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Highly hydroxylated or γ-cyclodextrin-bicapped water-soluble derivative of fullerene: The antioxidant ability assessed by electron spin resonance method and β-carotene bleaching assay

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ABSTRACT

Antioxidant ability of the water-soluble derivative of fullerene (C60), prepared by high-degree hydroxylation [C60-$(OH)_{32}$·$8H_2O$] or C60/γ-cyclodextrin (1:2 mol/mol) clathrate formation [C60/$(\gamma\text{-CD})_2$], was assessed by electron spin resonance method and β-carotene bleaching assay. These C60 derivatives have an ability to diminish a 1:2:2:1 quartet ESR spectrum attributed to hydroxyl radicals (·OH) as shown by DMPO-spin trap/ESR method. Meanwhile, a singlet radical-signal different from ·OH-attributed signals increased in a manner dependent on concentrations of C60-$(OH)_{32}$·$8H_2O$. This might suggest that C60-$(OH)_{32}$·$8H_2O$ scavenges ·OH owing to dehydrogenation of C60-$(OH)_{32}$·$8H_2O$, and is simultaneously oxidized to a stable radical species, which may be a dehydrogenated fullerenol radical (C60-O·). Furthermore, these water-soluble derivatives of C60 suppressed fading of yellowish color characteristic of intact β-carotene in β-carotene bleaching assay. Antioxidant abilities of these derivatives were assessed as retention of yellowish color (viz absorbance at 470 nm) for 180 min. Namely, β-carotene-attributed chromaticity (% relative absorbance at 470 nm compared with the control) after 180 min was 69% for C60-$(OH)_{32}$·$8H_2O$ (400 μM: C60-eq.), and 32% for C60/$(\gamma\text{-CD})_2$ (400 μM: C60-eq.), whereas it was 6% for L(+)-ascorbic acid (400 μM) which is hydrophilic, and 85% for (±)-α-tocopherol (400 μM) which is lipophilic, respectively. Thus C60-$(OH)_{32}$·$8H_2O$ and C60/$(\gamma\text{-CD})_2$ can scavenge ·OH, and have a distinct antioxidative activity in the aqueous system containing linoleic acid which is abundantly contained in the cell membrane together with other unsaturated lipids. These C60 derivatives have a potential to protect the cell membrane from oxidative stress due to ·OH.

Fullerene-C60 (C60) originally dissolve only in carbon disulfide or aromatic solvents, which has been disadvantageous for bioassay.[1,2] Therefore, a variety of water-soluble reagents or polar groups has been attempted to render C60 to become water-soluble, and to utilize the scavenging ability against free-radicals effectively.[3] For example, using less-toxic water-soluble reagents, polyvinylpyrrolidone (PVP)-entrapped C60[4,5] or cyclodextrin-bicapped C60,[6–10] hydroxylated C60,[11–16] namely C60-$(OH)_n$ (fullerenol, n = 18–24), and various other derivatives have already been examined. Recently, an antioxidant ability of PVP-entrapped C60 and γ-cyclodextrin-bicapped C60 has been measured by β-carotene bleaching assay.[17] However, the characteristic of antioxidant abilities of diverse water-soluble derivatives of C60 has not sufficiently analyzed.

In the present study, highly hydroxylated fullerene [C60-$(OH)_{32}$·$8H_2O$] [conventionally C60-$(OH)_{18-24}$][11–16] and γ-cyclodextrin/fullerene (1:2 mol/mol) clathrate [C60/$(\gamma\text{-CD})_2$] were used. With each C60 derivative, scavenging ability against hydroxyl radicals was evaluated by electron spin resonance measurement (DMPO-spin trap/ESR method), and antioxidative activity in aqueous system including linoleic acid was examined by β-carotene bleaching assay. The results were compared with those of naturally occurring antioxidants, L(+)-ascorbic acid and (±)-α-tocopherol.

