C_{60} and Water-Soluble Fullerene Derivatives as Antioxidants Against Radical-Initiated Lipid Peroxidation

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C_{60}, vitamin E, and three C_{60} derivatives (polar **1** and water-soluble C_3/D_3C_{60}s) were examined for their antioxidant effects on prevention of lipid peroxidation induced by superoxide and hydroxyl radicals. The protection effect on lipid peroxidation was found to be in the sequence: $C_{60} \geq$ vitamin E > **1** > none, for liposoluble antioxidants, and $C_3C_{60} \gg D_3C_{60}$ > none, for water-soluble ones. Fluorescence quenching of $PyCH_2COOH$ (Py = pyrene) by both C_3- and D_3C_{60}s shows that the Stern−Volmer constant, K_{SV}, is about the same for both quenchers in aqueous solution. Upon addition of liposomes, the fluorescence quenching becomes more efficient: 5-fold higher in K_{SV} for C_3C_{60} than for D_3C_{60}. When $Py(CH_2)_nCOOH$ (n = 1, 3, 5, 9, or 15) was incorporated in lipid membranes, the K_{SV}s all were small and nearly equal for D_3C_{60} but were quite large and different for C_3C_{60} with the sequence: $n = 1 < 3 < 5 < 9 < 15$. The better protection effect of C_3C_{60} on lipid peroxidation than that of D_3C_{60} is attributed to its stronger interaction with membranes. Overall, the antioxidation abilities of the compounds examined were rationalized in terms of the number of reactive sites, the location of antioxidant in lipid membranes, and the strength of interactions between antioxidants and membranes.

Introduction

Inspired by the high chemical reactivity of C_{60} toward various organic radicals,[1] many research laboratories have devoted great efforts in synthesizing water-soluble fullerene derivatives and actively pursuing their biomedical effects.[2−7] Friedman et al.[2] reported that a water-soluble bis(phenethylaminosuccinate) C_{60} acts as a competitive inhibitor of HIV protease with a K_i value of 5.3 μM. When the substituent group was modified, the K_i value was further improved to 0.32 μM.[3a] Fullerene−oligonucleotide conjugates were found to be able to photochemically induce sequence-specific DNA cleavage.[3] To study the distribution and metabolism of fullerene derivatives in biological systems, radioactive carbon-labeled C_{60} was synthesized.[4a,b] In vivo studies show that fullerene derivatives did not have acute toxicity to model animals (rats and rabbits).[4b,c] Upon UV irradiation, fullerene derivatives, however, can cause the formation of singlet oxygen, hydroxyl radical, and superoxide and become cytotoxic to cells.[5] Polyhydroxylated C_{60}s were reported to have effects on antiproliferation of vascular smooth muscle cells,[6a] attenuation of exsanguination-induced bronchoconstriction,[6b] and suppression of microsomal cytochrome P450-dependent monooxygenases.[6c] It was also reported that two hexa(carboxylic acid) C_{60} derivatives can inhibit the excitotoxic death of cultured cortical neurons induced by exogenous excitotoxins or by oxygen glucose deprivation.[7a] The same carboxylic acid C_{60} derivatives were also reported to be able to block the apoptosis signal of transforming growth factor-β (TFG-β) in human hepatoma cells.[7b] In the latter cases, the authors observed that the C_3 regioisomer has much stronger effects than the D_3 regioisomer.[7] In contrast, it was also observed that D_3C_{60} has a stronger effect than C_3C_{60} on the prevention of ceraminde-induced cell deaths.[8]

The above studies all show that water-soluble C_{60} derivatives indeed can protect cells from being attacked by reactive oxygen species (ROSs). In particular, two regioisomers of hexa(carboxylic acid) C_{60}s show different protection effects in different occasions.[7,8] The difference cannot be explained by their chemical reactivities toward ROSs, since their quenching efficiencies are about the same in homogeneous solution.[7a] In these studies, what remains unknown is how a water-soluble fullerene derivative interacts with various biological species (such as proteins, enzymes, DNA, membranes, etc.) and its subsequent protection effect on retaining a particular kind of biological function (such as enzyme activities, transmembrane potential, gene expression, etc.) upon radical attacks.

