Studies on Development of Water Soluble Fullerenes and Their Application as Antioxidants

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Preface

The studies presented in this thesis have been carried out under the guidance of Professor Takumi Oshima and Dr. Ken Kokubo at Osaka University during 2006-2009. This thesis deals with the antioxidant activity of fullerene (C_{60}) for life science application is focused on the fundamental studies comprised of the following four points: (1) the antioxidant activity of water-soluble fullerenes evaluated by β-Carotene bleaching assay, (2) the effects of oxygen on fullerenes for enhanced antioxidant activity, (3) the synthesis of highly water-soluble fullerenes more than half-covered by hydroxyl groups, and (4) the one-step synthesis of water-soluble fullerenols bearing nitrogen-containing substituents.

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Contents

Chapter 1. General Introduction

Chapter 2. The antioxidant activity of water-soluble fullerenes evaluated by β-Carotene bleaching assay

Chapter 3. The effects of oxygen on fullerenes for enhanced antioxidant activity

Chapter 4. The synthesis of highly water-soluble fullerenes more than half-covered by hydroxyl groups

Chapter 5. The one-step synthesis of water-soluble fullerenols bearing nitrogen-containing substituents

Chapter 6. General Conclusion

List of Publications

Acknowledgements

Chapter 1. General Introduction

Fullerene as an antioxidant

Fullerene (C_{60}) was discovered during a study of interstellar materials, trace substances in space, in 1985. Kroto *et al.* received the 1996 Nobel Prize in Chemistry for this discovery. Fullerene, like soccer ball, is a spherical carbon allotrope which consists of 60 carbons and, because of its simple and symmetric shape, is expected to be a basic molecular for various applications. In 1991, Krusic *et al*. reported that fullerene easily reacts radicals, i.e. reactive molecular species, such as active oxygen, which are hazardous to living organisms, like sponge absorbing much water[1] and initiated extinct studies of fullerene as an antioxidant for pharmaceutical application. In 2000, Dugan *et al.* found that water-soluble fullerene derivatives could protect human brain cells from harmful radicals and showed their potential to cure Parkinson's diseases[2]. This superior antioxidant feature of fullerene has also been anticipated to be applied to food additives and cosmetic ingredients.

Fullerene for cosmetic ingredient

The Japanese cosmetic industry not only has secured almost one third share of the world market but also is leading the cutting-edge technologies. Regardless of expense, current trend of the industry is pursuing higher functionality. More researchers have been engaged in the development of new efficient cosmetic ingredients with desirable functions. One of the most desirable functions is antioxidant activity so called anti-aging function. The stupendous success of Co-enzyme Q10 as a new anti-aging cosmetic ingredient in 2005 stimulated the market and accelerated the demand for brand-new antioxidants. Fig. 1-1 shows the molecular structures of typical antioxidants applied to cosmetic ingredients and fullerene for reference.

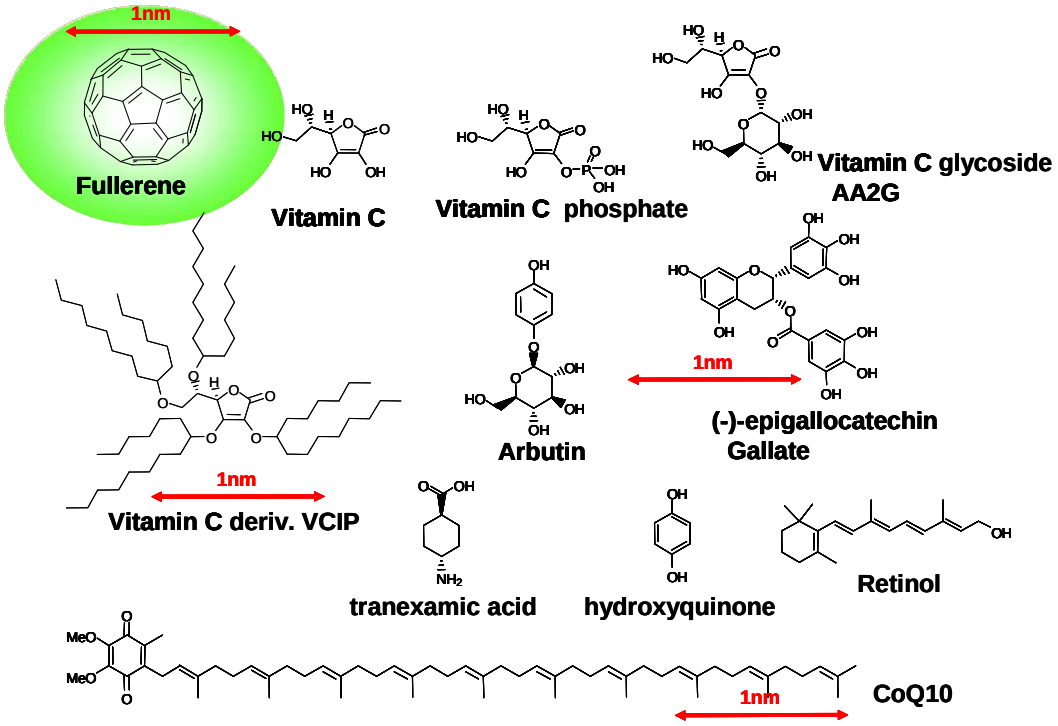

Fig. 1-1. Typical antioxidants applied as cosmetic ingredients and fullerene.

The application of fullerene to cosmetic ingredients has been tried by several leading cosmetic firms since its discovery. However, the practical implementation has not been successful until recently because of several obstacles such as its high price and poor supply with rarity, safety issue, insolubility in water, and a lack of its standard evaluation method of efficacy. At long last day, in 2002, the commercial production of fullerene was started in Japan and then, the stable supply of fullerene with reasonable price has been realized. Both this recent change of the availability and the strong demand for new antioxidants in Japanese cosmetic industry encouraged further research of fullerene as a cosmetic ingredient in practical use. No sooner than Sawaguchi *et al.* confirmed its safety in preclinical testing for external application[3] and Miwa *et al.* reported that water-soluble fullerenes were more effective than vitamin C derivatives as antioxidants on human skin cell[4], the first cosmetic products which contained water-soluble fullerene complex as an active ingredient were launched in

Fig. 1-2. The cosmetic products with fullerenes in current market.

2006. The available cosmetic products with fullerene are shown in Fig. 1-2. As a product made in Japan, the fullerene for cosmetic ingredients would be worthy of attention. To keep stable growth of the demand and establish the indisputable position in future, the further intense and creative researches increasing its water-solubility and antioxidant activity should be required.

Development of water soluble fullerenes and their application as antioxidants

In this series of study, the author worked on the development of new fullerene antioxidants taking two chemical approaches in the commercialization procedure of antioxidants as shown Fig. 1-3[5, 6]. One, solubilization in water, is the synthesis of highly water-soluble fullerene complexes and derivatives, and the other, evaluation of antioxidant activity, is the establishment of practical method and the evaluation of these antioxidant activities. In chapter 2, the author first applied β-Carotene bleaching assay (Fig.1-4) which has been commonly used as practical and simple measurement method of antioxidant activity in food science.

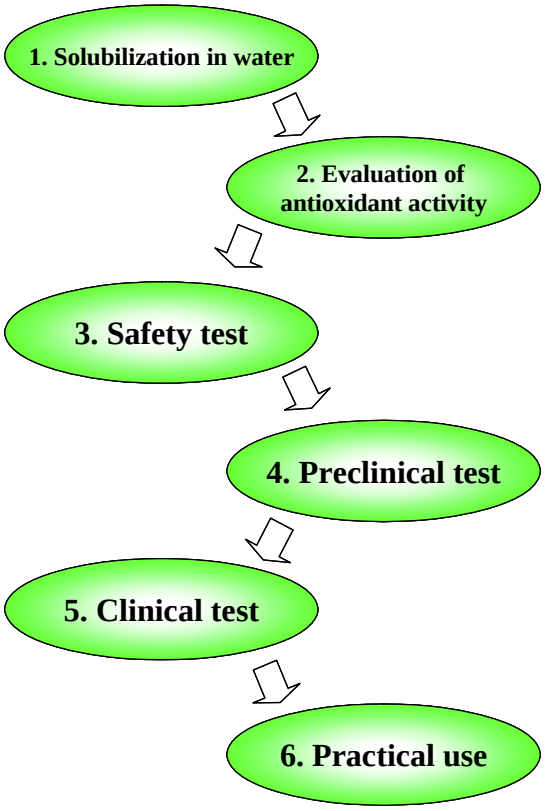

Fig. 1-3. The commercialization procedure of antioxidants as cosmetic ingredients.

For the evaluation of appropriateness to measure antioxidant activities of water-soluble fullerenes by β-Carotene bleaching assay, the author synthesized water-soluble fullerene complexes: PVP/C_{60} and γ-CD/C_{60}, and measured these antioxidant activities with comparison to typical antioxidants available in cosmetic industries. In chapter 3, to investigate the modification effects of oxygen on fullerenes, the author synthesized new water-soluble fullerene complexes: PVP/$C_{60}O$, PVP/$C_{60}On$, and γ-CD/$C_{60}O$, and evaluated these antioxidant activities by β-Carotene bleaching assay. In chapter 4, the author succeeded to synthesize highly water-soluble fullerenes more than half-covered by hydroxyl groups, with H_2O_2, from fullerenol ($C_{60}(OH)_{12}$) as starting compound. The antioxidant activities of the fullerene derivatives were also compared with others. In chapter 5, searching for conventional method to synthesize highly water-soluble

fullerene derivatives, the author found that the simple method of one-step synthesis of water-soluble fullerenols bearing nitrogen-containing substituents from C_{60} with H_2O_2 and NH_3. The reaction mechanism of the derivatives was also discussed and presumed.

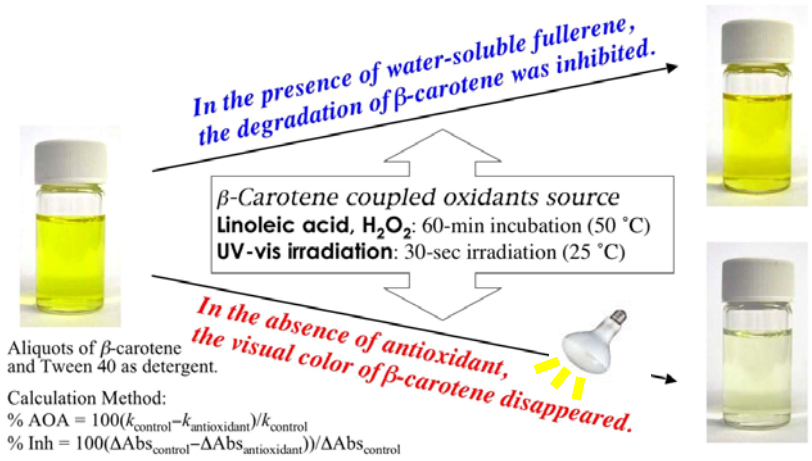

Fig. 1-4. The outline of *β*-Carotene bleaching assay.

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Chapter 2. The antioxidant activity of water-soluble fullerenes evaluated by β-Carotene bleaching assay

Introduction

Fullerenes are known to exhibit a wide range of biological activity, including their high reactivity with reactive oxygen species (ROS) such as superoxide and hydroxyl radicals and nitric oxide,[1-6] which attack lipids, proteins, DNA, and other biological macromolecules. It was demonstrated that fullerenes can be used as potent antioxidant and neuroprotective drugs against degenerative diseases related to oxidative stress.[7-16] The author has recently reported that a number of water-soluble fullerenes behave as potent ROS scavengers in cell cultures and can protect human skin keratinocytes from UV irradiation and oxidative damage by t-butyl hydroperoxide.[17,18] It was shown that the reaction rate for tris-malonic acid derivative of fullerene (C3) to remove superoxide radical was approximately equivalent to that of a manganese-based superoxide dismutase.[19] Antioxidant activity of polyhydroxylated fullerene was also estimated to be better than α-tocopherol (vitamin E) in inhibition of lipid peroxidation caused by superoxide and hydroxyl radicals.[20] Despite numerous studies on the radical scavenging activity of water-soluble fullerenes, little is known about the comparative assay of fullerenes versus β-Carotene, the latter has frequently served as a reference compound for determining the antioxidant activity with simplicity.[21-24]

In the present study, the antioxidant activity of supramolecular water-soluble fullerenes, namely polyvinylpyrrolidone (PVP)-entrapped C_{60}[25] and γ-cyclodextrin (CD)-bicapped C_{60},[26-29] as shown in Fig. 2-1, was kinetically evaluated by means of the coupled autoxidation of linoleic acid and β-Carotene. [20-24] Furthermore, the author performed a β-Carotene bleaching assay using other promoters such as hydrogen peroxide (H_2O_2) and photoirradiation for the first time.

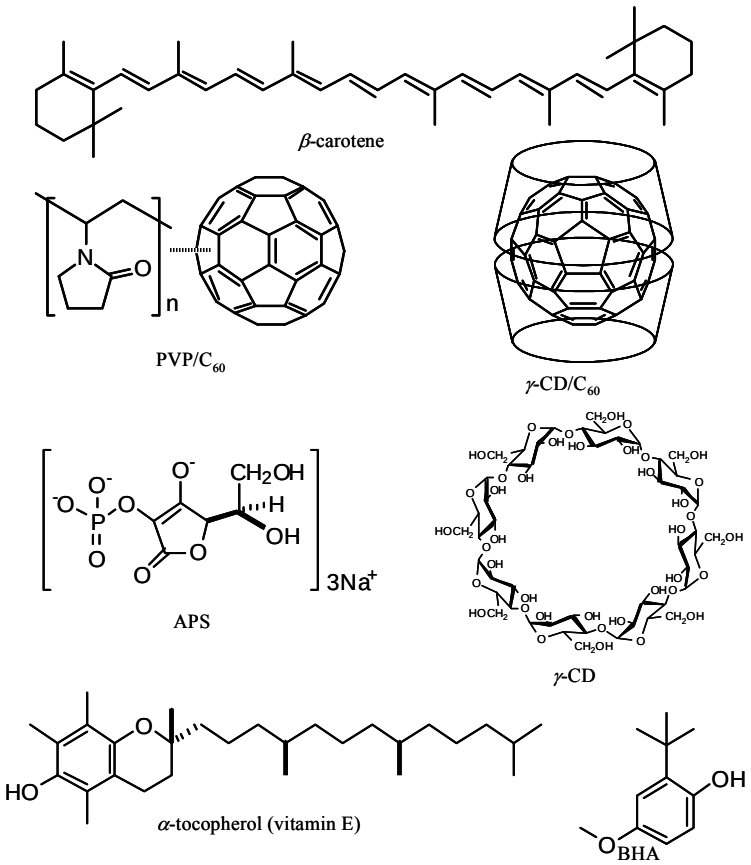

Fig. 2-1. Structures of β-Carotene, PVP/C_{60}, γ-CD/C_{60}, APS, γ-CD, α-tocopherol and BHA.

Experimental

The β-Carotene bleaching assay was performed according to the optimally modified procedure. To a solution of 22 μL of commercial surfactant Tween 40 (0.2 g/mL in chloroform) in a quartz cell equipped with a screw-on cap was added 11 μL of β-Carotene (1.0 mg/mL in chloroform) and 4.4 μL of linoleic acid (0.1 g/mL in chloroform), and then chloroform was removed in vacuo. Aliquot of the emulsion was immediately diluted with 2.4 mL of phosphate buffer solution (ca. 0.02 M, pH 7.0). To the mixture was added 0.1 mL of antioxidant (0.03 μmol, equivalent to C_{60}) in deionized water or in aqueous 80 % methanol. The solution was mixed well and heated at 50 ˚C in a UV spectrometer, and submitted for monitoring the decrease of absorbance of β-Carotene. As positive reference, α-tocopherol was used while

antioxidant-free deionized water was used as control. PVP/C_{60}, γ-CD/C_{60} and commercial antioxidant, Radical Sponge® (main components: PVP/C_{60} and 1,3-butylene glycol (1,3-BG)) were treated at the equivalent amount of C_{60} as active component. α-Tocopherol and *t*-butyl hydroxyanisole (BHA) in aqueous 80 % methanol, and sodium L-ascorbyl-2-phosphate (APS)[30] in aqueous solution were freshly prepared and used at the C_{60}-equivalent concentration. Aqueous solutions of PVP, γ-CD and 1,3-butylene glycol were prepared at the same amount of the corresponding component in PVP/C_{60}, γ-CD/C_{60} and Radical Sponge®, respectively. Similar treatment was also applied for β-Carotene H_2O_2 system. Aliquot of the emulsion of β-Carotene and Tween 40 prepared in a quartz cell was immediately diluted with 2.4 mL of phosphate buffer solution (ca. 0.02 M, pH 7.0). To the mixture was added 0.1 mL of antioxidant (0.03 μmol, equivalent to C_{60}) in deionized water or in aqueous 80 % methanol and 30 μL of ca. 30 % H_2O_2. The solution heated at 50 ˚C was consequently monitored at 460 nm for 37.5–60 min.