Butylated hydroxyanisol (BHA), linoleic acid, (±)-α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tween 40, β-carotene, hydrogen peroxide (30% solution in water), iron (II) sulfate heptahydrate, L(+)-ascorbic acid, disodium hydrogenphosphate 12-water, sodium dihydrogenphosphate dihydrate, and γ-cyclodextrin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec Co. (Tokyo, Japan). Highly hydroxylated fullerene [C60-$(OH)_{32}$·$8H_2O$ (estimated average composition)] was synthesized by Oshima Laboratory in Osaka University (Osaka)[18] and supplied from Vitamin C60 BioResearch Co. (Tokyo). Fullerene/γ-cyclodextrin (1:2 mol/mol) clathrate [C60/$(\gamma\text{-CD})_2$] were supplied from Vitamin C60 BioResearch Co. (Tokyo).

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Scavenging ability of water-soluble C60-fullerenes for chemically generated hydroxyl radicals by Fenton reaction was evaluated by DMPO-spin trap/ESR method. Reagents for Fenton reaction (1 mM H_2O_2 30 μL, 100 μM $FeSO_4$ 30 μL), sample solution [180 μL; C60-$(OH)_{32}$·$8H_2O$ or C60/(γ-CD)$_2$ or reversed-osmosis ultrapure water (control)], and spin trap reagent (1.79 M DMPO 60 μL) were mixed in a micro tube in this order.

After 30 s, the mixed solutions were poured in a flat quarts cell (Labotec Co., Tokyo), and started ESR measurement (FR-30, JEOL Ltd., Tokyo).

β-Carotene bleaching method is widely used to measure for antioxidant activity of plant extracts, etc.[19] It is an in vitro assay that measures the inhibition of coupled auto-oxidation of linoleic acid and β-carotene. β-Carotene (10 mg), linoleic acid (1 g), and Tween 40 (2 g) were dissolved in 10 mL of chloroform, respectively. Then each solution of β-carotene (0.25 mL), linoleic acid (0.1 mL), and Tween 40 (0.5 mL) was added to an Erlenmeyer flask. Chloroform was removed under a stream of N_2 gas. Reversed-osmosis ultrapure water (50 mL) and 0.2 M phosphate buffer (pH 7.0, 4.45 mL) were added and the solution was mixed. Aliquots (2.88 mL) of β-carotene and linoleic acid emulsion were mixed with the solution (0.12 mL) of C60-$(OH)_{32}$·$8H_2O$ or C60/(γ-CD)$_2$ in disposable cuvettes. The cuvettes were incubated at 50 °C in a water bath. Absorbance at 470 nm of each sample was measured immediately at 0 min and every 20 min up to 180 min with a double-beam spectrophotometer (U-2800, Hitachi High-Technologies Co., Tokyo). Retention of yellow-orange color attributed to β-carotene was calculated using the following formula:

β-Carotene-attributed chromaticity (%, relative absorbance at 470 nm compared with control) = $100 - \{(A_{\text{test at 0 min}} - A_{\text{test at t min}})/(A_{\text{control at 0 min}} - A_{\text{control at 180 min}})\} \times 100$.

Control: reversed-osmosis ultrapure water.

A four-peak ESR signal (1:2:2:1 quartet) which is indicative of a DMPO-OH adduct was detected in hydroxyl radicals generation system (Fenton reaction, Fig. 1). Relative intensities of DMPO-OH signals (DMPO-OH/MnO) were 11.1 for the control (reversed-osmosis ultrapure water), 0.62–0.90 for C60-$(OH)_{32}$·$8H_2O$, 2.39–3.64 for C60/(γ-CD)$_2$ (C60-eq. 2.5–200 μM), and 12.6–4.6 for the synthetic antioxidative reagent, butylated hydroxyanisol (BHA, 15–400 μM), meanwhile, γ-CD (without C60) did not diminish a DMPO-OH signal. Thus, these water-soluble C60 derivatives represented scavenging activity for hydroxyl radicals superior to BHA (Figs. 1 and 2). A distinct singlet signal was appeared with C60-$(OH)_{32}$·$8H_2O$, which was different from a DMPO-OH attributed one. This singlet signal remain without DMPO, and increased in a manner dependent on doses of C60-$(OH)_{32}$·$8H_2O$.

