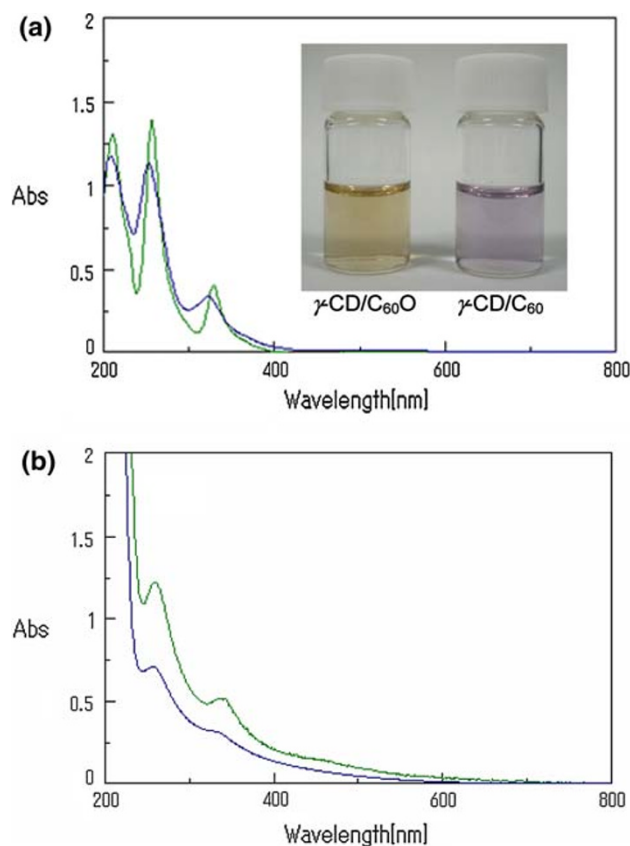


Fig. 2 UV-vis spectra of (a) γ -CD/ $C_{60}O$ (blue line) and γ -CD/ C_{60} (green line) and (b) PVP/ $C_{60}O$ (blue line) and PVP/ C_{60} (green line) in water (10 μM)

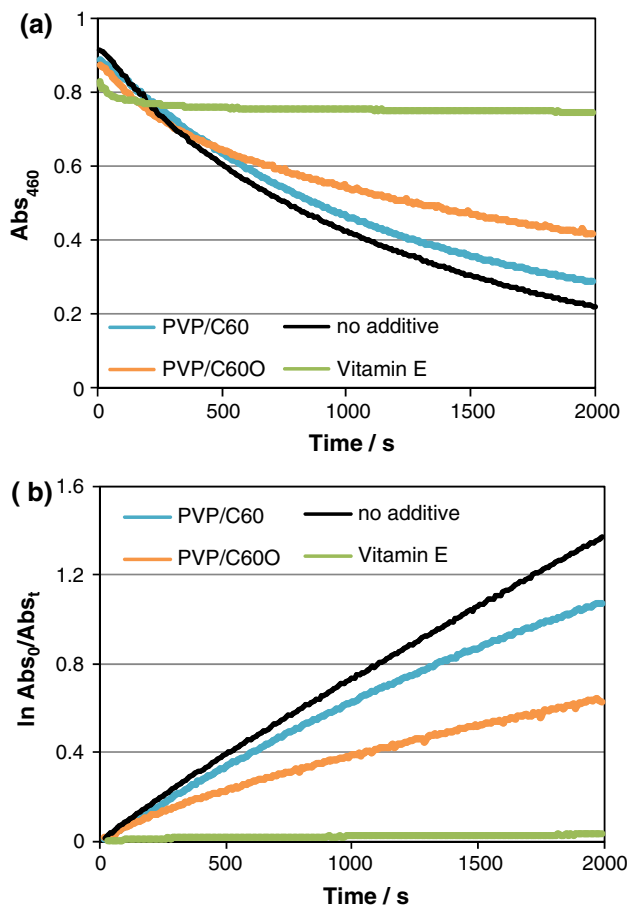


Fig. 3 β -Carotene bleaching assay with linoleic peroxy radical; (a) decay curves of absorbance at 460 nm (Abs_{460}) and (b) plots of $\ln(Abs_0/Abs_t)$ versus time in the presence of antioxidants (10 μ M), where Abs_0 is initial Abs_{460} and Abs_t is Abs_{460} at time t . Vitamin E was used as a positive control

linoleic acid under air atmosphere [16–18]. The assay was performed according to an optimally modified procedure (Fig. 3) [19].