Cell membrane is an integral part of biological processes. Many membrane-bound proteins are involved in biological energy transduction in mitochondria respiration processes (such as complexes I and II, the NADH and succinate dehydrogenases, the cytochrome $b-c_1$ complex (III), cytochrome oxidases (IV), and the Na^+-K^+-ATPase) as well as signal transduction[9] (such as various kinds of ion channels, neurotransmitter acceptors, protein kinase C). When lipid molecules were attacked by radicals (for example, hydroxyl radicals, see

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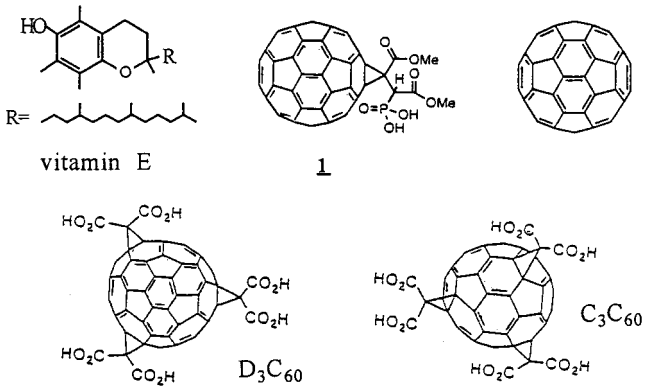
Chart 1



eqs 1−4), lipid alkyl radicals (L•) will be formed. The L•

$$LH + {}^{\bullet}OH \rightarrow L^{\bullet} + H_2O \quad (1)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2)$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad (3)$$

$$L^{\bullet} + L^{\bullet} \rightarrow L{-}L \quad (4)$$

(LH = lipid hydrocarbon chain)

can react with O_2 to form a peroxyl radical (eq 2), which further abstracts a hydrogen atom from neighboring lipid molecules to regenerate lipid alkyl radicals. Chain propagation (eqs 2 and 3) produces massive amount of lipid peroxides of which the polar peroxide group will move toward the aqueous phase and leads to break down of the membrane integrity. Chain termination (eq 4) requires encountering of two radicals and occurs with far less probabilities than the chain propagation steps. If lipid molecules are unsaturated, peroxidation will proceed much faster due to higher reactivity of the allylic protons.

In this paper, we report the interactions between five antioxidants (see Chart 1) and lipid membranes, as well as their protection effects on radical-induced lipid peroxidation (and thus retaining the transmembrane potential). We found that liposoluble C_{60} is a better antioxidant than vitamin E (i.e., α-tocopherol). Another three C_{60} derivatives show different protection effects on lipid peroxidation and can be potential antioxidation medicines for treating lipid peroxidation-related diseases.

Results and Discussion

In the studies, the radical species used are superoxide and hydroxyl radicals, which are generated by the xanthine−xanthine oxidase enzyme reaction[10] and the Fenton reaction[10] (see eqs 5 and 6), respectively. In the

$$\text{xanthine} + O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{uric acid} + O_2^{-\bullet} \quad (5)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (6)$$

experiments, a pH-sensitive dye, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium (HPTS), encapsulated in the internal aqueous phase of liposomes was used to report the membrane leakage (for details, see the Experimental Section). The absorbances of the HPTS dye at 403 and 454 nm are strongly pH-dependent. The outer bulk aqueous solution of a liposome has a pH value of 7.87, and 6.63 for the internal aquous phase. Upon membrane leakage (due to lipid peroxidation), the ratio of absorbances of HPTS at 454 vs 403 nm will increase (see Figure 1 of the Supporting Information). A higher ratio of A_{454}/A_{403} represents a larger extent of lipid peroxidation and thus membrane leakage. As shown in Figure 1a, the ratio of A_{454}/A_{403} of liposome-encapsulated HTPS increases at higher ferrous ion (or hydroxyl radical) concentrations, indicating more membrane leakage at higher hydroxyl radical concentrations. The increase in the A_{454}/A_{403} ratio for liposome alone (i.e., without addition of any antioxidant) is highest among all. In the presence of 0.5 mol % of **1**, vitamin E, or C_{60} in lipid bilayers, the membrane leakage (i.e., the A_{454}/A_{403} ratio) decreases in the sequence: liposome > **1** > vitamin E > C_{60} in the lipid bilayer. Vitamin E is the principal natural lipid-soluble antioxidant in biological systems. It can terminate the chain propagation processes by donating a hydrogen atom to lipid peroxyl radicals[11] (see