Result and Discussion

Fig. 2-2a shows the decreasing absorbance (Abs) of β-Carotene at 460 nm for various antioxidants under the influence of linoleic acid. As shown in Fig. 2-2b, plots of $\ln[(Abs_0)/(Abs_t)]$ (Abs_0: the initial absorbance; Abs_t: absorbance at time t) versus reaction time for each antioxidant gave a linear regression line after the short pre-steady state. The slope is the observed first-order rate constant k_{obs} for the decay of β-Carotene (C), because of the proportionality of Abs to the concentration of β-Carotene. The present antioxidant reaction consists of the following steps:

$$\mathbf{L + O2} \xrightarrow{k_1} \mathbf{LOO\cdot} \qquad \mathbf{(1)}$$

$$\mathbf{LOO\cdot + C} \xrightarrow{k_2} \mathbf{product\ 1} \qquad \mathbf{(2)}$$

$$\mathbf{LOO\cdot + Antioxidant} \xrightarrow{k_3} \mathbf{product\ 2} \qquad \mathbf{(3)}$$

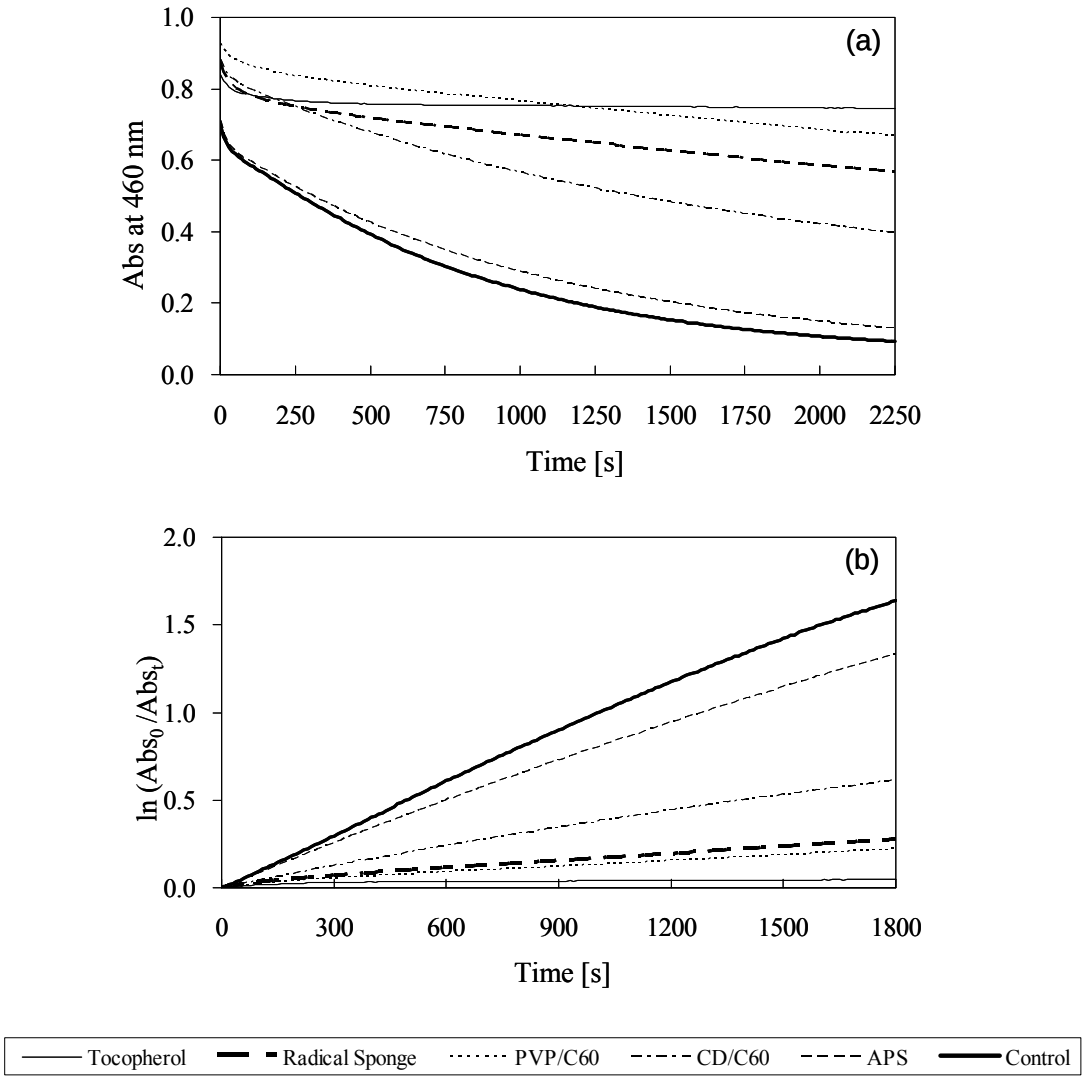

Fig. 2-2. a) Decay curves of absorbance at 460 nm during β-Carotene bleaching assay using linoleic acid. b) Radical scavenging activity in terms of the reduced degradation rate of β-Carotene. For clarity, the decreasing Abs and the $\ln(Abs_0/Abs_t)$ plots were omitted for BHA, PVP and γ-CD.

where, k_1, k_2 and k_3 are the rate constants for the formation of peroxyl radical (LOO•) from linoleic acid (L) with oxygen (eq 1), the reaction of β-Carotene with peroxyl radical (eq 2), and the reaction of antioxidant with peroxyl radical (eq 3), respectively. The pseudo first-order decay of β-Carotene can be rationalized in the presence of large excess of linoleic acid and O_2 by assuming that a generated small amount of peroxyl radical readily reacts with both β-Carotene and antioxidant (steady-state

approximation).[31]

Similar treatment was also applied for β-Carotene H_2O_2 system (Fig. 2-3). Obtained k_{obs} values for linoleic acid and H_2O_2 system were summarized in Table 2-1.

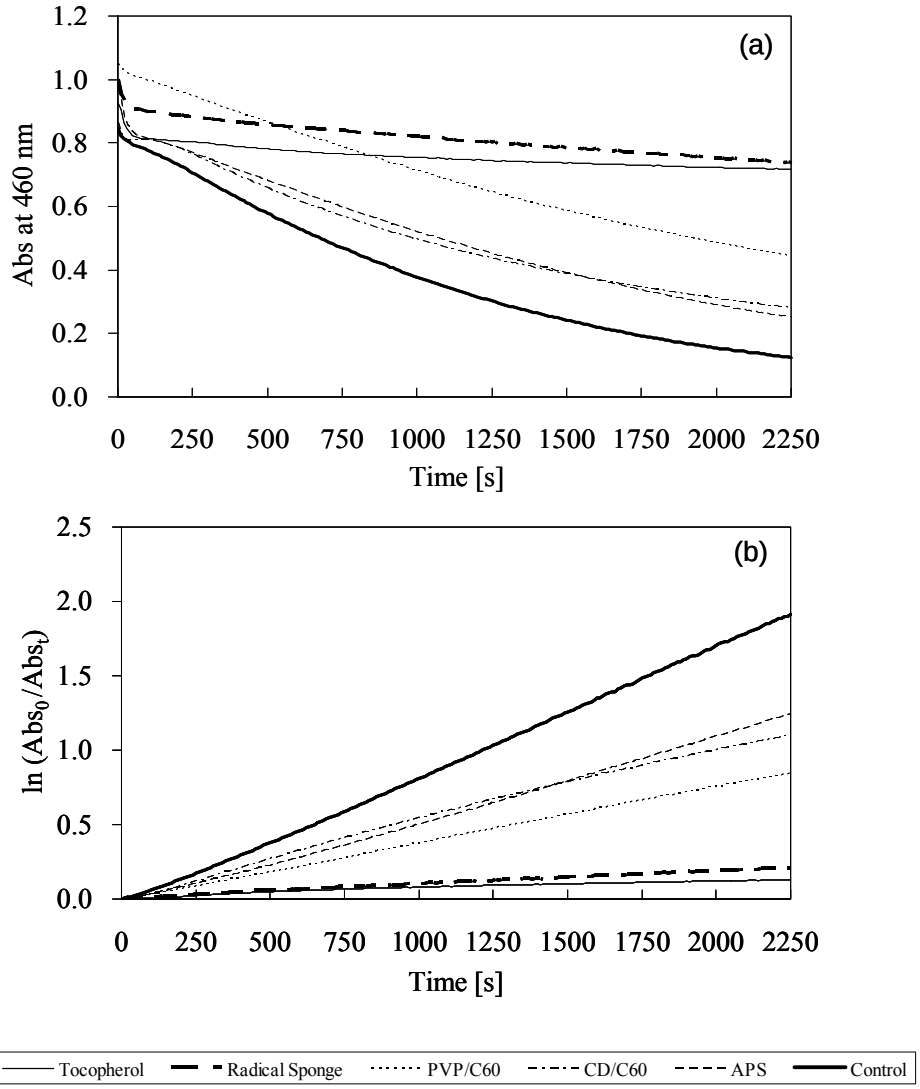

Fig. 2-3. a) Decay curves of absorbance (Abs) at 460 nm during β-Carotene bleaching assay using H_2O_2. b) Radical scavenging activity in terms of the reduced degradation rate of β-Carotene. For clarity, the decreasing Abs and the $\ln(Abs_0/Abs_t)$ plots were omitted for 1,3-BG, PVP and γ-CD.

Table 2-1. Observed first-order rate constant (k_{obs}) for β-Carotene degradation

Antioxidant	β-Carotene−linoleic acid system [$10^4 k_{obs}/s^{-1}$][a]	β-Carotene−H_2O_2 system [$10^4 k_{obs}/s^{-1}$][a]
α-Tocopherol	0.157	0.546
Radical Sponge®	1.42	0.888
PVP/C_{60}	1.14	3.82
γ-CD/C_{60}	3.39	5.01
APS	7.42	5.64
1,3-BG	n.a.[c]	1.47
BHA	0.341	n.a.[c]
PVP	9.96[b]	7.90
γ-CD	9.40[b]	8.91
Control	9.95[b]	8.71

[a] Analysis was carried out during total measuring time after system stabilization for 100 sec.
[b] Measuring time of 1200 sec was adopted.
[c] Not analyzed.

Antioxidant activity in the two different ROS generation systems, linoleic acid autoxidation and H_2O_2, can be quantitatively assessed as percent of antioxidant activity relative to control (%AOA)[21-24] using k_{obs} value (eq 4 and Table 2-2).

$$\%\text{AOA} = \frac{[(k_{obs}\text{ of control}) - (k_{obs}\text{ of sample})}{k_{obs}\text{ of control}} \times 100 \quad (4)$$

Although β-Carotene is easily degraded by photoirradiation under oxygen atmosphere, Yanagi et al. have demonstrated that an encapsulation technique using a carbon nanotube can protect β-Carotene on UVA light irradiation.[32] In order to investigate inhibitory activity of the water-soluble fullerene on oxidative degradation of β-Carotene, the author has measured the differential absorbance (ΔAbs) at 460 nm before and after 30 sec-irradiation with a 300 W high-pressure Hg lamp. In this case, it

is difficult to monitor the decreasing Abs under the photoirradiation. Instead, the author evaluated the antioxidant activity as percent of inhibition relative to control (%Inh)[33] using ΔAbs value (eq 5 and Table 2-2).

$$\%\text{Inh} = \frac{[(\Delta\text{Abs of control}) - (\Delta\text{Abs of sample})}{\Delta\text{Abs of control}} \times 100 \quad (5)$$

Table 2-2. Antioxidant activity of water-soluble fullerenes

	β-Carotene–coupled oxidants source		
Antioxidant	Linoleic acid [% AOA][a]	H_2O_2 [% AOA][a]	Irradiation [% Inh][b]
α-Tocopherol	98.4%	93.7%	-3.8%
Radical Sponge®	85.7%	89.8%	79.1%
PVP/C_{60}	88.5%	56.2%	79.2%
γ-CD/C_{60}	65.9%	42.5%	79.8%
APS	25.4%	35.2%	3.5%
1,3-BG	n.a.[c]	83.2%	n.a.[c]
BHA	96.6%	n.a.[c]	n.a.[c]
PVP	-0.1%	9.3%	n.a.[c]
γ-CD	5.5%	-2.2%	n.a.[c]

[a] % AOA = $100(k_{control}-k_{sample})/k_{control}$.
[b] % Inh = $100(\Delta Abs_{control}-\Delta Abs_{sample})/\Delta Abs_{control}$.
[c] Not analyzed.

Water-soluble fullerenes PVP/C_{60} and γ-CD/C_{60} exerted their strong inhibitory effects on the discoloration of β-Carotene, which was approximately equal to α-tocopherol. In the β-Carotene-H_2O_2 system, %AOA of Radical Sponge® (the mixture of PVP/C_{60} and 1,3-BG) was much higher than the others. It could be presumed that the degradation of β-Carotene was induced by the coupled oxidants, lipid peroxyl radicals (LOO•) from autoxidation of linoleic acid,[20-24] H_2O_2,[34] and possible

superoxide anion radicals ($O_2^{\bullet-}$)[35,36] on irradiation of β-Carotene under oxygen atmosphere. Owing to the lack of antioxidant activity of PVP and γ-CD, it can be verified that fullerene protects β-Carotene from attack by reactive oxidants as an eminent radical scavenger. Upon UV irradiation, however, fullerene may be inferred to lead to the generation of ROS which causes cytotoxicity through a pathway of intracellular oxidative stress. Nevertheless, the result revealed that PVP/C_{60}, γ-CD/C_{60}, Radical Sponge® inhibited the degradation of β-Carotene even on UV-vis irradiation. Therefore, it could be considered that the C_{60}-induced photooxidation did not occur under the ambient air atmosphere and water-soluble fullerenes strongly exhibited detoxification ability.

Conclusions

The present data provide the first evidence that supramolecular water-soluble fullerenes, PVP/C_{60}, γ-CD/C_{60} and commercial antioxidant, Radical Sponge® can significantly protect β-Carotene from the discoloration by lipid peroxyl radicals, H_2O_2, and possible superoxide anion radicals upon irradiation and this means that fullerene directly detoxifies a variety of ROS[37, 38], where fullerene captures radical species more rapidly than β-Carotene.[1-3] These water-soluble fullerenes also demonstrated higher antioxidant activity than APS and these new results are consistent with those of previous report by generally accepted ESR measurements[39] that showed the higher radical scavenging efficacy of PVP/C_{60} than that of APS. Therefore it is concluded that β-Carotene bleaching assay could apply with enough appropriateness to measure antioxidant activities of water-soluble fullerenes. Further work needs to be done to investigate the radical scavenging mechanisms of water-soluble fullerene with β-Carotene-coupled oxidant sources.

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31) If a small amount of LOO• generated under the presence of large excess of linoleic acid and O_2 is readily captured by both *β*-Carotene (C) and antioxidant, steady-state approximation can be applied;

$$\frac{d[\mathrm{LOO}\cdot]}{dt} = k_1[\mathrm{L}][\mathrm{O}_2] - k_2[\mathrm{LOO}\cdot][\mathrm{C}] - k_3[\mathrm{LOO}\cdot][\text{antioxidant}] = 0 \qquad (6)$$

From eq 6, eq 7 can be obtained.

$$[\mathrm{LOO}\cdot] = \frac{k_1[\mathrm{L}][\mathrm{O}_2]}{k_2[\mathrm{C}] + k_3[\text{antioxidant}]} \qquad (7)$$

Since the decay rate of C is written by eq 8, insertion of eq 7 into eq 8 gives the pseudo-first order eq 9, when k_3[antioxidant] >> k_2[C] and [L], [O_2] >> [C] are attained.

$$-\frac{d[\mathrm{C}]}{dt} = k_2[\mathrm{LOO}\cdot][\mathrm{C}] \qquad (8)$$

$$-\frac{d[\mathrm{C}]}{dt} = \frac{k_1k_2[\mathrm{L}][\mathrm{O}_2][\mathrm{C}]}{k_2[\mathrm{C}] + k_3[\text{antioxidant}]} \cong k_{\mathrm{obs}}[\mathrm{C}] \qquad (9)$$

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Chapter 3. The effects of oxygen on fullerenes for enhanced antioxidant activity

Introduction

Fullerenes and its derivatives are well known as a new class of antioxidants and they have attracted considerable attention in biological applications due to their high reactivity toward radicals[1], especially reactive oxygen species (ROS) such as superoxide[2], hydroxyl radical[3], peroxyl radicals[4], and nitric oxide[5]. These harmful radicals attack lipids, proteins, DNA, and other biological 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 paper, the author first reports 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_{rrs} was kinetically determined using a β-Carotene bleaching assay in the presence of water-soluble polyvinylpirrolidone (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. 3-1).