Figure 4 shows the dependency of the pseudo-first-order rate constants, k_{obs} , for the discoloration of β -carotene on the antioxidant concentration of PVP and CD complexes of C_{60} and oxidized $C_{60}O$. Here, the rate (R_f) of discoloration of β -carotene by the LOO^\bullet radical is given by Eq. 1 [18], where k_c and k_f denote the second-order rate constants for the radical scavenging of β -carotene and fullerene antioxidant, respectively.

$$R_f = \frac{-d[\beta\text{-carotene}]}{dt} = k_{obs}[\beta\text{-carotene}] = k_c[\beta\text{-carotene}] \left(\frac{k_c[\beta\text{-carotene}]}{k_c[\beta\text{-carotene}] + k_f[\text{fullerene}]} \right) [LOO^\bullet] \quad (1)$$

It was found that the β -carotene bleaching was significantly suppressed by the increasing amount of antioxidants,

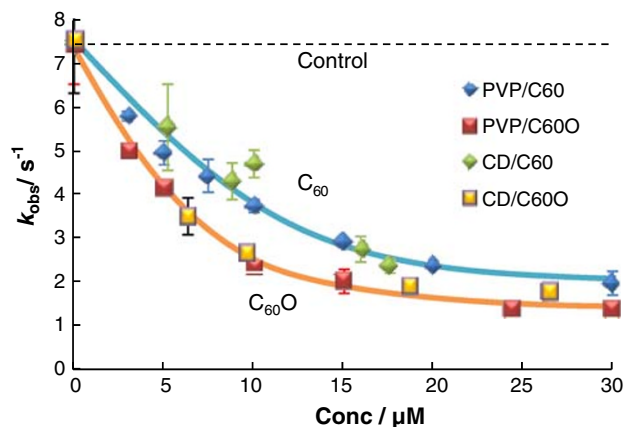


Fig. 4 Effects of antioxidant concentration on the observed pseudo-first-order rate constants k_{obs} of β -carotene bleaching with linoleic acid peroxy radical at 50°C. Values of k_{obs} were obtained by monitoring the absorbance of β -carotene aqueous solution (8.2 μ M) at 460 nm. The dotted horizontal line indicates the value of k_{obs} in the absence of antioxidants as a control

although $C_{60}O$ was more effective than C_{60} in all tested ranges of concentration. It was also noted that the entrapped PVP and CD exerted no appreciable effect on the antioxidant activity of guest fullerenes. To the best of our knowledge, this is the first result of the higher antioxidant activity of $C_{60}O$ in comparison with pristine C_{60} , despite the decreasing of π -conjugation. The concentration-dependent antioxidant activities %AOA [19] ($= 100 \times \{k_{obs} \text{ of control} - k_{obs} \text{ of fullerene}\} / k_{obs} \text{ of control}$) of PVP/ C_{60} and $C_{60}O$ were 50% and 68% in 10 μ M for antioxidant, and 73% and 81% in 30 μ M, respectively.

Here, it is more convenient to define the absolute antioxidant activity of fullerenes toward the LOO^\bullet radical by considering the relative radical scavenging rate constants k_{rrs} ($= k_f/k_c$) of fullerenes versus β -carotene, as given in Eq. 2 [18], where R_0 is the bleaching rate in the absence of antioxidants ($[\text{fullerene}] = 0$ in Eq. 1).

$$\frac{R_0}{R_f} = \frac{k_{obs} \text{ of control}}{k_{obs} \text{ of fullerenes}} = \frac{k_c[\beta\text{-carotene}] + k_f[\text{fullerene}]}{k_c[\beta\text{-carotene}]} = 1 + \frac{k_f[\text{fullerene}]}{k_c[\beta\text{-carotene}]} \quad \left(\frac{k_f}{k_c} = k_{rrs} \right) \quad (2)$$

As shown in Fig. 5, the plots of the ratio R_0/R_f versus the ratio of $[\text{fullerene}]/[\beta\text{-carotene}]$ gave a good regression line with intercept = 1 for each of the antioxidants, C_{60} , $C_{60}O$, and a commercially available mixture of fullerene oxide $C_{60}O_n$.¹ The dotted line indicates the value in the absence

¹ The $C_{60}O_n$, instead of $C_{60}O_2$ due to the difficulty in availability, was used to investigate the effect of the number of pin-up oxygen on C_{60} as well as the scope for the practical use. The component ratio of $C_{60}O_n$ was determined by LCMS (mass spectra and peak area) as follows: C_{60} , 22; $C_{60}O$, 33; $C_{60}O_2$, 27; $C_{60}O_3$, 14; $C_{60}O_4$, 5%.