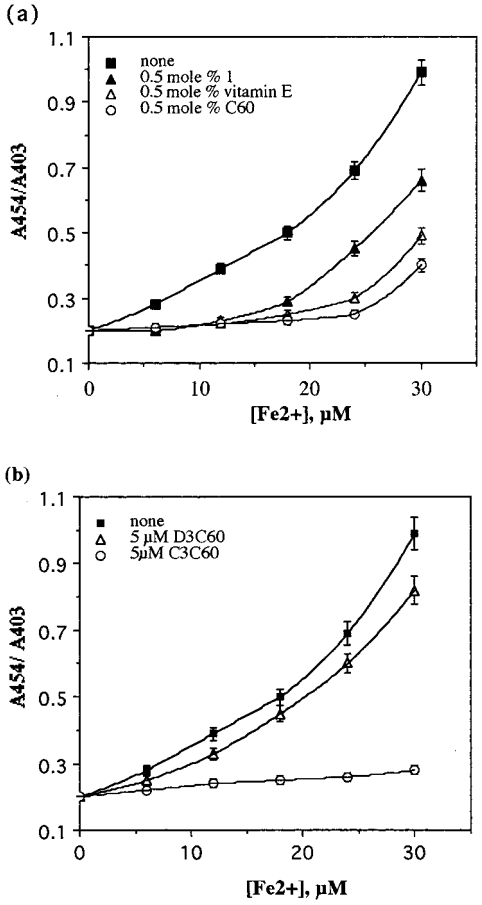
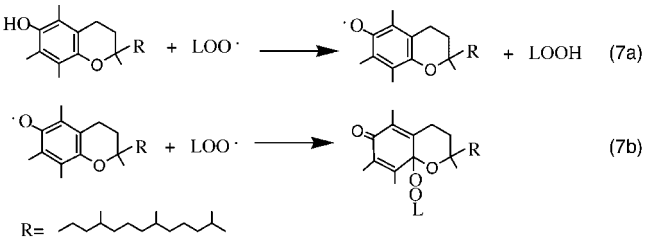


Figure 1. Absorbance ratio A_{454}/A_{403} as a function of the concentration of $FeSO_4$. The membrane contains 35 mol % unsaturated PE lipids, 1 mM of H_2O_2 was added to bulk aqueous phase first, and aliquots of Fe^{2+} solution were added: (a) 0.5 mol % of liposoluble antioxidant present in the lipid membrane; (b) 5 μM water-soluble antioxidant (C_3C_{60} or D_3C_{60}) added to the bulk aqueous phase of liposome solution and equilibrated for 15 h before addition of Fe^{2+} ions.

eq 7). The vitamin E radical produced is quite stable



due to resonance stabilization of the phenoxyl radical by the aromatic ring and will not abstract hydrogen atoms from lipid molecules. It, however, can further react with another lipid peroxyl radical to form a stable product. A C_{60} molecule contains 30 C=C double bonds.[12] A bridged-type substitution group will use up 1 C=C double bond. For example, both tris(malonate)-substituted C_3- and D_3C_{60} have 27 double bonds on the fullerene cage. For C_{60} and its derivatives, the chemical reactivity is due to its C=C double bonds. C_{60} is known to be able to react with up to 34 methyl radicals.[1] In terms of the number of reactive sites, C_{60} and **1** are certainly far better than vitamin E, which explains the superior antioxidation effect of C_{60} than vitamin E. The less effective radical scavenging property of **1** in membrane could not be explained by the number of reactive sites. The major difference between C_{60} and **1** is the polar substituent group in **1**, which tends to drive **1** toward the polar water−bilayer interface. The distant location of **1** away from the reactive allylic protons rationalizes why **1** cannot efficiently terminate the chain propagation processes as does the nonpolar C_{60}. The result in Figure 1a clearly shows that to have good protection effects, the antioxidant has to reside itself in close proximity to the chain propagation field, in addition to the number of reactive sites.

The above antioxidants examined are liposoluble. To be easily absorbed by biological systems, water-soluble antioxidants are required. Two water-soluble C_{60} derivatives, namely C_3- and D_3C_{60}s, are also investigated for their protection effects on lipid peroxidation. When the water-soluble antioxidant, C_3- or D_3C_{60}, was added to the aqueous phase before generation of hydroxyl radicals, the membrane leakage decreases in the sequence: none > D_3C_{60} ≫ C_3C_{60} (see Figure 1b). The C_3C_{60} regioisomer is a far better antioxidant against lipid peroxidation than its counterpart D_3 isomer, which is in sharp contract to their equal number of C=C double bonds and same free radical scavenging abilities in homogeneous aqueous solution (vide infra).[7a] The difference ought to be related to the existence of the nonpolar bilayer environment. The C_3C_{60} has a nonpolar end which could intercalate into the nonpolar bilayer phase and leads to a better protection effect than the D_3 regioisomer (vide infra).