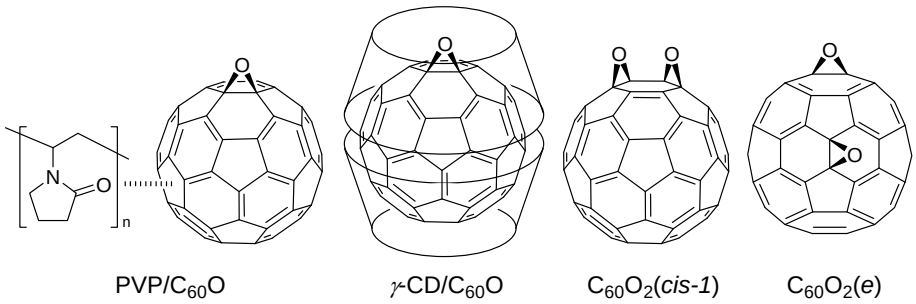

Fig. 3-1. Plausible Structure of Water-Soluble Complexes of [60]Fullerene Monoepoxide $C_{60}O$ and Structure of Major Isomers of $C_{60}O_2$(*cis-1* and *e*).

Experimental

Materials and apparatuses: Fullerene C_{60} and oxidized fullerene $C_{60}O_n$ were purchased from Frontier Carbon Corporation. Polyvinylpyrrolidone (PVP K30) 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.

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 hours 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 g ×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 ε = 5.06×10^4 $M^{-1}cm^{-1}$ 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 ($\varepsilon = 3.25\times10^4$ $M^{-1}cm^{-1}$ 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, its formation (obtained as a brownish water solution including an excess of free *γ*-CD) was confirmed 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. 3-2a). 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. 3-2b). 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

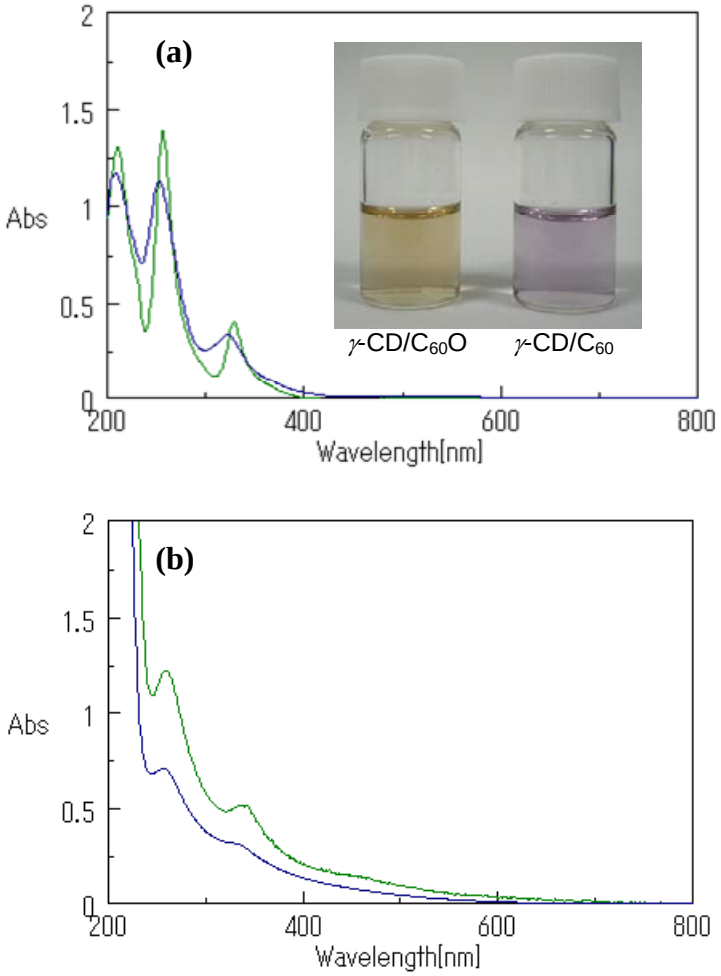

Fig. 3-2. UV-vis spectra of (**a**) *γ*-CD/C_{60}O (black line) and *γ*-CD/C_{60} (gray line) and (**b**) PVP/C_{60}O (black line) and PVP/C_{60} (gray line) in water (10 μM).

the addition of lipid peroxyl radical (LOO•) generated from the autoxidation of linoleic acid under air atmosphere[16–18]. The assay was performed according to an optimally modified procedure (Fig. 3-3) [19].

Fig. 3-4 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• 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.

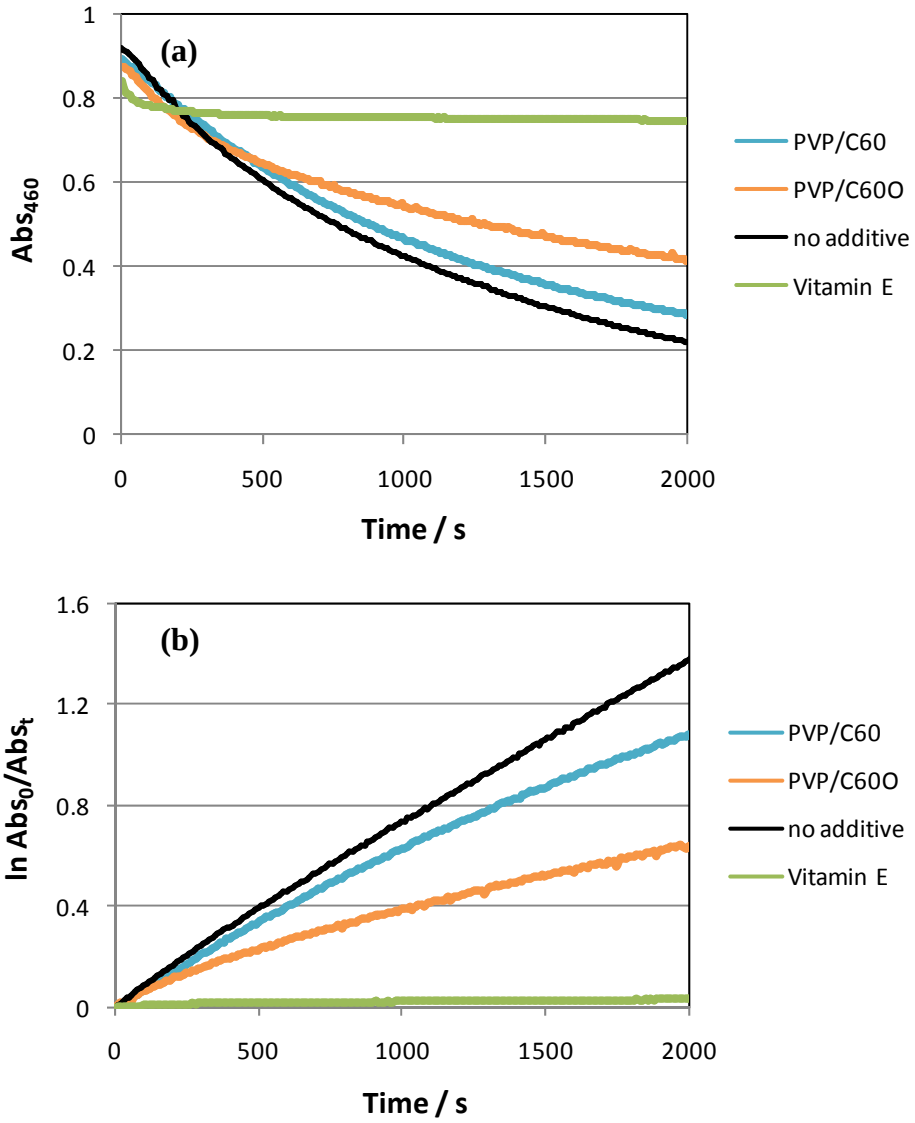

Fig. 3-3. β-Carotene bleaching assay with linoleic peroxyl radical; (**a**) decay curves of absorbance at 460 nm (Abs_{460}) and (**b**) plots of ln (Abs_0/Abs_t) *vs* time in the presence of antioxidants (10 μM), where Abs_0 is initial Abs_{460} and Abs_t is Abs_{460} at time t. Vitamin E was used as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

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

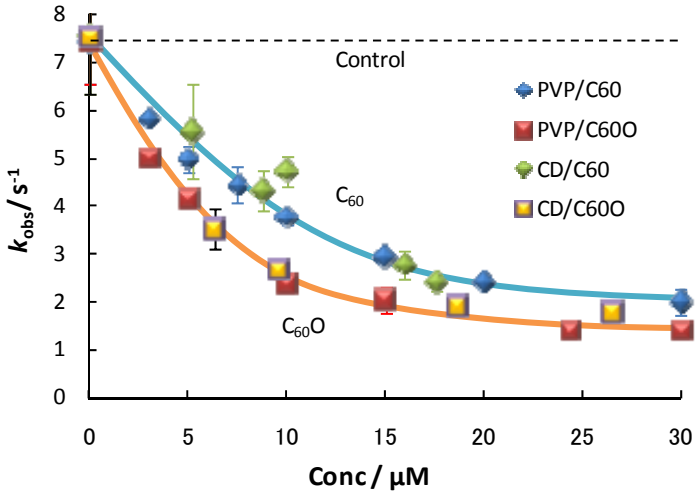

Fig. 3-4. Effects of antioxidant concentration on the observed pseudo-first-order rate constants k_{obs} of β-Carotene bleaching with linoleic acid peroxyl radical at 50 ºC. Values of k_{obs} were obtained by monitoring the absorbance of β-Carotene aqueous solution (8.2 μM) at 460 nm. The dotted horizontal line indicates the value of k_{obs} in the absence of antioxidants as a control.

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

To the best of the author's 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 × {k_{obs} of control – k_{obs} of fullerene}/ k_{obs} of control) of PVP/C_{60} and $C_{60}O$ were 50 % and 68 % in 10 μM for antioxidant, and 73 % and 81 % in 30 μM, respectively. Here, it is more convenient to define the absolute antioxidant activity of fullerenes toward the LOO• radical by considering the relative radical scavenging rate constants k_{rrs} (= k_f/k_c) of fullerenes *vs* β–Carotene, as given in eq 2[18], where R_0 is the

bleaching rate in the absence of antioxidants ([fullerene] = 0 in eq 1).

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

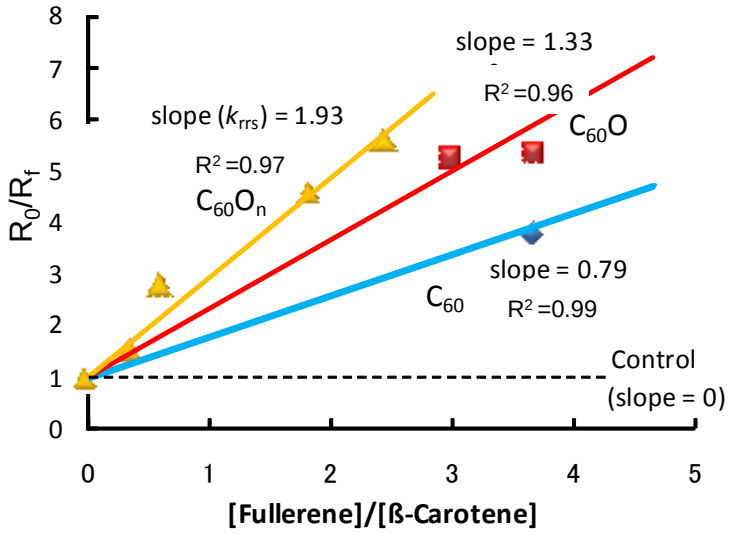

Fig. 3-5. Plots of the ratio of *β*-Carotene bleaching rates in the presence (R_f) or absence (R_0) of fullerene antioxidants R_f/R_0 *vs* ratio of concentration [fullerene] / [*β*-Carotene] for PVP-entrapped C_{60}, $C_{60}O$, and $C_{60}O_n$. The slope of each linear regression line corresponds to the relative radical scavenging rate constant k_{rrs} relative to that of *β*-Carotene. The dotted horizontal line indicates the value in the absence of antioxidants as a control.

As shown in Fig. 3-5, the plots of the ratio R_0/R_f *vs* the ratio of [fullerene] / [*β*-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 $C_{60}O_n$[20]. The dotted line indicates the value in the absence of antioxidants as a control (slope = 0). The slopes, k_{rrs} = 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• 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}, the author 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• and L• radical (Fig. 3-6).

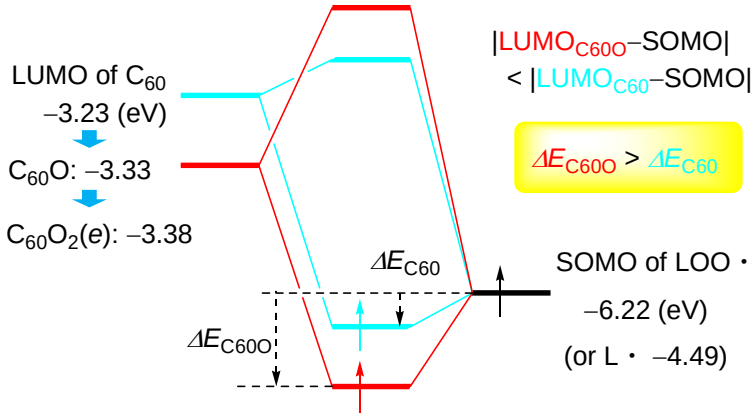

Fig. 3-6. Frontier molecular orbital interaction between LUMO of fullerenes C_{60}, $C_{60}O$, and $C_{60}O_2(e)$ and SOMO of linoleic acid peroxyl radical (LOO•) or linoleic acid radical (L•) calculated by B3LYP/6-31G* level of theory.

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[21] 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|. Thus, $C_{60}O$ can enjoy greater stabilization than C_{60} in capturing LOO• ($\Delta E_{C60O} > \Delta E_{C60}$), or possibly linoleic acid radical L• first formed in autoxidation, thus enhancing the antioxidant activity[22].

Conclusion

The author has 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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20) 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 $C_{60}O_n$ were 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 %.

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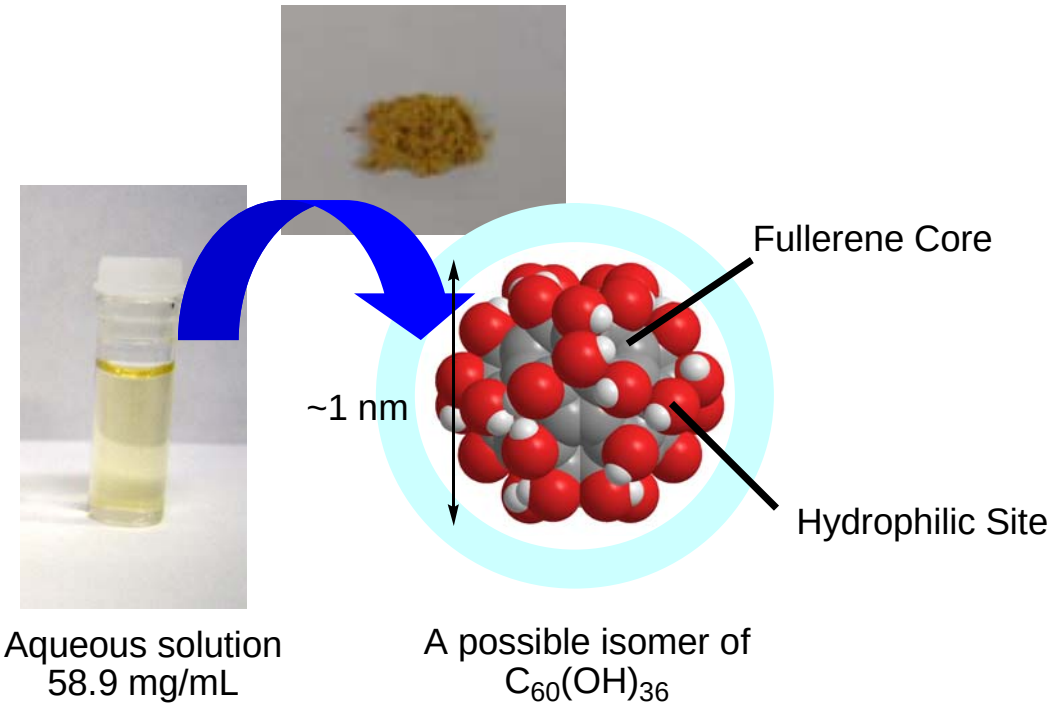
22) Calculated HOMO levels of C_{60}, $C_{60}O$, and $C_{60}O_2$(*e*) are –5.99, –5.95, and –5.99 eV, respectively, and less related to the present observation.