Na(+)-fullerenol (OH = 12–15) is reported to exist as stable radical anion at extremely low temperature (1.5–5.0 K).[20] Our results

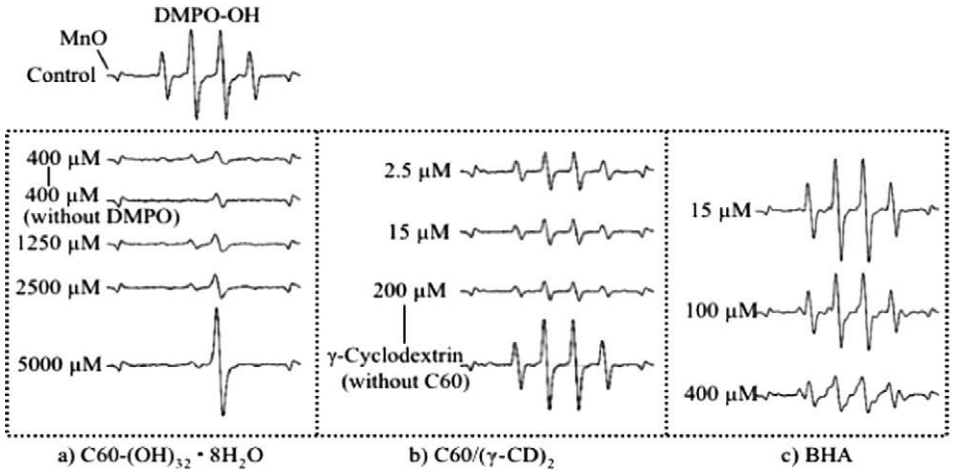


Figure 1. ESR spectra which show quenching effects on hydroxyl radicals with highly hydroxylated fullerene [C60-$(OH)_{32}$·$8H_2O$], fullerene/γ-cyclodextrin (1:2 mol/mol) clathrate [C60/(γ-CD)$_2$] and butylated hydroxyanisol (BHA). Control: reversed-osmosis ultrapure water. Molarity: C60-fullerene equivalent.

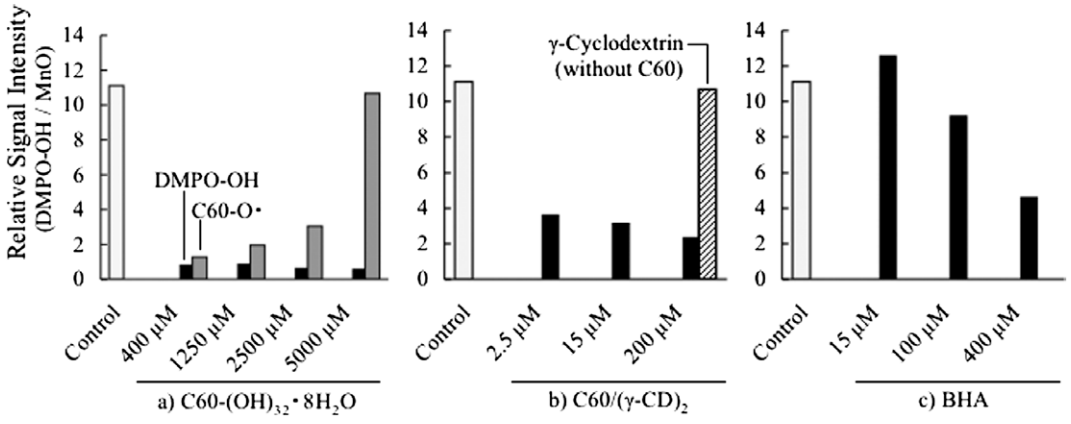


Figure 2. Quenching effects on hydroxyl radicals with (a) Highly hydroxylated fullerene [C60-$(OH)_{32}$·$8H_2O$], (b) fullerene/γ-cyclodextrin (1:2 mol/mol) clathrate [C60/(γ-CD)$_2$], and (c) butylated hydroxyanisol (BHA), Control: Reversed-osmosis ultrapure water. Molarity: C60-fullerene equivalent.

might suggest that C60-$(OH)_{32}$·$8H_2O$ scavenges hydroxyl radicals owing to dehydrogenation of C60-$(OH)_{32}$, and simultaneously might be oxidized itself to a stable radical species, assumedly a dehydrogenated fullerenol radical (C60-O·).