When xanthine−xanthine oxidase is added to induce lipid peroxidation, similar results were obtained (see Figure 2 of Supporting Information). That is, membrane leakage decreases in the sequence: none > **1** > vitamin E > C_{60} for liposoluble antioxidants and none > D_3C_{60} ≫ C_3C_{60} for water-soluble antioxidants. The lipsoluble C_{60} again shows an equal or better protection effect on lipid peroxidation against superoxide radicals than vitamin E. And the water-soluble C_3C_{60} shows a better

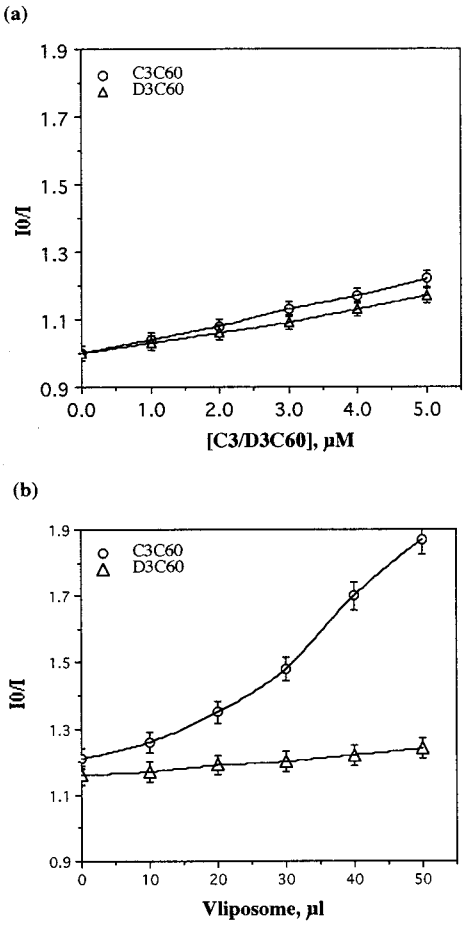


Figure 2. (a) Fluorescence quenching of 0.5 mM 1-pyrenecarboxylic acid as a function of the quencher (C_3C_{60} or D_3C_{60}) concentration. (b) To the solution in (a) at 5 μM C_3C_{60} or D_3C_{60} were sequentially added aliquots of a liposome solution. The concentration of the liposome concentration is arbitrary. Addition of liposomes brings together both the lipophilic fluorophore and quenchers, and therefore the fluorescence quenching becomes more efficient (see text for detail discussion).

protection effect than its D_3 counterpart. The differences in the protection effect essentially can be rationalized in terms of the number of reactive sites, the locations of antioxidants in the lipid bilayer, and the strength of antioxidant−membrane interactions, as in the case of hydroxyl radicals (vide supra).

To prove the hypothesis that the difference in protection effect between water-soluble D_3- and C_3C_{60} is due to their proximity to the lipid peroxidation field, we studied the interactions between membranes and these two antioxidants. As shown in Figure 2a, the slopes of the Stern−Volmer fluorescence quenching of 1-pyrenecarboxylic acid by both C_3- and D_3C_{60}s are roughly the same (K_{SV} = (4.4 ± 0.1) × 10^4 and (3.4 ± 0.1) × 10^4 M^{-1} for C_3- and D_3C_{60}, respectively). When aliquots of liposome solution was added to the pyrene−C_{60} solution, the I_0/I ratio increases proportionally (see Figure 2b). The increasing rate is ∼5 times higher for C_3C_{60} than for D_3C_{60}. Upon addition of liposomes, the nonpolar pyrene and fullerene cage moieties will tend to intercalate into the nonpolar bilayer phase, which makes the encounter between fluorophores and quenchers two-dimensional, instead of 3-dimensional, and leads to more efficient fluorescence quenching. The 5-fold higher rate of C_3C_{60} than D_3C_{60} indicates that C_3C_{60} has a