Chapter 4. The synthesis of highly water-soluble fullerenes more than half-covered by hydroxyl groups



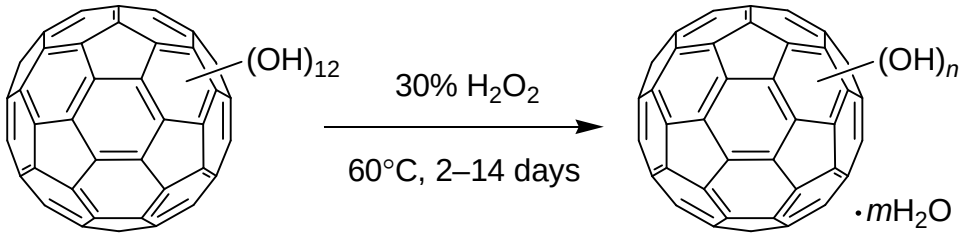
Introduction

Water-soluble fullerenes are promising materials in the field of life science, as medicines or cosmetics, in the light of their expected high scavenging ability of radical species[1 – 16]. Thus, the development of facile synthetic methods for biocompatible water-soluble fullerenes in an industrial scale has been eagerly desired. Since the polyhydroxylated fullerene, fullerenol, has been considered to be one of the possible candidates for the highly water-soluble fullerenes due to its simple structure[7, 8] and negligible toxicity[2, 9], several synthetic methods have been already reported.[16–26] The fullerenol with less than 12 hydroxyl groups on a fullerene cage still showed very poor

water solubility[16], whereas the fullerenols with more larger number of hydroxyl groups (16 or 20−24) exhibited good water solubility[21, 26]. However, these water-soluble fullerenols may be restricted for the utilization by unfavorable contamination of Na ion which is inevitably introduced under the treatment with NaOH in these synthetic methods[22, 27]. In this study, the facile synthetic method of the novel milky white colored and no-contaminated fullerenol which has 36−40 hydroxyl groups was found. This compound showed high water solubility of 58.9 mg/ml.

Experimental

Fullerene has been called 'radical sponge' due to its extremely high reactivity toward radical species[28]. Such a high radical reactivity is very attractive for the synthetic approach to polyfunctionalized fullerene derivatives, because the radical addition can be a sophisticated strategy to introduce a large number of functional groups in one step[29, 30]. The addition of the hydroxyl radical to the double bonds of C_{60} is known to take place with a diffusion-controlled rate constant of *ca*. 10^{10} $M^{-1}s^{-1}$ [31]. Therefore, first the reaction of fullerene C_{60} with hydroxyl radicals prepared by Fenton condition was investigated[32]. However, the reaction did not proceed even for several weeks probably because of the insolubility of C_{60} in an aqueous solution. Then the author applied the similar reaction to 12-hydroxylated fullerenol instead of C_{60} and found that the homogeneous yellow aqueous solution was obtained after 2−4 days heating at 60°C even in the absence of Fe^{2+} catalyst. Although the Fe^{2+} catalyst is effective to accelerate the reaction, the complete removal of the catalyst from the product fullerenol is somewhat complicated[33]. Therefore, the above reaction conditions were adequately optimized without the catalyst.



1

2: n = 36, m = 8
3: n = 40, m = 9

(Estimated average structure)

Scheme 4-1

Fullerenol $C_{60}(OH)_{12}$ **1** employed was prepared by L. Y. Chiang's oleum method[2a] and the structure was confirmed by IR spectroscopy. The number of hydroxyl group was determined by FABMS as well as elemental analysis. Then, the suspension of fullerenol **1** (0.100 g) in 30 % aqueous H_2O_2 (10 mL) was vigorously stirred at 60 ˚C under air for several days (Scheme 4-1). The deep red suspension was turned into clear yellow solution within 2–4 days, although a slight difference in the color or the number of hydroxyl groups was observed depending on the difference of a lot number of the starting 12-hydroxylated fullerenol, scale of reaction, heating condition, and/or efficiency of stirring due to an inevitable solid–liquid heterogeneous reaction. To this solution, a mixture of 2-propanol, diethyl ether and hexane (each 50 mL) was added as poor solvents to afford a yellow brown to milky white precipitation of desirable fullerenol. After careful centrifuge and decantation, the residual solid was washed twice with 50 mL of diethyl ether by the general ultrasonic–centrifuge–decantation procedure. Drying of the residue under vacuum at room temperature for 18 h gave novel water soluble fullerenol **2** (0.097 g, 67 %[34]) as yellow brown to milky white powder. The fullerenol **3** was similarly obtained as milky white powder on the prolonged reaction time up to 2 weeks (0.103 g, 68 %[34]).

To evaluate the antioxidant activities of newly synthesized water-soluble fullerene, *β*-Carotene bleaching assay was applied for fullerenol **2**. 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 fullerenol **2** (20 μM) 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 IR spectra of fullerenols **2** and **3** were shown in Fig. 4-1 in comparison with that of starting fullerenol **1**[16]. These spectral shapes were very similar to each other, although the relative peak intensities somewhat differ, suggesting the difference in the numbers of introduced hydroxyl groups. With a broad O–H band around 3400 cm^{-1}, the spectra showed three characteristic bands at 1080, 1370, and 1620 cm^{-1} assigned for νC–O, δ_SC–O–H and νC=C absorption. These four broad bands are invariably reported as the diagnostic absorptions of various fullerenols.[16-26] A small peak around 1720 cm^{-1} implies the existing of carboxylic group which may be formed by further oxidation of hydroxyl group associated with C–C bond cleavage of fullerene nucleus or carbonyl group formed by known Pinacol rearrangement of vicinal hydroxyl groups of fullerenol[35, 36]. However, such a partial decomposition must be not so crucial because of the generally strong C=O absorption is relatively small compared to generally weak or medium other C=C or C–O absorption. In order to determine a number of hydroxyl groups introduced for these fullerenols **2** and **3**, the molecular weight was measured by FABMS and MALDI-TOFMS spectroscopy. However, the mass spectra of these fullerenols were not clearly obtained in contrast to that of **1**[16, 37].

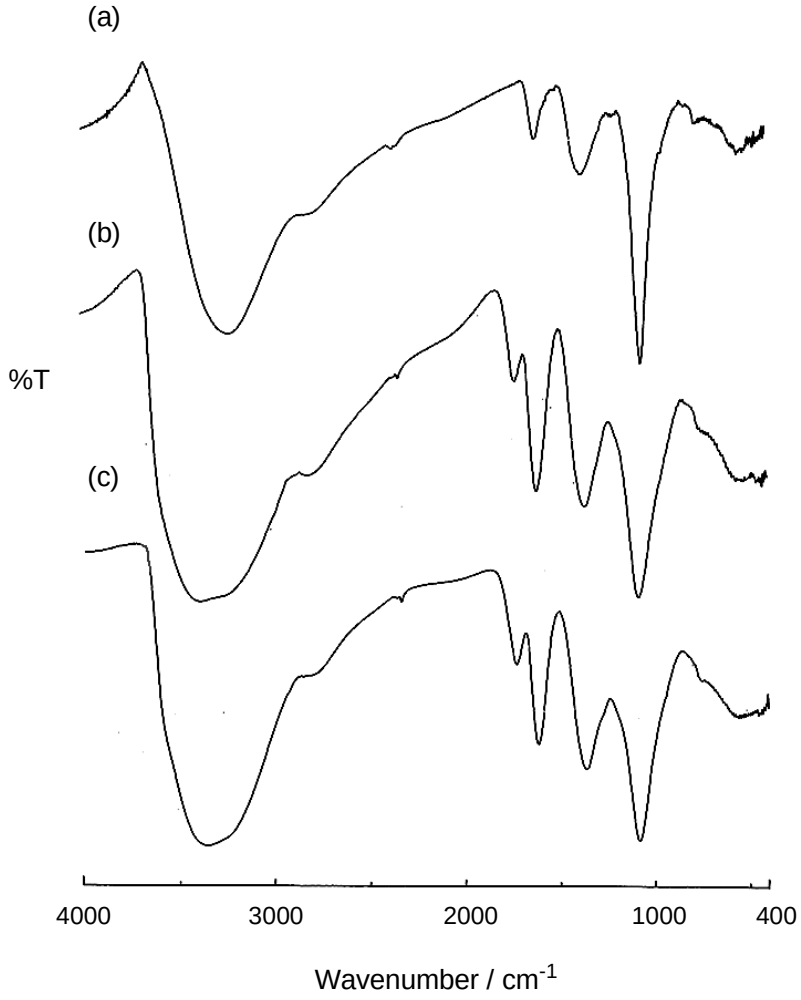


Fig. 4-1. IR spectra of (a) fullerenol $C_{60}(OH)_{12}$ **1**, (b) $C_{60}(OH)_{36}•8H_2O$ **2**, and (c) $C_{60}(OH)_{40}•9H_2O$ **3**.

Then, the author estimated their average structure by elemental analysis along with a water content measurement. Most fullerenols reported so far were estimated their average structure only by elemental analysis[21, 26], most fullerenols were not calculated as hydrated formulas[16-26]. However, especially for the fullerenols with larger number of substituted hydroxyl groups, one can easily imagine the existence of a substantial amount of secondary bound water to the plural hydroxyl groups on fullerene surface. Such tightly entrapped water could not be dissociated by usual heating up to about 150

ºC[35]. Therefore, the author conducted the thermal gravity analysis of fullerenol **2** by TGA spectrometer (Fig. 4-2).

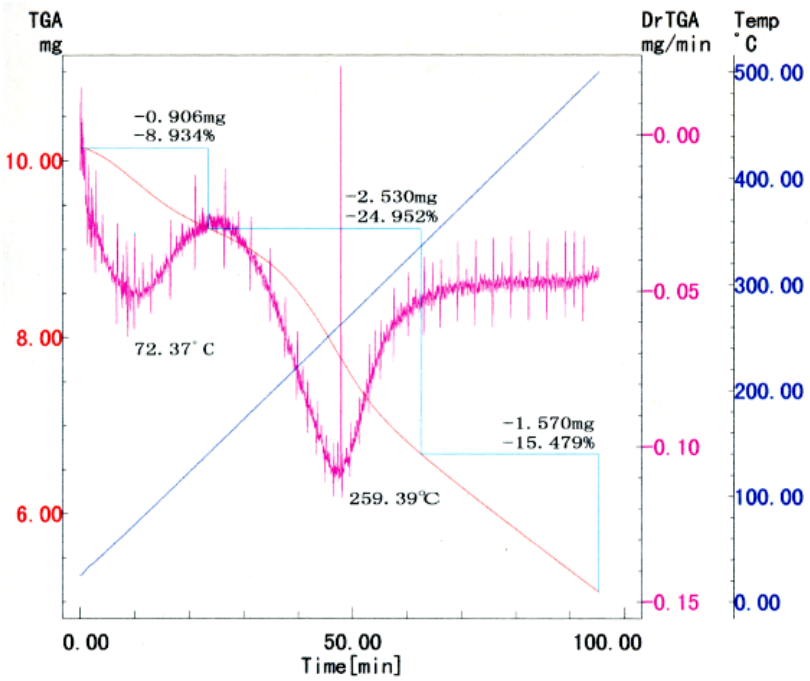


Fig. 4-2. Thermal gravity analysis of fullerenol **2**.

The weight loss of fullerenol **2** was observed in the three ranges of temperature; i.e., room temperature to 130 ºC, 130–350 ºC, and >350 ºC. The first loss (8.9 wt%) is assigned to the secondary bound water as reported by Chiang *et al.*[35] The second reduction (25 wt%) may be attributed to the dehydration of introduced hydroxyl groups by possible thermal Pinacol rearrangement. The reduction in the higher temperature (>350 ºC) may be attributable to the decomposition of fullerene nucleus. The weight reduction profile observed for fullerenol **3** was similar to that of the starting fullerenol **1**. The first weight losses are 9.6 (rt-130 ºC) and 12.0 wt% (rt-250 ºC), respectively, although the pristine C_{60} is quite stable (no weight loss) up to 400 ºC [38]. The water content of ca. 9 wt% is satisfactory consistent with the value obtained by a Karl-Fisher moisture titrator.

Table 4-1. Elemental analysis, water content, water solubility and average structure of fullerenol **2** and **3**

Average Structure	Elemental Analysis [%][a]	Water Cont. [wt%][a,b]	Solubility [mg/mL][c]
$C_{60}(OH)_{36}$ $8H_2O$ (**2**)	C: 48.06, H: 3.61 (C: 48.79, H: 3.54)	8.9 (9.7)	17.5
$C_{60}(OH)_{40}$ $9H_2O$ (**3**)	C: 46.26, H: 3.68 (C: 46.11, H: 3.74)	9.6 (10.4)	58.9

[a] Values in parentheses are calculated data. [b] Water content was determined by TGA analysis. [c] Water solubility at 25°C in neutral water (pH7).

As shown in Table 4-1, the average structure of fullerenol **2** was deduced as $C_{60}(OH)_{36}$•$8H_2O$ by elemental analysis along with water content measurement. If the average structure was estimated only by elemental analysis, it would be calculated as $C_{60}(OH)_{44}$. Thus, the measuring the secondary bound water is quite important to evaluate the incorporated OH groups. Similarly, the average structure of fullerenol **3** was determined as $C_{60}(OH)_{40}$•$9H_2O$. It is reasonably conceivable that the increased reaction time results in the further introduction of hydroxyl groups. Furthermore, it is noteworthy that the water solubility of fullerenol **3** was much higher than that of **2** due to the increased number of hydroxyl group, whereas the number of the bound water molecule is essentially the same. Wang *et al* have reported the high water solubility of fullerenol $C_{60}(OH)_{16}$ over 200 mg/mL[21]. However, such a type of fullerenol may slightly include sodium ions because of (1) the synthetic process using NaOH and (2) the difficulty in complete removal of sodium ion from the weakly acidic or chelation natured fullerenol[22, 27]. Because the simple acidification of fullerenol must induce the known Pinacol rearrangement, unfortunately, thus it is difficult to remove the sodium ion without any column chromatography process.

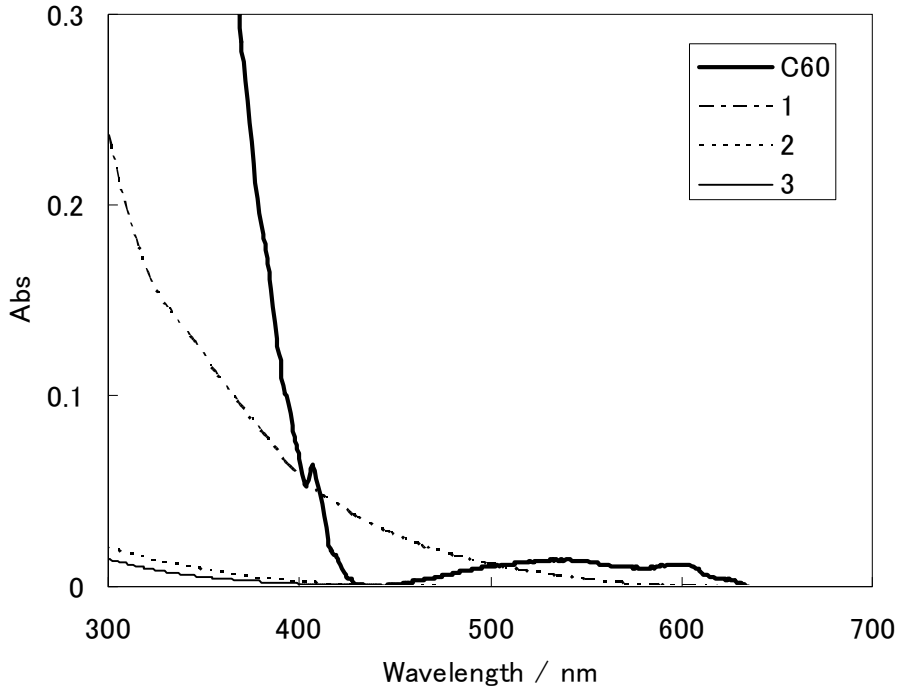


Fig. 4-3. UV-vis spectra of C_{60} and fullerenols **1**–**3**. C_{60} in toluene, $C_{60}(OH)_{12}$ **1** in 1,4-dioxane, and aqueous solution of $C_{60}(OH)_{36}$•$8H_2O$ **2** and $C_{60}(OH)_{40}$•$9H_2O$ **3** (each 3.33 mg/L).