β-Carotene bleaching method is based on that lipid radicals as auto-oxidation products of linoleic acid attack double bonds of β-carotene, but antioxidative substance can retain β-carotene (yellowish-orange color) depend on their antioxidant ability. Water-soluble C60 [C60-$(OH)_{32}$·$8H_2O$, C60/(γ-$CD)_2$] restrained fading of yellowish color of β-carotene, so antioxidant ability of these derivatives were assessed by retention of yellowish color (viz absorbance at 470 nm) for 180 min (Fig. 3). Namely, β-carotene-attributed chromaticity [% relative absorbance at 470 nm = {($A_{\text{test at 0 min}} - A_{\text{test at t min}}$)/($A_{\text{control at 0 min}} - A_{\text{control at 180 min}}$)} × 100] was 69% for C60-$(OH)_{32}$·$8H_2O$ (400 μM: C60-eq.), and 32% for C60/(γ-$CD)_2$ (400 μM: C60-eq.). Meanwhile, in the case of naturally occurring antioxidants, it was 6% for L(+)-ascorbic acid (400 μM) which is hydrophilic, and 85% for (±)-α-tocopherol (400 μM) which is lipophilic (Fig. 4). γ-Cyclodextrin did not show an antioxidative activity (Scheme 1).

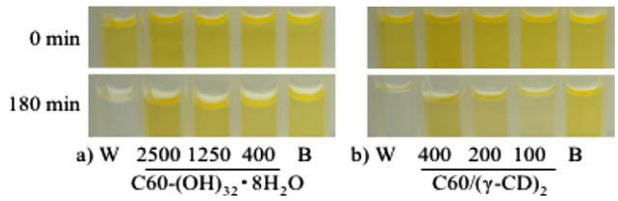
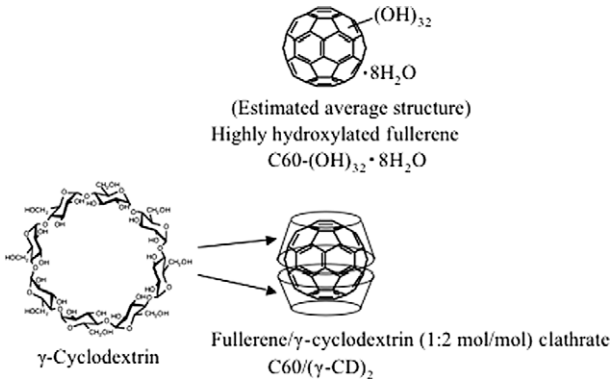


Figure 3. Discoloration of β-carotene/linoleic acid aliquots by addition of: (a) highly hydroxylated fullerene [C60-$(OH)_{32}$·$8H_2O$], (b) fullerene/γ-cyclodextrin (1:2 mol/mol) clathrate [C60/(γ-$CD)_2$], reversed-osmosis ultrapure water (W), and butylated hydroxyanisol (B), incubated at 50 °C in a water bath for 180 min, values represent C60-eq. molarity (μM) in each water-soluble C60-fullerenes.



Scheme 1. Water-soluble fullerenes.

These results suggest that C60-$(OH)_{32}$·$8H_2O$ and C60/(γ-$CD)_2$ exert superior antioxidative activity to L(+)-ascorbic acid though their water-solubility in the aqueous system with linoleic acid.