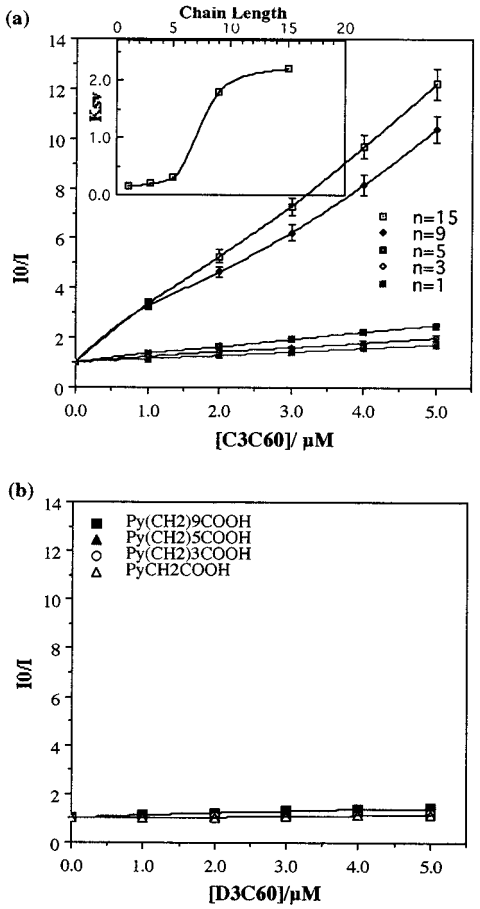


Figure 3. Stern−Volmer plot (I_0/I) as a function of the (a) C_3C_{60} and (b) D_3C_{60} concentration for different pyrene fluorescence probes with different chain lengths (n = 1, 3, 5, 9, and 15). Inset: Stern−Volmer constant K_{sv} as a function of the chain length. The fluorescence probe is membrane-incorporated Py$(CH_2)_n$COOH (n = 1, 3, 5, 9, 15).

stronger interaction with membranes than D_3C_{60}. To study how deep the fullerene cage intercalates into the membrane, *n*-pyrenealkylcarboxylic acids with different methylene chain length (n = 1, 3, 5, 9−15) were used as fluorescence probes. When the fluorophore and the quencher are in close proximity, the quenching efficiency will be the best and therefore the location of the quencher can be estimated. As shown in Figure 3a, the quenching efficiency for C_3C_{60} increases from n = 1, 3, 5, 9−15, whereas the quenching rates for D_3C_{60} all are low and almost the same for all pyrene probes (see Figure 3b). The results are consistent with those in Figure 2b that C_3C_{60}, but not D_3C_{60}, has a very strong interaction with lipid membranes. The difference between C_3- and D_3C_{60}s in interactions with membranes is understandable from a structural point of view. The three malonic groups of D_3C_{60} locate in the equator of the fullerene sphere, whereas those of C_3C_{60} are in one side of the half-sphere of the bucky ball. Therefore, the nonpolar end of the C_3C_{60} will tend to intercalate into the nonpolar bilayer phase. The results are also consistent with the better water solubility of D_3C_{60} than C_3C_{60}.

The fluorescence quenching results in Figure 3a indicate that the location of C_3C_{60} is still deeper than the average locations of the pyrene moieties of different conformers of 1-pyrenehexadecanoic acids. In the literature, Flory[13] and others[14] have shown that a D-$(CH_2)_n$-A (D: donor; A: acceptor) molecule will tend to adopt various kinds of conformations. The number of various conformers versus the D−A distance, *r*, has a distribution maximum at ca. 80% of the most extended distance at 300 K.[14] The distribution of these conformations is negative-temperature-dependent: i.e., a higher temperature shifts the distribution maximum toward conformations of shorter A−D distances. In other words, the extended conformers are favored by low temperatures, and the closed chain conformers are favored by high temperatures. In the current pyrene-$(CH_2)_n$-COOH system, the carboxylic group is located at the membrane−water interface, and the pyrene moiety in the membrane phase can adapt various distances, *r*, away from the bilayer−water interface. For n = 15 at room temperature, the fluorescence quenching approaches the plateau (see Figure 3a, inset), indicating that the *r* of maximum populated conformers (∼15 Å) is about the depth of the fullerene cage of C_3C_{60} inside membranes.

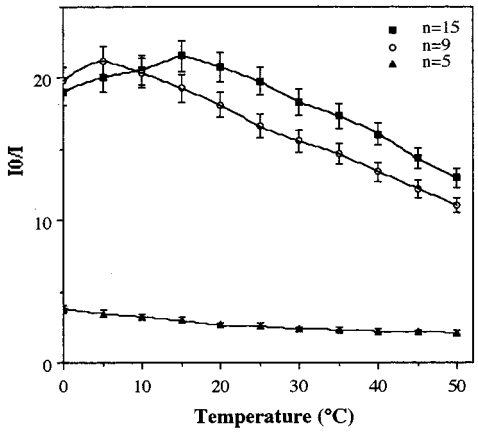


Figure 4. Stern−Volmer plot (I_0/I) as a function of temperature for n = 5, 9, and 15 pyrene probes. The quencher is 5 μM C_3C_{60} in the bulk aqueous solution.