The UV-visible absorption spectra of the fullerenols **2** and **3** in water were shown in Fig. 4-3. As compared to those of pristine C_{60} in toluene and fullerenol **1** in dioxane, the fullerenols **2** and **3** are almost transparent in visible region, implying their considerable disappearance of C=C double bond character and the incidental π-conjugation (average number of C=C double bonds in C_{60}, **1**, **2** and **3** are 30, 24, 12, and 10, respectively). Indeed, the color of these water soluble fullerenols **2** and **3** is blue-shifted and far different from C_{60} as shown as powder or in solution (Fig. 4-4). The particle size of the 0.1 wt% aqueous solution[41] of **2** was measured by DLS analysis.

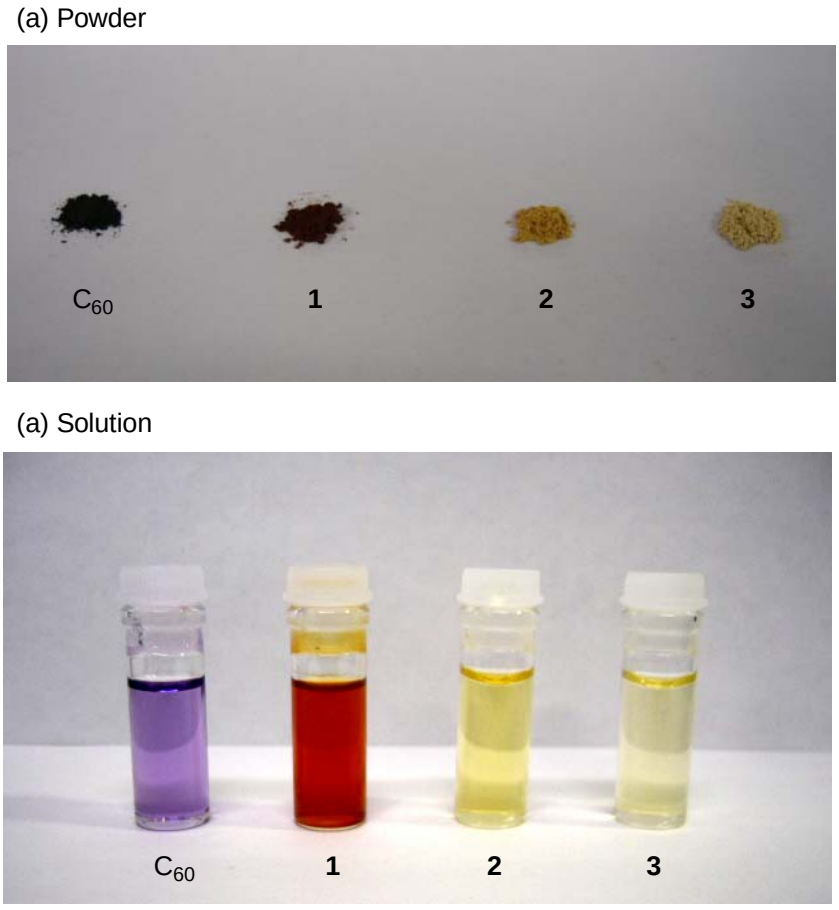


Fig. 4-4. Colors of water-soluble fullerenols **1**–**3** (a) and its solution (b). C_{60} in toluene $C_{60}(OH)_{12}$ **1** in 1,4-dioxane, and aqueous solution of $C_{60}(OH)_{36}$•$8H_2O$ **2** and $C_{60}(OH)_{40}$•$9H_2O$ **3**.

The DLS chart of size distribution in number is shown in Fig. 4-5. By the MOPAC PM3 calculation, the diameter of a possible isomer of $C_{60}(OH)_{36}$[42], which was estimated from the O–O atomic distance between the furthest hydroxyl groups on both poles, was almost 1.0 nm. Thus, the relatively narrow distribution of particle size of 0.7–2.0 nm clearly indicates that the fullerenol **2** is highly dispersed in a molecule level and that the expected aggregation of fullerenols, as is well known for may fullerene derivatives[43, 44], and even for some fullerenols[7, 8, 4]5, is not so substantially formed. This is because that the fullerenol **2** may be surrounded by solvent water molecule due to the strong

hydrogen bonding with the introduced hydroxyl groups and hence the intermolecular hydrophobic interaction (or π– π interaction) between C_{60} cores is overwhelmed.

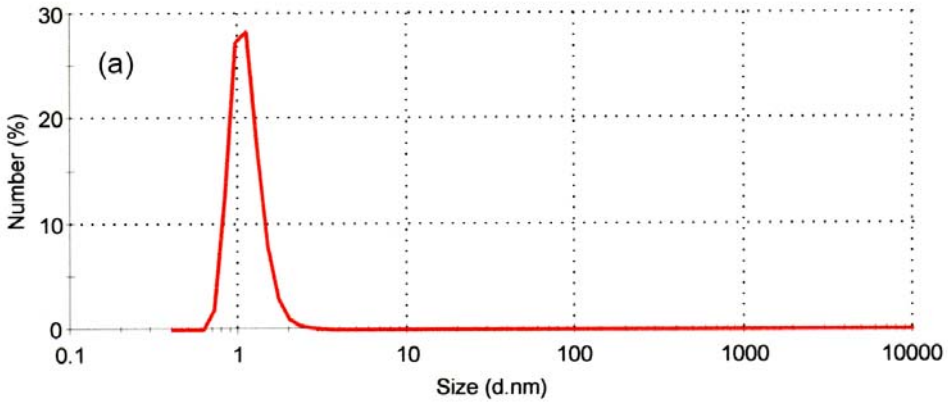


Fig. 4-5. DLS analysis of fullerenol **2** in water (0.1 wt%) as produced expressed by size distribution in number.

Interestingly, the aggregation phenomenon was observed in the deposition process. Addition of a mixture of 2-propanol, diethyl ether, and hexane (5:5:5 to fullerenol aqueous solution in *v/v*) as a poor solvent into the aqueous solution of the fullerenol **2** or **3** gradually brought about a deposition of fullerenol. The SPM surface analysis of the powder of fullerenol **2** revealed the nano-sized spherical structure in a range of about 50 nm implying the formation of fullerenol aggregation (Fig. 4-6). Therefore, the addition of poor solvent probably reduce the solvation of the fullerenol by water molecular and increase the hydrophobic interaction between intermolecular fullerene cores to afford the spherical aggregation, which finally grows into a deposition. Thus, it seems that the aggregation size of the fullerenol may be controlled by the polarity and the nature of solvent.

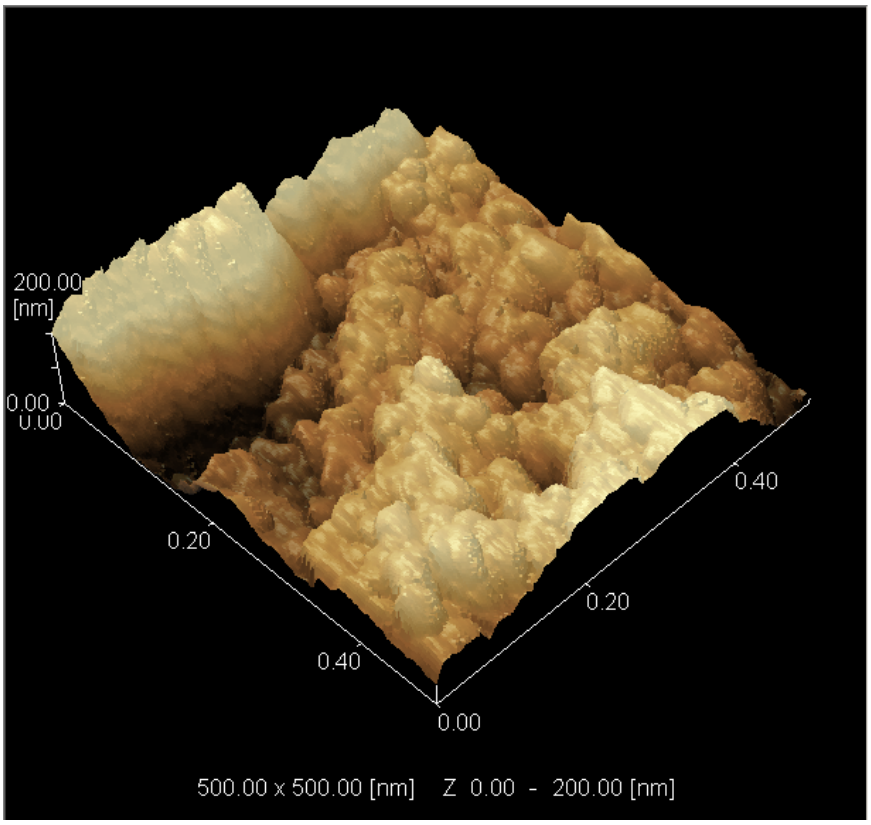


Fig. 4-6. SPM surface analysis for a powder of fullerenol **2**.

Although the average structure of fullerenol **2** is investigated as much as possible in the present stage, the exact structure of each fullerenol constructing an isomeric mixture with various numbers and positions of hydroxyl groups is still under investigation. The actual structure may contain some epoxy oxygens on fullerene surface as previously proposed in some fullerenols[24, 25, 27, 35]. Or the synthetic method using the hydrogen peroxide may partly introduce a -OOH group[46] along with a -OH group. It is hard to rule out these possibilities because these functional groups are generally difficult to distinguish by IR spectroscopy. However, the elemental analysis along with the TGA analysis suggests that the number of these impure groups[25] introduced is quite small (*e.g.*

calculated elemental analysis for $C_{60}(OH)_{36}O_4.8H_2O$ is C: 46.76 %, H: 3.40 %). And the obtained good water solubility apparently supports the higher number of hydroxyl groups introduced.

To collect initial knowledge for future application of the fullerenols to cosmetic ingredient, the concentration-dependent antioxidant activity, %AOA (= 100 × {k_{obs} of control – k_{obs} of fullerene}/ k_{obs} of control) of fullerenol **2** ($C_{60}(OH)_{36}O_4$•$8H_2O$) in comparison with fullerenol **1** ($C_{60}(OH)_{12}$) and PVP/C_{60} were measured by β-Carotene bleaching assay. The obtained results are shown in Fig. 4-7.

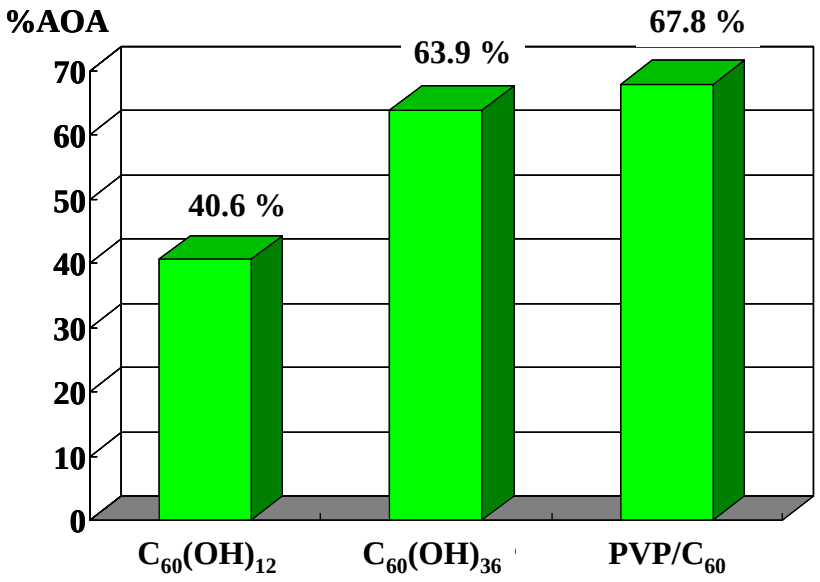


Fig. 4-7. Antioxidant Activities of fullerenols and PVP/C_{60} in 20 μM.

The observed %AOA of fullerenol **1** ($C_{60}(OH)_{12}$), fullerenol **2** ($C_{60}(OH)_{36}O_4$•$8H_2O$), and PVP/C_{60} as a water-soluble pristine fullerene complex in 20 μM were 40.6 %, 63.9 %, and 67.8 %, respectively. Interestingly, the newly synthesized water-soluble fullerene derivative more than half covered by hydroxyl groups, fullerenol **2,** showed

relatively high antioxidant activity which was almost the same level as PVP/C_{60}. The reason why the antioxidant activity of fullerenol **2** is higher than that of fullerenol **1** may be attributed to the number of hydroxyl group. Therefore, the mechanism of radical scavenging reaction is supposed to be different from the water-soluble fullerene complex such as PVP/C_{60}.

Conclusions

The author developed the facile synthetic method of water-soluble polyhydroxylated [60]fullerenes, fullerenols, bearing more than 30 hydroxyl groups without using sodium hydroxide. The estimated average structure of these fullerenols was determined as $C_{60}(OH)_{36}$•$8H_2O$ or $C_{60}(OH)_{40}$•$9H_2O$, depending on the reaction time. The obtained fullerenol showed a high water solubility up to 58.9 mg/mL and a high dispersion property in a molecule scale. In the preliminary experiment by β-Carotene bleaching assay, the fullerenol, $C_{60}(OH)_{36}$•$8H_2O$, as same as water-soluble fullerene complex, also exhibit a potent antioxidant activity as cosmetic ingredient. The further studies for these mechanisms of antioxidant activities are now in progress.

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Chapter 5. The one-step synthesis of water-soluble fullerenols bearing nitrogen-containing substituents.

Introduction

How to dissolve fullerenes into water effectively is a fundamental and inevitable problem not only in the field of chemical industry for the utilization of this valuable nanocarbon as well as carbon nanotubes[1] but also in the field of life science for the application to medicines or cosmetic ingredients in light of the expected scavenging ability toward radical species[2]. Thus, the development of facile synthetic methods of water-soluble fullerene derivatives has been eagerly pursued. Although polyhydroxylated fullerenes, fullerenols, have been considered one of the promising candidates for the highly water-soluble fullerene in addition to their radical scavenging ability [3], generally, a fullerenol with fewer than 12 hydroxyl groups on a fullerene cage shows very poor water solubility [4]. Others with more numerous hydroxyl groups (16 or 20−24) exhibited good solubility [5, 6], whereas these fullerenols might be restricted for practical use by unfavorable contamination of Na^+ ion, which is inevitably introduced during treatment with NaOH and which is difficult to remove by the usual manner [7]. In the previous work, the author succeeded in the facile synthesis of novel fullerenols, which have 36−40 hydroxyl groups with sodium free, showed high solubility of 58.9 mg/ml to water [8]. The simple reaction of C_{60} in H_2O_2 aqueous solution did not proceed even for several weeks and probably due to insolubility of C_{60} to the aqueous solution. Then the method with 12-hydroxylated fullerenol as a reactant instead of C_{60} was conducted and succeeded in obtaining the desired water-soluble fullerenol. However the availability of 12-hydroxylated fullerenol is quite limited and the method using oleum is

not sufficient in terms of practical use. Therefore the author has strained to find a one-step and efficient synthetic method directly from C_{60} as a commercially available starting material. On the other hand, highly polar amino group ($-NH_2$) can be an alternate candidate to design a new water-soluble fullerene derivative. Only one example having both amino and hydroxyl groups on fullerene cage has been already known[9] but water-soluble one has not been developed yet. As the well known polynitro-substituted fullerenes[10], the additional nitrogen-containing group must provide an interesting feature for water-soluble fullerenes.

In this study, it is aimed to synthesize water-soluble fullerenols in one-step directly from C_{60} with the hydrogen peroxide method. By the simple addition of NH_3 aqueous solution, the newly synthesized fullerenols having nitrogen-containing groups were obtained and one of them showed the highest water solubility (>200 mg/ml) as same as the one reported[5]. The obtained fullerene derivatives, with their spherical shape and functional groups on the surface, are also anticipated to be one of the useful nanomaterials in versatile fields.

Experimental

Materials and Analysis

Fullerene C_{60} (Frontier Carbon Corporation), other reagents, and solvents were all commercially available and used without further purification. Fullerenol $C_{60}(OH)_{12}$ **1** was prepared by L. Y. Chiang's oleum method[4] and fullerenols $C_{60}(OH)_{36}\cdot 8H_2O$ **2** and $C_{60}(OH)_{40}\cdot 9H_2O$ **3** were prepared by the previously reported method (8). IR spectra were measured with a JASCO FT/IR-300E instrument, and TGA spectra were obtained with a Shimadzu TA-50 instrument. Water content measurement was recorded on

Kyoto Electronics Karl Fischer Moisture Titrator MKA-610-ST equipped with evaporator ADP-611.