Thus C60-$(OH)_{32}$·$8H_2O$ and C60/(γ-$CD)_2$ can scavenge ·OH, and have a distinct antioxidative activity. Linoleic acid and other unsaturated lipids are contained in the cell membrane, and reactive oxygen species such as hydroperoxides, hydrogen peroxides, and·OH are generated in human epidermal keratinocytes undergoing UVB radiation.[21,22] It has been already reported that PVP-entrapped fullerene exerts a cytoprotective effect in human skin keratinocytes (HaCaT) against oxidative stress induced by the UVA irradiation.[23] Therefore, these derivatives of C60 are also expected to have a potential to protect the cell membrane from oxidative stress due to

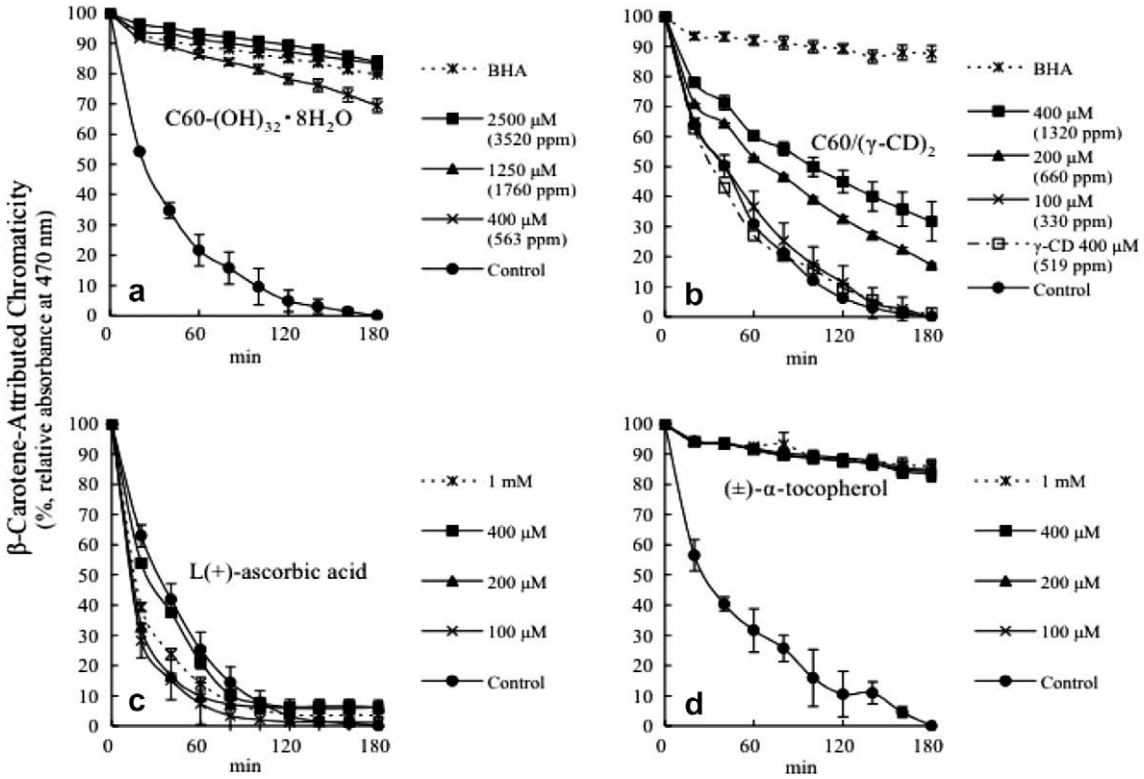


Figure 4. β-Carotene-attributed chromaticity (%, relative absorbance at 470 nm) by addition of (a) highly hydroxylated fullerene [C60-$(OH)_{32}$·$8H_2O$], (b) fullerene/γ-cyclodextrin (1:2 mol/mol) clathrate [C60/(γ-$CD)_2$], (c) L(+)-ascorbic acid, d) (±)-α-tocopherol, reversed-osmosis ultra pure water (Control), and butylated hydroxyanisol (BHA), incubated at 50 °C in a water bath for 180 min, values represent C60-eq. molarity (μM) in each water-soluble C60-fullerenes, and whole molecular weight (ppm). β-Carotene-attributed chromaticity % = 100 − {($A_{\text{test at 0 min}} - A_{\text{test at t min}}$)/($A_{\text{control at 0 min}} - A_{\text{control at 180 min}}$)} × 100. Mean ± SD ($n$ = 3).