To testify whether the above conformation distribution model is correct, fluorescence quenching experiments were conducted as a function of temperature. As shown in Figure 4, the fluorescence quenching efficiency for n = 15 increases to reach a maximum at T = 15 °C and then drops at higher temperatures. Similar trend was also observed for n = 9 except that the most efficient quenching temperature shifts to a lower temperature (T = 5 °C). For n = 5, the quenching efficiency increases monotonically as temperature decreases, which is consistent with the negative-temperature-dependent conformation distribution model that low temperatures favor more extended conformations.[13,14] The fact that maximum quenching can still be observed for both n = 9 and 15 at temperatures slightly below 300 K, indicates that the location of C_3C_{60} is slightly deeper than 80% of the most extended distance, *r*, for n = 9 and shallower than 80% of the most extended distance, *r*, for n = 15. Therefore, the depth of the fullerene cage of C_3C_{60} in membrane can be estimated[15] to be ca. 14 ± 4 Å away from the water−bilayer interface.

To further prove that the superior effect of C_3C_{60} over D_3C_{60} on protection of ROS-initiated lipid peroxidation is truly due to differences in their proximity in membrane, but not due to differences in their chemical reactivities toward ROS, the reaction rates of both C_3-

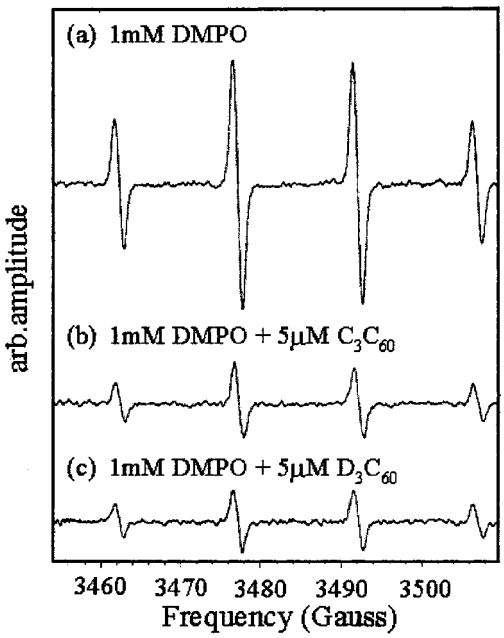


Figure 5. ESR spectra of DMPO-hydroxyl radical adduct. The hydroxyl radical was generated by the Fenton reaction (20 μM $FeSO_4$ and 200 μM H_2O_2). The concentrations of spin-trapping reagents are as follows: (a) 1 mM DMPO, (b) 1 mM DMPO and 5 μM C_3C_{60}, and (c) 1 mM DMPO and 5 μM D_3C_{60}.

and D_3C_{60} toward hydroxyl radicals were measured by competition reaction by ESR. In the literature, it is known that the spin-trapping reagent, DMPO, has a reaction rate of 1.8×10^9 M^{-1} s^{-1} toward hydroxyl radicals[16] and 10 M^{-1} s^{-1} toward superoxide radicals.[16] Upon coexistence of C_3C_{60} or D_3C_{60} in solution (see Figure 5), part of hydroxyl radicals will react with the fullerene moiety and lead to a decrease in the DMPO signal. From the percentage of decrease of the DMPO signal (area obtained by double integration of the ESR bands), the reaction rate toward hydroxyl radicals is $6.3 \pm 0.9 \times 10^{10}$ M^{-1} s^{-1} for C_3C_{60} and $5.6 \pm 0.8 \times 10^{10}$ M^{-1} s^{-1} for D_3C_{60}.[17] Both the reaction rates are basically diffusion-controlled, and there is no clear difference between C_3C_{60} and D_3C_{60} in the chemical reactivities toward hydroxyl radicals. In a similar way, the reaction rates toward superoxide radicals are measured to be $6.0 \pm 0.7 \times 10^2$ M^{-1} s^{-1} for C_3C_{60} and $5.0 \pm 0.4 \times 10^2$ M^{-1} s^{-1} for D_3C_{60} (see Figure 3 of Supporting Information for relative ESR intensities).