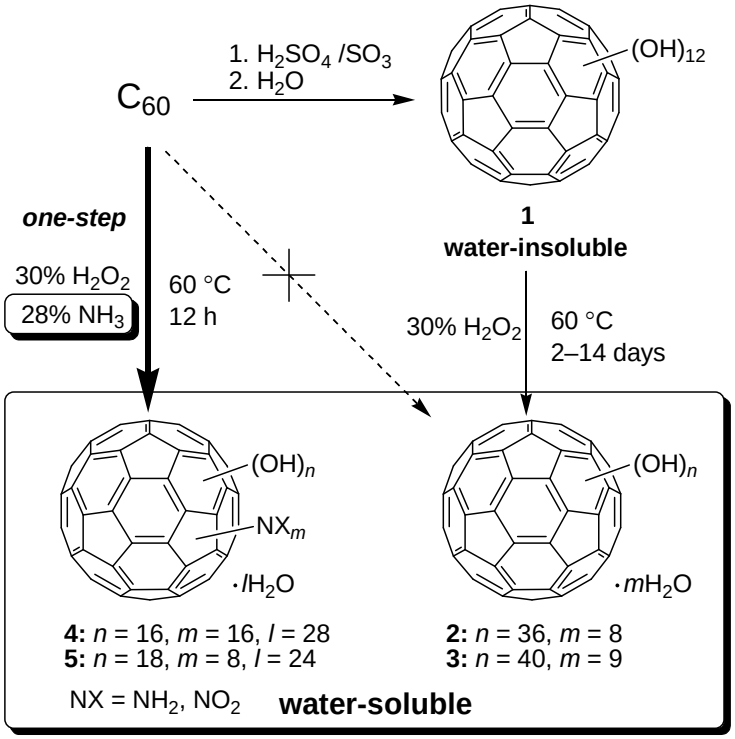
Preparation of Highly Water-Soluble Fullerene Derivatives 4 and 5 from C_{60}

The suspension of C_{60} (0.100 g) in 30 % aqueous H_2O_2 (10 mL) and 28 % aqueous NH_3 (4 mL) was prepared and vigorously stirred at 60 ˚C under air for 12 hours. The black suspension was turned into dark yellow suspension. After careful centrifugation and decantation, the solution was separated into two portions: clear solution **A** and residual solid **B**. To the solution **A**, 30 mL of ethanol was added as poor solvent to afford a precipitation and obtained residual solid. Treatment of residual solid with water (5 mL) and then ethanol (35 mL) gave reprecipitation. The precipitated residual solid was washed ethanol (40 mL) twice under ultrasonic irradiation and dried under vacuum at room temperature for 18 hours. Finally, novel water-soluble fullerene derivative **4** as a yellowish powder (0.074 g) was obtained. The residual solid **B** was dried for 24 hours to remove remaining NH_3 and dissolved in deionized water (20 mL). After careful centrifugation and decantation of the solution, the residual solid was removed and the rest, pure clear solution, was mixed with ethanol (20 mL) to afford precipitation. The precipitated solid was washed twice by ethanol (40 mL) under ultrasonic irradiation and dried under vacuum at room temperature for 18 hours. The other novel water-soluble fullerene derivative **5** was also obtained as a yellowish powder (0.077 g).

Results and Discussion

Characterization of the Highly Water-Soluble Fullerene Derivatives 4 and 5

Firstly the reaction of fullerene C_{60} with NH_3 in its aqueous solution (28 %) both by heating at 60 °C and stirring at room temperature was investigated. However, the reaction did not proceed even after several days. Then the author conducted a similar reaction of C_{60} (0.100 g) in the presence of both 30 % H_2O_2 and 28 % NH_3 in their aqueous solution at 60 °C (Scheme 5-1). After only 12 hours, the black suspension was turned into a dark yellow suspension (although the reaction of $C_{60}(OH)_{12}$ **1** with H_2O_2 to give fullerenols **2** and **3** required typically 2–14 days as shown in Scheme 5-1) and the complete disappearance of the starting C_{60} was confirmed by HPLC. After careful centrifugation and decantation, the solution was separated into two portions: clear solution **A** and residual solid **B**. By reprecipitation work up from the solution **A**, the water-soluble fullerene derivative **4** as a yellowish powder (0.074 g) was obtained. The other water-soluble fullerene derivative **5** was also obtained from the similar treatment



Scheme 5-1

with the residue **B** as a yellowish powder (0.077 g). Thus, the total amount of water-soluble products obtained was 0.151 g from 0.1 g of the starting pristine C_{60}. As found from the precipitation work up process, the water solubility of product **4** was much higher than that of product **5** even though its solubility was high enough. The IR spectra of water-soluble fullerenes **4** and **5** are shown in Fig. 5-1 along with those of the authentic fullerenols **1** and **2**. Despite their difference in water-solubility, the IR spectra of **4** and **5** were essentially the same.

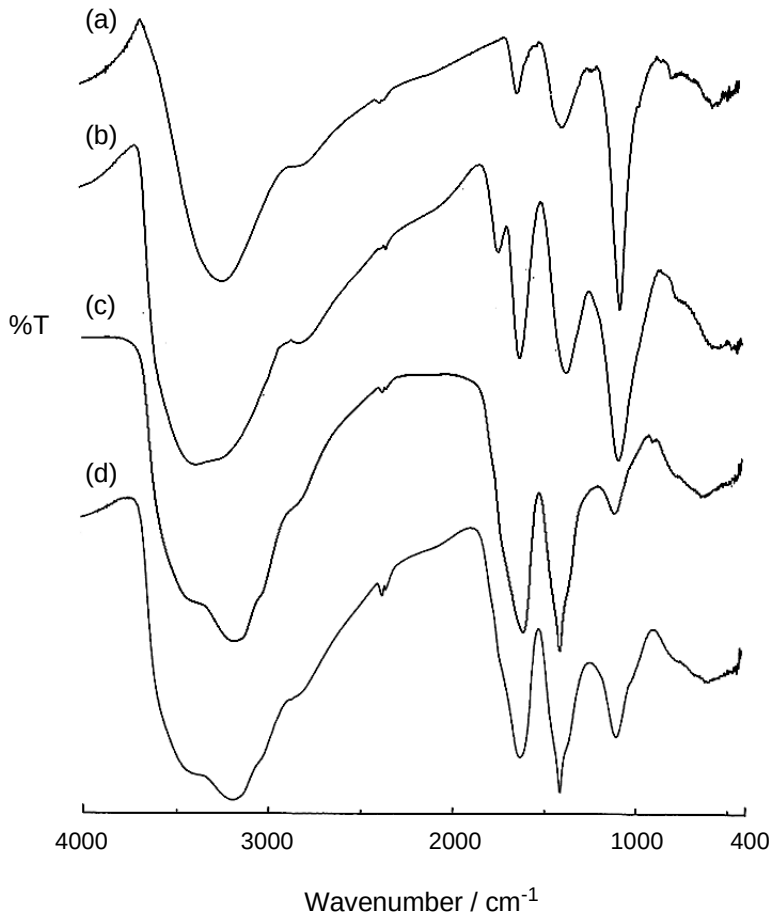


Fig. 5-1. IR spectra of fullerenol (a) $C_{60}(OH)_{12}$ (**1**), (b) $C_{60}(OH)_{36}$ (**2**), N-containing fullerenol (c) $C_{60}(OH)_{16}(NH_2)_8(NO_2)_8$ (**4**), and (d) $C_{60}(OH)_{18}(NH_2)_2(NO_2)_6$ (**5**).

With a broad band around 3000–3500 cm^{-1} consisting with νO–H, the spectra of **4** and **5** showed three characteristic bands at 1080, 1370, and 1620 cm^{-1} assigned for νC–O, δ_S C–O–H and νC=C absorption. These four broad bands are invariably reported as diagnostic absorption of various fullerenols[4, 8]. Such similar patterns of fullerenols **1** and **2** were found in both the spectra of **4** and **5**, although the relative peak intensities differ somewhat, suggesting a difference in the numbers of introduced hydroxyl groups. The small shoulder absorption observed at 3170 cm^{-1} in the spectra of both **4** and **5** was probably caused by νN–H, implying the introduction of amino groups from NH_3 employed. Additionally, the distinctive narrow and sharp peaks overlapped at 1600 and 1400 cm^{-1}, respectively, in both the spectra of **4** and **5** were observed. It is difficult to characterize these absorptions only by IR spectroscopy, however, nitro groups show strong two absorptions in these regions. Therefore it is inferred from the spectra that the newly synthesized fullerene derivatives **4** and **5** have some amino groups and nitro groups (or other nitrogen-containing substituents like -NHOH and -NO may also be possible to exist) in addition to abundant amount of hydroxyl groups. In order to obtain further information for these derivatives **4** and **5**, FABMS and MALDI-TOFMS spectroscopy were applied to measure their molecular weight. However the mass spectra of these compounds were not clearly obtained as well as the previously synthesized fullerenols **2** and **3**[8, 11]. Therefore their average structures were estimated by the results of elemental analysis along with a water content measurement by TGA as previously reported method[8]. It is known that the existence of a substantial amount of secondary water bound to the plural hydroxyl groups on a fullerene surface and such tightly entrapped water cannot be dissociated by the usual heating up to about 150 ºC[12].

The results of thermogravimetric analysis of fullerene derivatives **4** and **5** using a TGA spectrometer under nitrogen flow were shown in Fig. 5-2 and 5-3.

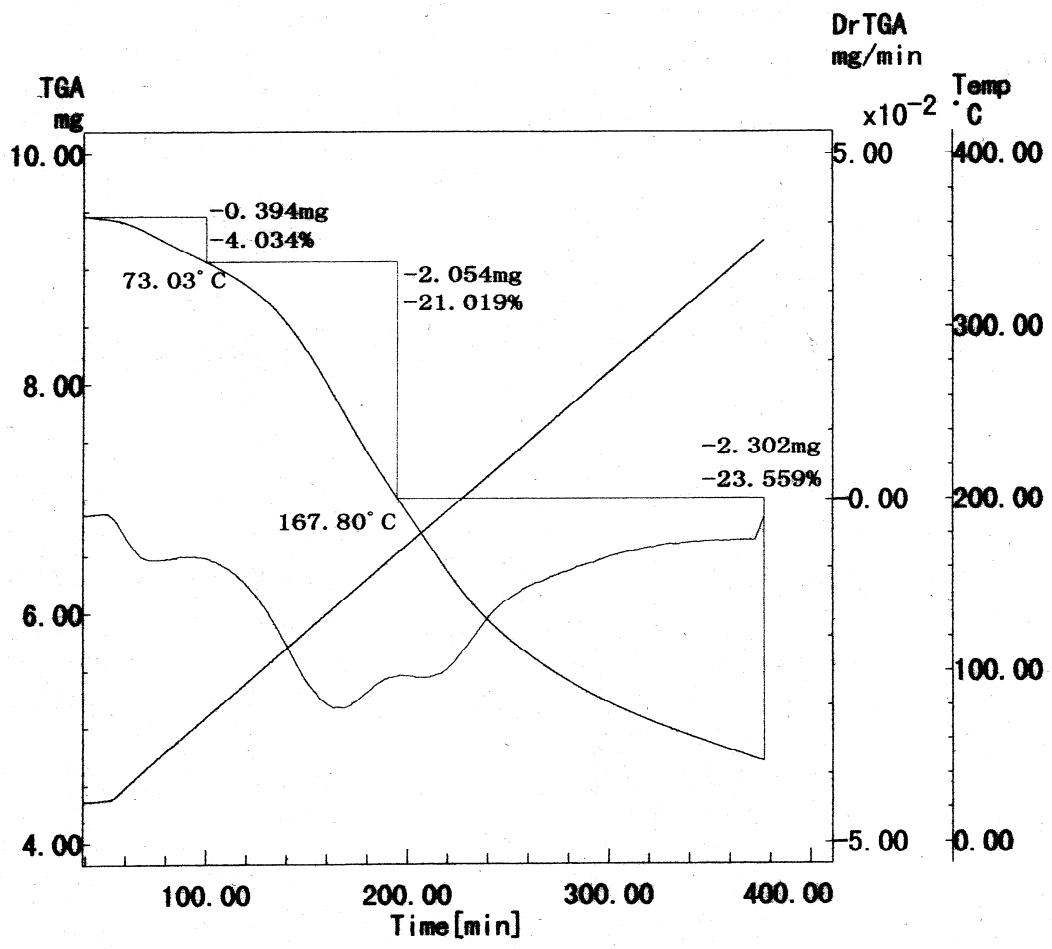


Fig. 5-2. Thermogravimetric analysis of N-containing fullerenol **4**.
Heating rate was 1 ºC/min under N_2 flow.

Although these samples were dried up under vacuum at room temperature for overnight, the weight loss of derivative **4** was still observed in three temperature ranges; *i.e.,* room temperature to 73 ºC, 73–168 ºC, and >168 ºC. The first (4 wt%) and the second weight loss (21 wt%) are both assigned to the secondary bound water, as reported by Chiang *et al* [12]. In the case of fullerenol **2**, only one derivative peak (Dr TGA) from room

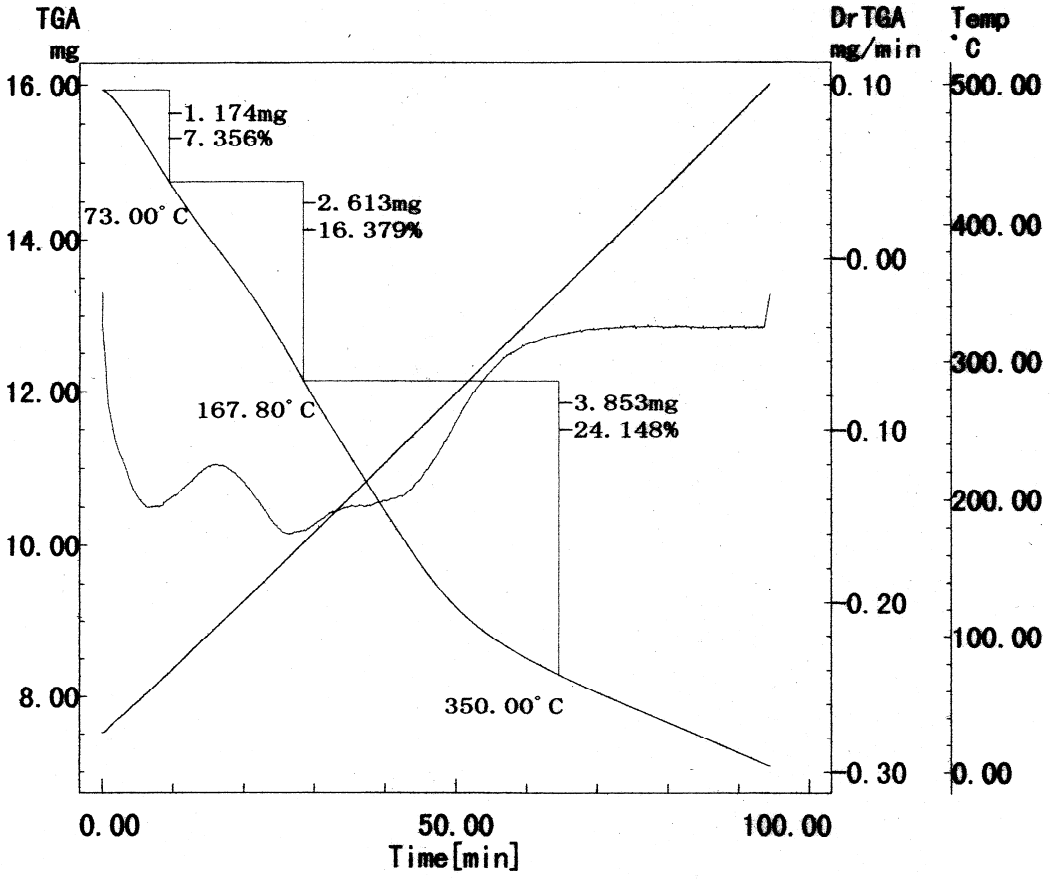


Fig. 5-3. Thermogravimetric analysis of N-containing fullerenol **5**.
Heating rate was 1 ºC/min under N_2 flow.

temperature to 130 ºC was observed[8]. The observed two reduction peaks for the present derivative **4**, corresponding to two kinds of secondary bound water, is explained by the effect of possible amino groups. The difference of strength of hydrogen bonding between amino and hydroxyl group (especially in their chelating position) may afford at least two kinds of bound water. The third weight loss may be attributed to the dehydration of introduced hydroxyl groups by possible thermal Pinacol rearrangement [12, 13] as well as the decomposition of fullerene nucleus. The similar spectrum was

obtained for the TGA measurement of derivative **5**. The estimated water contents, 25.0 wt% for derivative **4** and 23.7 wt% for derivative **5**, are shown in Table 5-1.