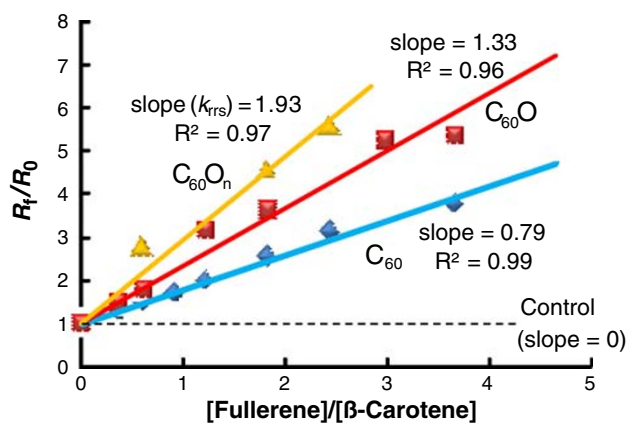


Fig. 5 Plots of the ratio of β -carotene bleaching rates in the presence (R_t) or absence (R_0) of fullerene antioxidants R_t/R_0 versus ratio of concentration [fullerene]/[β -carotene] for PVP-entrapped C_{60} , $C_{60}O$, and $C_{60}O_n$. The slope of each linear regression line corresponds to the relative radical scavenging rate constant k_{trs} relative to that of β -carotene. The dotted horizontal line indicates the value in the absence of antioxidants as a control

of antioxidants as a control (slope = 0). The slopes, $k_{trs} = 0.79$ (for C_{60}), 1.33 (for $C_{60}O$), and 1.93 (for $C_{60}O_n$), represent the efficiency of the antioxidants and thus $C_{60}O$ and $C_{60}O_n$ react with the LOO^\bullet radical approximately 1.7 and 2.4 times faster than C_{60} . There is a clear tendency that the introduction of pin-up oxygen on C_{60} increases its antioxidant activity.

In order to clarify the reason for the significant effect of the pin-up oxygen on the antioxidant activity of C_{60} , we calculated the energy level of LUMO and HOMO for the C_{60} , $C_{60}O$, and $C_{60}O_2$ as well as the energy level of SOMO for the LOO^\bullet and L^\bullet radical (Fig. 6). It was found that the pin-up oxygen lowers the LUMO level relative to those of pristine C_{60} . According to the Klopman and Salem equation [20] as well as the frontier molecular orbital (FMO) theory, the energy (ΔE) gained in the orbital interactions is inversely proportional to the energy difference |LUMO–SOMO|.

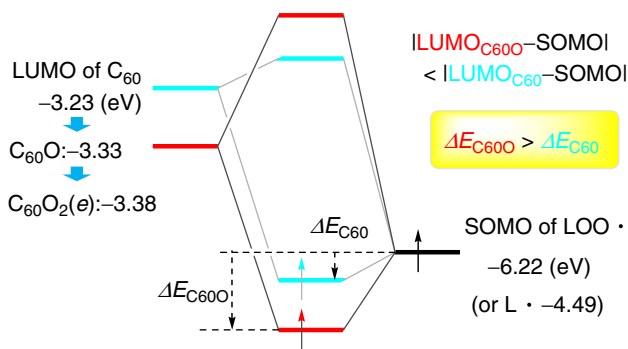


Fig. 6 Frontier molecular orbital interaction between LUMO of fullerenes C_{60} , $C_{60}O$, and $C_{60}O_2(e)$ and SOMO of linoleic acid peroxy radical (LOO^\bullet) or linoleic acid radical (L^\bullet) calculated by B3LYP/6-31G* level of theory

Thus, $C_{60}O$ can enjoy greater stabilization than C_{60} in capturing LOO^\bullet ($\Delta E_{C_{60}O} > \Delta E_{C_{60}}$), or possibly linoleic acid radical L^\bullet first formed in autoxidation, thus enhancing the antioxidant activity.²

Conclusion

In conclusion, we have found a meaningful key in developing new applicable antioxidants using fullerenes by means of a simple and conventional technique that can enhance their antioxidant activity by simply introducing pin-up oxygen on the fullerene cage.

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² Calculated HOMO levels of C_{60} , $C_{60}O$, and $C_{60}O_2(e)$ are -5.99 , -5.95 , and -5.99 eV, respectively, and less related to the present observation.

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