For all C_{60} derivatives, the number of reactive sites is about the same (27 C=C double bonds for both C_3- and D_3C_{60} and 29 C=C double bonds for **1**), and in homogeneous solution their chemical reactivities toward either superoxides or hydroxyl radicals are essentially the same. A biological system, however, is a two-phase (nonpolar membrane and polar aqueous phase) system. The polarity of antioxidants and thus their interactions with the species being protected therefore become very crucial to their in vivo antioxidation abilities against ROSs. Scavenging of radical initiators, however, is not the most important step in the prevention of lipid peroxidation. It is the termination of the chain propagation processes that significantly retards lipid peroxidation and thus avoids the breaking down of the membrane integrity. The D_3C_{60} in bulk aqueous phase cannot terminate the lipid peroxidation chain propagation effectively as does the C_3C_{60}. Therefore, D_3C_{60} has nearly no effect in prevention of lipid peroxidation. Figure 1 also shows that membrane leakage is nonlinearly proportional to the concentration of radical initiators, indicating that lipid peroxides might have a cooperative effect on induction of membrane leakage.

Studies have shown that upon attack by free radicals, the cell membrane of the vesicular vessel was damaged, which will trigger the precipitation of monocytes and macrophage cells and proliferation of smooth muscle cells on the vessel wall and leads to atherosclerosis.[18] Our observation that lipid peroxidation can be avoided by fullerene derivatives can rationalize previous report of antiproliferation of vascular smooth muscle cells by polyhydroxylated C_{60}.[6a] Occurrence of lipid peroxidation will lead to the breakdown of the membrane integrity, loss of the transmembrane potential, inactivation of these membrane-bound proteins/enzymes, and malfunction of external signal molecules (such as antigens, growth factors, polypeptide hormones, GTP-binding proteins, second-messenger-generating enzymes, soluble intracellular second messengers—Ca^{2+}, cAMP, inositol phosphates, regulatory proteins, etc.).[9] Eventually, nearly all biological processes stop, and cell death occurs. Diseases related to lipid peroxidation are numerous:[19] to name a few, multiple sclerosis, atherosclerosis, hemolytic diseases, Parkinson's disease, Alzheimer's disease, porphyria, chronic inflammation, etc. C_{60} and its water-soluble derivatives can protect membranes from ROS-initiated lipid peroxidation and thus have great potential as medicines for treating the above diseases.

Conclusion

We have shown that both liposoluble and water-soluble C_{60} derivatives can effectively prevent lipids from radical-initiated peroxidation and breakdown of membrane integrity. To have protection effects, the antioxidant has to reside in close proximity to the membrane interior region where lipid peroxidation chain propagation occurs. Liposoluble C_{60} shows stronger effects than the most effective natural antioxidant, vitamin E, in prevention of lipid peroxidation. The differences in antioxidation effects between D_3- and C_3C_{60}s originate from their interactions with membranes. Maintenance of membrane integrity (and therefore the transmembrane potential) is essential to a large part of biological activities. The current study has shown that both lipophilic (C_{60}) and hydrophilic C_{60} (C_3C_{60}) derivatives have great potential as medicines for treating lipid peroxidation-related diseases.

Experimental Section

Materials. C_{60} (99.9+%) was purchased from MER Corp. Vitamin E (i.e., α-tocopherol) was from Lancaster and used as received. The water-soluble C_3- and D_3C_{60}s were synthesized according to the procedure reported by Lamparth and Hirsch.[20] The purities of the compounds were confirmed by NMR. The synthetic procedure of polar C_{60} derivative **1** will be published elsewhere.[21] The lipids used in this study were saturated 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC; Avanti Polar Lipid) and unsaturated 1,2-dilionleoyl-*sn*-glycero-3-phosphoethanolamine (PE; Avanti Polar Lipid). A water-soluble, pH-sensitive dye, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium (HPTS; Molecular Probes), was encapsulated inside liposomes to detect leakage of membranes upon radical at-

tacks. Liposoluble fluorescence probes used in this study were 1-pyrenecarboxylic acid (Aldrich, recrystallized twice from ethanol), 1-pyrenebutyric acid (Eastman, recrystallized twice from ethanol), 1-pyrenehexanoic acid (Molecular Probes), 1-pyrenedecanoic acid (Molecular Probes), and 1-pyrenehexadecanoic acid (Molecular Probes).