Table 5-1. Elemental analysis, water content, water solubility, and average structure of fullerenols **2**, **4**, and **5**

Estimated Average structure	Elemental analysis (%) [a]	Water cont. (wt%) [b]	Solubility (mg/mL) [c]
$C_{60}(OH)_{36}\cdot 8H_2O$ (**2**) [d]	C:48.06, H:3.61 (C:48.79, H:3.54)	8.9 (9.7)	17.5
$C_{60}(OH)_{16}(NH_2)_8(NO_2)_8\cdot 28H_2O$ (**4**)	C:35.87, H:4.22, N:10.97 (C:36.15, H:4.45, N:11.24)	25.0 (25.3)	>200
$C_{60}(OH)_{18}(NH_2)_2(NO_2)_6\cdot 24H_2O$ (**5**)	C:40.82, H:3.86, N:6.67 (C:40.78, H:3.99, N:6.34)	23.7 (24.5)	30

[a]Values in parentheses are calculated data. [b]Water contents were determined by thermogravimetric analysis. [c]Water solubility at 25 ºC in neutral water (pH 7). [d]Data from ref 8.

These weight loss might be attributed not only to dehydration but also to dissociation of NH_3. Additionally, the estimated water contents were considerably higher than those of fullerenols **2** and **3**. To compare the results of TGA measurement, the water content measurement for derivative **4** by using Karl Fischer titrator were also applied. By heating the sample with oven up to 190 ºC, the water content of **4** was determined as 20 wt%. The value is fairly consistent with the result of TGA considering that the rapid raising of oven temperature might be rather insufficient to vaporize the bound water from the solid sample. The considerably high water content of **4** and **5** as compared with fullerenols **2** and **3** is not inconsistent with their high water solubility. The color, water solubility, and IR spectra of fullerene derivatives **4** and **5** closely resemble to those of water-soluble fullerenols **2** and **3**. However, the critical difference was found in the nitrogen atom content. The results of elemental analysis for this, along with water

contents and water solubility, of **4** and **5** are also summarized in Table 5-1. The nitrogen atom content of **4** and **5** were 11 % and 7 %, respectively, and these values cannot be excluded for the estimation of their average structure in order to survey their chemical properties. Because of the difficulties of further characterization for the nitrogen-containing substituents in **4** and **5**, their average structures were deduced from the results of elemental analysis and water content measurement by postulating that the nitrogen (N-) containing groups were only composed of the most plausible amino and nitro groups. The results of the deduction on average structure of the derivatives **4** and **5** were $C_{60}(OH)_{16}(NH_2)_8(NO_2)_8 \cdot 28H_2O$ and $C_{60}(OH)_{18}(NH_2)_2(NO_2)_6 \cdot 24H_2O$, respectively, using the calculating method reported for fullerenols **2** and **3**[8]. The determination processes of the estimated average structure for fullerenols **4** and **5** are partly exemplified in Table 5-2 and 5-3, respectively. More than 50 calculations were systematically executed (representative 5 results are shown in each Table). By the criteria that water content % H_2O should be within 0.5 % and that all elemental analysis values (% C, % H, % N) should be within 1 % between those calculated and experimental values, the estimated distribution for fullerenol **4** was ranged as $C_{60}(OH)_{15-17}(NH_2)_{5-10}(NO_2)_{7-11} \cdot 27–29H_2O$. Thus, its estimated average structure was determined as $C_{60}(OH)_{16}(NH_2)_8(NO_2)_8 \cdot 28H_2O$ (Calc #4 in Table 5-2). The total number of substituent groups was 32 (with enough 24 polar OH and NH_2 groups for observed water solubility), being well consistent with the number of hydroxyl groups in fullerenol **2**. Similarly, the estimated average structure of fullerenol **5** was determined as $C_{60}(OH)_{18}(NH_2)_2(NO_2)_6 \cdot 24H_2O$.

Table 5-2. Determination of estimated average structure of N-containing fullerenol **4**[a]

Group	Calc #1	Calc #2	Calc #3	**Calc #4**	Calc #5	Exp[b]
-OH	36	30	24	**16**	12	
$-NH_2$	11	10	9	**8**	7	
$-NO_2$	5	6	7	**8**	9	
Total#[c]	52	46	40	**32**	28	
$OH+NH_2$	47	40	33	**24**	19	
H_2O	16	20	24	**28**	32	
%C	35.55	35.55	35.55	**36.15**	35.55	**35.87**
%H	4.47	4.47	4.47	**4.45**	4.47	**4.22**
%N	11.05	11.05	11.05	**11.24**	11.05	**10.97**
$\%H_2O$	14.2	17.8	21.3	**25.3**	28.4	**20–25**

[a]The value of elemental analysis of $C_{60}(OH)_n(NH_2)_m(NO_2)_m \cdot lH_2O$ was calculated for various number of OH, NH_2, and NO_2 groups as well as the number of secondary bound water. [b]The experimental value. [c]Total number of OH, NH_2, and NO_2 groups.

Table 5-3. Determination of estimated average structure of N-containing fullerenol **5**.[a]

Group	Calc #1	Calc #2	Calc #3	**Calc #4**	Calc #5	Exp[b]
-OH	29	26	20	**18**	12	
$-NH_2$	4	3	2	**2**	1	
$-NO_2$	5	5	6	**6**	7	
Total#[c]	38	34	28	**26**	20	
$OH+NH_2$	33	29	22	**20**	13	
H_2O	16	17	21	**24**	27	
%C	40.12	41.25	41.25	**40.78**	41.20	**40.82**
%H	3.87	3.81	3.81	**3.99**	3.92	**3.86**
%N	7.01	6.41	6341	**6.34**	6.40	**6.67**
$\%H_2O$	16.0	17.5	21.6	**24.5**	27.8	**20–24**

[a]The value of elemental analysis of $C_{60}(OH)_n(NH_2)_m(NO_2)_m \cdot lH_2O$ was calculated for various number of OH, NH_2, and NO_2 groups as well as the number of secondary bound water. [b]The experimental value. [c]Total number of OH, NH_2, and NO_2 groups.

Although these chemical formulas are average structure of a mixture of various constitutional isomers, the author can obtain many interesting features from these: (1) the most abundant group is hydroxyl group (16–18) in both derivatives **4** and **5**,

(2) the higher water-soluble **4** (>200 mg/mL) has larger number of amino groups than that of less soluble **5** (30 mg/mL), (3) the number of secondary bound water is slightly increased by increasing number of amino group, (4) the numbers of hydroxyl group and nitro group are essentially the same each other, and (5) the total numbers of substituents are 32 for **4** and 26 for **5**, respectively. Although the fullerene derivatives bearing either amino or nitro group are well known[9, 10], the fullerenols bearing these N-containing groups, such as amino or nitro group, have not reported yet to the best of the author's knowledge. The observed water solubility (>200 mg/mL) of fullerenol **4** is the highest value among the reported fullerene derivatives as high as the value reported for $C_{60}(OH)_{16}$ [5, 14].

Optimization of Reaction Condition: The Effect and Role of Ammonia

To optimize the reaction condition of the one-step synthesis for fullerenols **4** and **5** from C_{60} and to elucidate the role of NH_3, some reactions of C_{60} were carried out by addition of various amounts of NH_3 aqueous solution (Table 5-4). The maximum total yield of **4** and **5** (0.151 g) from 0.1 g of C_{60} was attained when 4 mL of NH_3 aqueous solution (28 %) was added together with 10 mL of H_2O_2 aqueous solution (30 %). A smaller amount of NH_3 aqueous solution (2 mL) resulted in the exclusive formation of **4**. By increasing amount of NH_3 solution, the yield of **4** gradually decreased and the formation of **5** became dominant. The IR spectra and the result of elemental analysis for both **4** and **5** obtained in each run were essentially the same character each other. This concentration effect of NH_3 is somehow interesting because nitrogen atom content in fullerenol **4** is slightly higher than that in **5**. In other words, these results suggest that initially formed fullerenol **4** can gradually convert to fullerenol **5**, decreasing the

number of N-containing groups. Indeed, such a mechanism was supported by time course experiment of this reaction (Fig. 5-4).

Table 5-4. Effects of amount of ammonia aqueous solution on the yield of N-containing fullerenols **4** and **5**[a]

28% NH_3 aq	Yield (mg, %)[b]		
(mL)	Fullerenol **4**	Fullerenol **5**	Total
2	87 (31)	trace	87 (31)
4	74 (27)	77 (31)	151 (58)
6	62 (22)	80 (33)	142 (55)

[a]The reaction of C_{60} (100 mg) was carried out in 30% hydrogen peroxide aqueous solution (10 mL) in the presence of 28% ammonia aqueous solution at 60 ºC under air for 12 hours. [b]The weight yield (mg) obtained from 100 mg of starting C_{60} and %yield (in parentheses) calculated by their estimated average structures **4** (M = 1992) and **5** (1766), respectively.

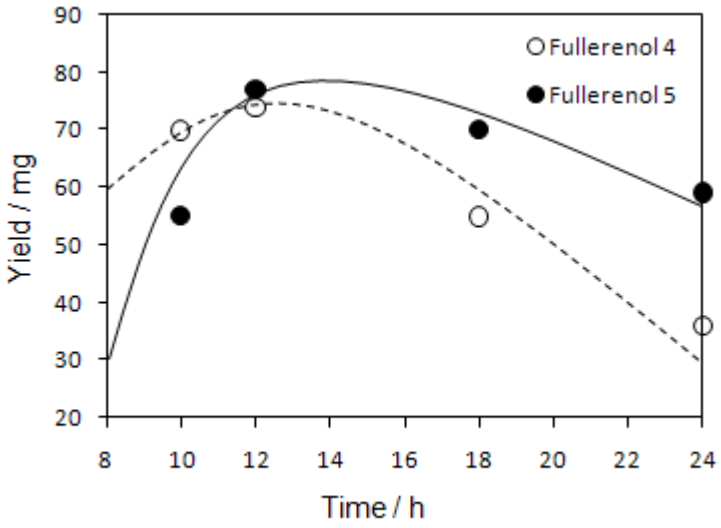


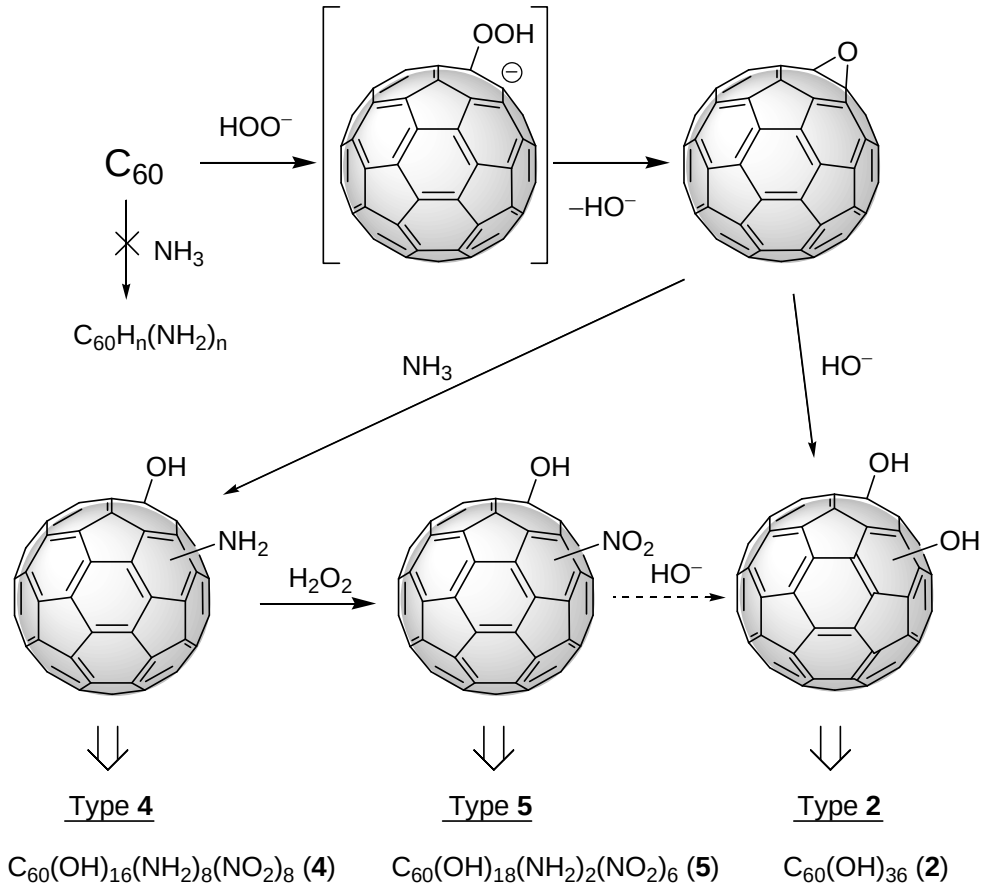
Fig. 5-4. Time course of the reaction of C_{60} (100 mg) with hydrogen peroxide (30 %, 10 mL) in the presence of ammonia (28 %, 4 mL) in aqueous solution at 60 ^{0}C under air.

As shown in Fig. 5-4, the yield of highly N-contained fullerenol **4** first increased as the major product till 12 hours, and then it gradually decreased after a prolonged time.

The similar time tendency was also observed in the yield of fullerenol **5**, but after the peak point at 12 hours fullerenol **5** became the major product. Therefore, the conversion of fullerenol **4** into **5** was again confirmed from this result.

Possible Reaction Path

To examine the influence of NH_3 toward H_2O_2, the independent experiments using each single reagent on C_{60} were conducted. C_{60} (0.1 g) were added into 10 mL of either 30 % H_2O_2 or 28 % NH_3 aqueous solution with all the same other conditions as described in experimental section. By monitoring with HPLC, C_{60} both in aqueous H_2O_2 and in aqueous NH_3 were remained >98 % unchanged even after 12 hours. These results ensured that the formation of derivatives **4** and **5** required the coexistence of both H_2O_2 and NH_3. Although the reaction of γ-cyclodextrin-bicapped C_{60} with NH_3 gas under high pressure[15] as well as the reaction of C_{60} with various amines[16] were reported, the experiment excluded the possibility of the preformation of aminofullerene like $C_{60}H_n(NH_2)_n$[17]. Thus the formation of the derivatives **4** and **5** would be presumed to be initiated by the attack of HOO^-, effectively formed from the equilibrium of H_2O_2 by the assistance of NH_3 to produce intermediacy oxidized fullerene $C_{60}O$ (Scheme 5-2). Then the nucleophilic attack of either NH_3 or HO^- gave amino group (NH_2)-substituted fullerenol (as Type **4** shown in Scheme 5-2) or simple fullerenol (as Type **2**), respectively[18]. As seen in the results in Tables 5-1 and 5-2, the Type **5** fullerenol bearing nitro group (NO_2) can be obtained by oxidation of Type **4** fullerenol due to the presence of a large excess amount of H_2O_2[19]. It is also known that such a nitro group on fullerene can be converted to various substituents by the substitution with corresponding nucleophiles[10].



Scheme 5-2

Therefore the total number of N-containing groups in derivative **5** may be decreased by following these possible reactions in comparison with that in derivative **4**.

Polyhydroxylation Reaction from Other Fullerene Derivatives

To confirm the proposed reaction mechanism including a possible intermediate of oxidized fullerene $C_{60}O$, the similar reaction of $C_{60}O_n$ (n = 0–4)[20] was studied in aqueous H_2O_2 without adding ammonia. The reaction conditions and the work-up procedure are essentially the same as previously reported for the reaction of $C_{60}(OH)_{12}$[5]. Although the reaction mixture was not converted into clear solution completely even

after two weeks due to the contamination of unreactive C_{60} in a mixture of starting $C_{60}O_n$, the yield of water-soluble yellowish compound, its IR spectra, and the result of elemental analysis were almost the same as those of fullerenol **2**, which was synthesized from $C_{60}(OH)_{12}$ (Table 5-5 and Fig. 5-5a). This compound may still remain epoxide oxygens on fullerene cage, however, they could not be detected from IR spectroscopy and elemental analysis. Such a good similarity of qualitative and quantitative analysis is well consistent with the author's proposed mechanism. The reason for the observed fact that $C_{60}O_n$, as well as $C_{60}(OH)_{12}$, can react in a heterogeneous H_2O_2 solution, but pristine C_{60} cannot, may be due to their slightly higher reactivity toward nucleophilic attack derived from their strain energy as well as their lower crystallinity and slightly higher polarity than those of C_{60}.

Table 5-5. Yield and elemental analysis data for some water-soluble fullerenols starting from various fullerene derivatives [a]

Starting Materials	Time (days)	Yield (mg)	Elemental analysis (%)
$C_{60}(OH)_{12}$ [b]	4	97	C:48.06, H:3.61
$C_{60}O_n$	14	94	C:46.36, H:3.61
$C_{60}H_{36}$	7	96	C:52.61, H:3.76
			(C:52.34, H:3.51) [c]
C_{60} + Et_2NH	3 [e]	176 [d]	C:53.14, H:4.46, N:2.56
$C_{60}(Et_2NH)_n$	1 [e]	19 [f]	C:52.73, H:4.24, N:2.86

[a]The reactions of each starting material (100 mg) were carried out in 30% H_2O_2 aqueous solution (10 mL) at 60 ºC. [b]Data from ref 8. [c]Value in parentheses is calculated data for the estimated average structure as $C_{60}H_4(OH)_{32}\cdot 6H_2O$ in conjunction with the measured water content of 7.9 wt%. [d]Diethylamine (5.76 mL, 400 equiv to C_{60}) was used instead of ammonia. [e]The reaction was carried out at room temperature. [f]The previously prepared $C_{60}(Et_2NH)_n$ (172 mg) by the reaction of C_{60} with diethylamine (ref 21) was used as a starting material.