·OH, and contribute to retention of functions and integrity of human skin cells.

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References and notes

1. Corona-Morales, A. A.; Castel, A.; Escobar, A.; Drucker-Colin, R.; Zhang, L. *J. Neurosci. Res.* **2003**, *71*, 121.
2. Bosi, S.; Da Ros, T.; Spalluto, G.; Prato, M. *Eur. J. Med. Chem.* **2003**, *38*, 913.
3. Nakamura, E.; Isobe, H. *Acc. Chem. Res.* **2003**, *36*, 807.
4. Ungurenasu, C.; Airinei, A. *J. Med. Chem.* **2000**, *43*, 3186.
5. Piotrovskii, L. B.; Kozeletskaia, K. N.; Medvedeva, N. A.; Dumpis, M. A.; Pozniakova, L. I.; Kiselev, O. I. *Vopr. Virusol.* **2001**, *46*, 38.
6. Tseng, W. Y.; Chen, Y. H.; Khairullin, II.; Cheng, S.; Hwang, L. P. *Solid State Nucl. Magn. Reson.* **1997**, *8*, 219.
7. Murthy, C. N.; Choi, S. J.; Geckeler, K. E. *J. Nanosci. Nanotechnol.* **2002**, *2*, 129.
8. Fillippone, S.; Heimann, F.; Rassat, A. *Chem. Commun. (Camb).* **2002**, *21*, 1508.
9. Ikeda, A.; Sato, T.; Kitamura, K.; Nisiguchi, K.; Sasaki, Y.; Kikuchi, J.; Ogawa, T.; Yogo, K.; Takeya, T. *Org. Biomol. Chem.* **2005**, *3*, 2907.
10. Liu, Y.; Liang, P.; Chen, Y.; Zhao, Y. L.; Ding, F.; Yu, A. *J. Phys. Chem. B. Condens. Matter Mater Surf. Interfaces Biophys.* **2005**, *109*, 23739.
11. Dugan, L. L.; Gabrielesen, J. K.; Yu, S. P.; Lin, T. S.; Choi, D. W. *Neurobiol. Dis.* **1996**, *3*, 129.
12. Lai, HS.; Chen, W. J.; Chiang, L. Y. *World J. Surg.* **2000**, *24*, 450.
13. Kamat, J. P.; Devasagavam, T. P.; Privadarsini, K. I.; Mohan, H. *Toxicology* **2000**, *155*, 55.
14. Isakovic, A.; Markovic, Z.; Todorovic-Marcovic, B.; Nikolic, N.; Vranies-Djuric, S.; Mirkovic, M.; Dramicanin, M.; Harhaji, L.; Raicevic, N.; Nicolic, Z.; Trajkovic, V. *Toxicol. Sci.* **2006**, *91*, 173.
15. Yamawaki, H.; Iwai, N. *Am. J. Physiol. Cell. Physiol.* **2006**, *290*, C1495.
16. Djordjevic, A.; Bogdanovic, G.; Dobric, S. *J. Buon.* **2006**, *11*, 391.
17. Takada, H.; Kokubo, K.; Matsubayashi, K.; Oshima, T. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 3088.
18. Kokubo, K.; Matsubayashi, K.; Tategaki, H.; Takada, H.; Oshima, T. *ACS Nano* **2008**, *2*, 327.
19. Emmons, C. L.; Peterson, D. M. *Cereal Chem.* **1999**, *76*, 902.
20. Husebo, L. O.; Sitharaman, B.; Furukawa, K.; Kato, T.; Wilson, L. J. *J. Am. Chem. Soc.* **2004**, *126*, 12055.
21. Xiao, L.; Takada, H.; Maeda, K.; Haramoto, M.; Miwa, N. *Biomed. Pharmacother.* **2005**, *59*, 351.
22. Pelle, E.; Huang, X.; Mammone, T.; Marenus, K.; Maes, D.; Frenkel, K. *J. Invest. Darmatol.* **2003**, *121*, 177.
23. Xiao, L.; Takada, H.; Gan, X.; Miwa, N. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1590.