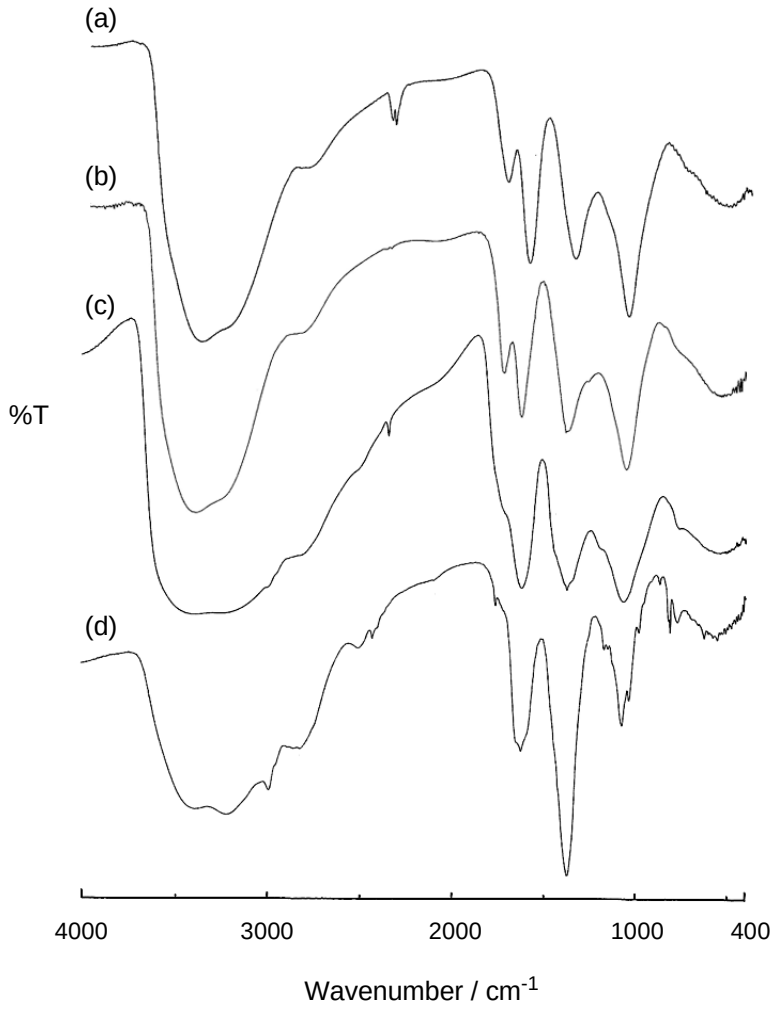


Fig. 5-5. IR spectra of water-soluble fullerenols from various starting materials (a) $C_{60}O_n$, (b) $C_{60}H_{36}$, (c) C_{60} in the presence of Et_2NH, and (d) $C_{60}(Et_2NH)_n$.

The similar reaction of $C_{60}H_{36}$ also afforded a water-soluble yellowish compound. The IR spectrum clearly indicated the similar fullerenol structure along with the nearly disappearance of strong νC–H absorption, which can be observed in the IR spectrum of starting material $C_{60}H_{36}$ (Fig. 5-5b). In this reaction, hydroxylation must be induced by substitution of hydrogen atom on fullerene cage. Therefore the reaction mechanism is somewhat different from the above one shown in Scheme 5-2. Taking into account the

assumption that hydroxyl group is introduced by substitution of hydrogen atom, the average structure is estimated as $C_{60}H_4(OH)_{32}\cdot 6H_2O$ from the data of elemental analysis along with their water content of 7.9 wt%, which is well consistent with the calculated value (Table 5-5). The water solubility of this compound was 20.2 mg/mL, again being well consistent with the value of fullerenol **2** (17.5 mg/mL).

The additive effect of secondary amine instead of ammonia on the hydroxylation reaction of pristine C_{60} was also investigated. Using excess amount of diethylamine (400 equiv to C_{60}), the reaction of C_{60} in H_2O_2 aqueous solution was carried out at room temperature. After three days, the suspension became a clear yellowish solution and a yellowish water-soluble compound was obtained after usual work-up. The IR spectrum of this compound suggested the fullerenol structure with some differences as compared to fullerenol **2**, implying the introduction of N-containing group as similar to the case of ammonia addition (Fig. 5-5c). Indeed, the data of elemental analysis of this compound revealed the introduction of a certain amount of nitrogen atom (2.56 %). To check whether this introduction of nitrogen occurs prior to hydroxylation or not, firstly, the amine adduct $C_{60}(Et_2NH)_n$ was prepared by reported method[21] and conducted the same reaction but in the absence of diethylamine at room temperature. In this reaction, the similar water-soluble compound was also obtained after 1 day. Interestingly, the data of elemental analysis closely resemble each other especially for the content of nitrogen atom (2.86 %). Even the IR spectrum showed characteristic features of fullerenol, however, the IR spectrum of this compound is somewhat different from that of the above fullerenol obtained from the reaction of C_{60} in the presence of diethylamine (Fig. 5-5d). Therefore, the hydroxylation with H_2O_2 by the assistance of basic amine may not initiate after the completion of amination but might occur simultaneously during the

amination. The nucleophilicity of NH_3 is much higher than that of diethylamine so that it can be introduced more on the fullerene cage as a certain N-containing substituent group.

Conclusions

Novel one-step and facile synthesis of water-soluble fullerenes from pristine C_{60} was developed using aqueous H_2O_2 upon coexistence of NH_3. The obtained two new derivatives were characterized by IR spectroscopy and elemental analysis along with TGA to demonstrate their novel fullerenols structure bearing some nitrogen-containing substituent groups. Although the further specification of these nitrogen-containing group is difficult for these mixed materials, by assumption that these groups are the most plausible amino and nitro groups, these average structures were deduced as $C_{60}(OH)_{16}(NH_2)_8(NO_2)_8 \cdot 28H_2O$ and $C_{60}(OH)_{18}(NH_2)_2(NO_2)_6 \cdot 24H_2O$. The former derivative showed its water solubility as high as the highest one reported (>200 mg/mL). The reaction mechanism is also proposed and explained by the observations on the optimization of reaction conditions as well as some control experiments started from various fullerene derivatives with or without basic additives. These newly synthesized water-soluble fullerene derivatives are expected to be an interesting nanomaterial as a functionalized water-soluble nanoparticle in the field of nanotechnology as well as life science.

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11) The positive ion FAB mass spectrum of fullerenol **1** was clearly obtained, showing the distribution of 8–14 hydroxyl groups with the maximum average at 11 (*m/z* 907).

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14) Although the number of hydroxyl groups of $C_{60}(OH)_{16}$ is rather low, the solubility is quite high as >200 mg/mL. According to the report of Wilson *et al* (7), the synthesis of this fullerenol was carried out using sodium hydroxide, *e.g.* $C_{60}(OH)_{24}$ and the above $C_{60}(OH)_{16}$, might result in some contamination of sodium salt. The extremely high water solubility of $C_{60}(OH)_{16}$ and the fullerenol **4** may due to a partial salt form of fullerenol, such as sodium and ammonium salt, respectively.

15) K. Fukui, N. Mori, S. Takekuma, H. Takekuma, Z. Yoshida, K. Sato, D. Shiomi, T. Kato, T. Takui, Synth. Met., **2001**, *121*, 1171–1172

16) (a) A. Hirsch, Q. Li, F. Wudl, *Angew. Chem. Int. Eng*., **1991**, *30*, 1309–1310, (b) K. D. Kampe, N. Egger, M. Vogel, *Angew. Chem. Int. Eng*., **1993**, *32*, 1174–1176, (c) H. Isobe, T. Tanaka, W. Nakanishi, L. Lemiègre, E. Nakamura, *J. Org. Chem*., **2005**, *70*, 4826–4832, (d) L. Lemiègre, T. Tanaka, T. Nanao, H. Isobe, E. Nakamura, *Chem*. *Lett*., **2007**, *36*, 20–21

17) Indeed, such aminofullerene $C_{60}H_n(NH_2)_n$ has not been reported yet.

18) Although the reaction of $C_{60}O_n$ with ammonia has not been explored, the reaction of cationic C_{60} with ammonia has been reported: (a) J. J. Stry, T. Coolbaugh, E. Turos, J. E. Garvey, *J. Am. Chem. Soc.*, **1992**, *114*, 7914–7916, (b) S. Petrie, *Int. J. Mass Spectromet*., **2006**, *255–256*, 213–224.

19) Oxidation of amino group to nitro group by hydrogen peroxide has been reported: (a) W. D. Emmons, *J. Am. Chem. Soc.*, **1957**, *79*, 5528–5530, (b) T. P. Kofman, E. A. Paketina, *Russ. J. Org. Chem*., **1997**, *33*, 1125–1132

20) A mixture of oxidized fullerenes $C_{60}O_n$ was purchased from Frontier Carbon Corporation. The component ratio 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%.

21) J. Janaki, M. Premila, P. Gopalan, V. S. Sastry, C. S. Sundar, *Thermochimica Acta*, **2000**, *356*, 109–116.

Chapter 6. General Conclusions

This thesis deals with the development of new antioxidant, which has high water-solubility and high antioxidant activity, using fullerene (C_{60}) for life science application. It is focused on the fundamental studies in applied chemistry comprised of the following four points, (1) the antioxidant activity of water-soluble fullerenes evaluated by β-Carotene bleaching assay, (2) the effects of oxygen on fullerenes for enhanced antioxidant activity, (3) the synthesis of highly water-soluble fullerenes more than half-covered by hydroxyl groups, and (4) the synthesis of one-step synthesis of water-soluble fullerenols bearing nitrogen-containing substituents. From the series of studies, the author obtains seminal results that demonstrate promising nature of the water-soluble fullerenes as superior antioxidants. The results, providing not only their standard evaluation method of antioxidant activities but also their effective enhancement methods of antioxidant ability and water-solubility, promise to contribute to, over and above cosmetic industry, the further research of fullerene and antioxidant chemistry. Conclusion in each chapter of the body is collected.

In chapter 2, the author applies β-Carotene breaching assay to the evaluation of antioxidant activities of water-soluble fulerenes for the first time and the results demonstrate that water-soluble fullerene complexes such as PVP/C_{60}, γ-CD/C_{60} and commercial antioxidant, Radical Sponge® can exhibit significant antioxidant activity, more than APS, and protect β-Carotene from the discoloration by lipid peroxyl radicals, H_2O_2, and possible superoxide anion radicals upon irradiation. These results are similar to those of generally accepted ESR measurements and, therefore, it is

concluded that β-Carotene breaching assay could apply with enough appropriateness to measure antioxidant activities of water-soluble fullerenes.

In chapter 3, the author found that water-soluble fullerene complexes with oxygen such as PVP/$C_{60}O$ and PVP/$C_{60}O_n$ showed 1.7–2.4 times higher antioxidant activities than PVP/C_{60} which was without oxygen. Therefore intoroducing pin-up oxygen on fullerene cage could be a meaningful key in developing new applicable antioxidants using fullerenes by means of a simple and conventional technique that can enhance their antioxidant activity.

In chapter 4, Using aqueous H_2O_2, the author developed the facile synthetic method of water-soluble polyhydroxylated [60]fullerenes, fullerenols, bearing more than 30 hydroxyl groups without using sodium hydroxide. The estimated average structure of these fullerenols was determined as $C_{60}(OH)_{36}\bullet 8H_2O$ or $C_{60}(OH)_{40}\bullet 9H_2O$, depending on the reaction time. The obtained fullerenol showed a high water solubility up to 58.9 mg/mL and a high dispersion property in a molecule scale. In the preliminary evaluation of $C_{60}(OH)_{36}\bullet 8H_2O$ by β-Carotene breaching assay, the fullerenol also exhibit a potent antioxidant activity similar to PVP/C_{60} for cosmetic ingredient and the antioxidation mechanism of fullerenols will be further studied.

In chapter 5, one-step synthesis of water-soluble fullerene derivatives from pristine C_{60} was developed using aqueous H_2O_2 upon coexistence of NH_3. The author deduced these average structures as $C_{60}(OH)_{16}(NH_2)_8(NO_2)_8\cdot 24H_2O$ and $C_{60}(OH)_{18}(NH_2)_2(NO_2)_6\cdot 24H_2O$. The former derivative showed its water solubility as high as the highest one reported (>200 mg/mL).

List of Publications

1) Antioxidant activity of supramolecular water-soluble fullerenes evaluated by β-Carotene bleaching assay

Hiroya Takada, Ken Kokubo, Kenji Matsubayashi, and Takumi Oshima *Bioscience, Biotechnology, and Biochemistry,* **2006***, 70*(12), 3088–3093.

2) Facile Synthesis of Highly Water-Soluble Fullerenes Over Half-Covered by Hydroxyl Groups

Ken Kokubo, Kenji Matsubayashi, Hiroshi Tategaki, Hiroya Takada, and Takumi Oshima.
ASC Nano, **2008,** *2***,** 327–333.

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

Kenji Matsubayashi, Tadashi Goto, Kyoko Togaya, Ken Kokubo, and Takumi Oshima
Nanoscale Research Letters, **2008,** *3*, 237–241.

4) One-Step Synthesis of Water-Soluble Fullerenols bearing Nitrogen-Containing Substituents

Kenji Matsubayashi, Ken Kokubo, Hiroshi Tategaki, Sayako Kawahama, and Takumi Oshima,
Fullerenes, Nanotubes and Carbon Nanostructures, **2009**, in press.

List of Supplementary Publications

1) Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis

Tomohisa Mori, Hiroya Takada, Shinobu Ito, Kenji Matsubayashi, Nobuhiko Miwa, Toshiko Sawaguchi
Toxicology, **2006** *225*(1)**,** 48–54.

2) Innovative antioxidant: fullerene (INCI #7587) is as Radical Sponge on the skin. Its high level of safety, stability and potential as premier antiaging and whitening cosmetic ingredient

Hiroya Takada, Hitoshi Mimura, Xiao Li, R. M. Islam, Kenji Matsubayashi, Shinobu Ito, Nobuhiko Miwa
Fullerenes, Nanotubes, and Carbon Nanostructures, **2006**, *14*(2–3), 335–341.

3) Inhibitory effect of the water-soluble polymer-wrapped derivative of fullerene on UVA-induced melanogenesis via downregulation of tyrosinase expression in human melanocytes and skin tissues

Xiao Li, Kenji Matsubayashi, Nobuhiko Miwa
Archives of Dermatological Research, **2007***, 299*(5–6)**,** 245–257.

4) Water-soluble C_{60} fullerene prevents degeneration of articular cartilage in osteoarthritis via down-regulation of chondrocyte catabolic activity and inhibition of cartilage degeneration during disease development

Kazuo Yudo, Kiyoshi Shishido, Hideki Murayama, Mitsunobu Yano, Kenji Matsubayashi, Hiroya Takada, Hiroshi Nakamura, Kayo Masuko, Tomohiro Kato, Nishioka, Kusuki.
Arthritis & Rheumatism, **2007***, 56*(10)**,** 3307–3318.

5) Comparison of nitric oxide synthase inhibitors, phospholipase A2 inhibitor and free radical scavengers as attenuators of opioid withdrawal syndrome

Tomohisa Mori, Shinobu Ito, Kenji Matsubayashi, Toshiko Sawaguchi,
Behavioural Pharmacology, **2007***, 18*(8)**,** 725–729.

6) Involvement of free radicals followed by the activation of phospholipase A2 in the mechanism that underlies the combined effects of methamphetamine and morphine on subacute toxicity or lethality in mice: Comparison of the therapeutic potential of fullerene, mepacrine, and cooling

Tomohisa Mori, Shinobu Ito, Mizuho Mizuho, Tadashi Suzuki, Shizuko Kobayashi, Kenji Matsubayashi, Toshiko Sawaguchi,
Toxicology, **2007***, 236*(3)**,** 149–157.

7) Oxidative stress in methamphetamine-induced self-injurious behavior in mice

Tomohisa Mori, Shinobu Ito, Taizo Kita, Minoru Narita, Tsutomu Suzuki, Kenji Matsubayashi, Toshiko Sawaguchi,
Behavioural Pharmacology, **2007***, 18*(3)**,** 239–249.