General Procedure. Liposomes were prepared according to the literature procedure.[22] Briefly, 5 mg of lipids was dissolved in chloroform and coated onto the test tube wall by purging away the solvent. Residual solvent was further removed by a vacuum line. Then, 4 mL of buffer solution (0.05 mM MOPS, 10 mM NaCl, 0.2 mM HPTS, pH 6.63) was added. The solution was bubbled with argon gas for 40 min to remove molecular oxygen, then sonicated in a bath-type ultrasonicator (Heat Systems, model XL2020, 200 W power) with a 15 s on−15 s off cycle for 12 min. During sonication, the solution was maintained in an Ar atmosphere. The HPTS dye in the outer bulk aqueous phase of the liposome solution was removed by flowing the liposome solution through a Sephadex G-50 column with a buffer solution (0.05 mM MOPS, 10 mM NaCl, pH 7.87). Liposomes containing liposoluble antioxidants or fluorescence probes were prepared in a similar way, except that solution containing additives (C_{60} in toluene, vitamin E in methanol, **1** in THF, or pyrene in ethanol) was added to the lipid chloroform solution at the beginning of the process. In the cases of water-soluble antioxidants, aliquots of C_3C_{60} (or D_3C_{60}) were added to liposome solutions and equilibrated for 15 h before generation of free radicals. Addition of H_2O_2 into the C_3C_{60}−liposome solution in the absence of Fe^{2+} ions does not cause any changes in the excitation spectrum of liposome-encapsulated dyes. In the case of generation of superoxides, 5 mU of freshly prepared xanthine oxidase solution was added to the C_3C_{60}(or D_3C_{60})−liposome solution before each addition of aliquot of xanthine (in 0.05 N NaOH) solution. Fluorescence excitation spectrum was measured 30 min after addition of Fe^{2+}-H_2O_2 or xanthine−xanthine oxidase solution. In general, 35% unsaturated PE (with two unsaturated C═C double bonds) was mixed with saturated DPhPC for the preparation of liposomes. The presence of unsaturated lipids makes lipid peroxidation much more sensitive to radical attacks and membrane leakage easier to be detected.

The extent of membrane leakage was detected by monitoring the change in the excitation spectrum of the encapsulated pH-sensitive dye, HPTS. The absorption coefficients (ϵ: $M^{-1}\,cm^{-1}$) of HPTS at 403 and 454 nm are listed as below: (19 976, 1 680 at pH 6.63), (19 850, 2 013 at pH 6.7), (19 650, 2 562 at pH 6.8), (19 235, 3 629 at pH 7.0), (17 928, 5 169 at pH 7.2), (16 573, 7 129 at pH 7.4), (15 054, 9 095 at pH 7.6), (13 069, 12 343 at pH 7.8), and (12 881, 12 953 at pH 7.87), respectively (see Figure 1 of Supporting Information for full spectra and the A_{454}/A_{403} ratio). The encapsulated dye is under an environment of pH 6.63, while the outer bulk aqueous phase has a pH value of 7.87. The ratio of A_{454}/A_{403} increases at higher pH environments. The pH difference between the inner and outer aqueous phases of liposomes does not cause any membrane leakage, since the excitation spectrum of the encapsulated dye after column elution is the same as that at pH 6.63. Upon occurrence of membrane leakage (due to lipid peroxidation), the encapsulated dye senses the pH change, and the A_{454}/A_{403} ratio increases. A higher A_{454}/A_{403} ratio represents a larger extent of lipid peroxidation (and thus membrane leakage).

In the case of incorporation of liposoluble fluorescence probes, Py-$(CH_2)_n$-COOH (n = 1, 3, 5, 9, and 15), into membranes, the pyrene probe in chloroform was mixed with the liposome-forming solution before sonication. The pyrene-to-lipid mole ratio was maintained at 0.5% for all fluorescence probes. After sonication, the liposome solution flowed through a Sephadex G-50 column to remove the residue pyrene molecules in the bulk aqueous solution. The concentration of liposomes with different pyrene probes used in each C_3/D_3C_{60} quenching experiment was adjusted to be the same by checking the light scattering of the liposome solution at 600 nm. Aliquot of C_3/D_3C_{60} was added to the liposome solution and equilibrated for 30 min before the fluorescence quenching measurement.

ESR experiments were conducted on a Bruker EMX-12 ESR spectrometer. A DPPH sample in a second resonator chamber was used as an external reference for g value measurements. A modulation amplitude of 0.5 G and microwave power of 10 mW were used in all experiments. In the spin trapping reaction, 20 μM $FeSO_4$ was added into a premixed solution containing 1 mM 5,5-dimethylpyrrolidine *N*-oxide (DMPO), 5 μM C_3C_{60} (or D_3C_{60}), and 200 μM H_2O_2. Upon addition of Fe^{2+} ions, hydroxyl radicals will be generated. In the case of superoxide quenching experiments, xathine (250 μM)−xanthine oxidase (20 mU/mL) was used to produce the superoxide radicals. The pH value of the enzyme solution was maintained at 7.5 by a 0.1 M Na_2HPO_4 buffer. The substrate, xanthine, was added into the premixed spin-trapping reagent solution last to initiate the superoxide production.

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Supporting Information Available: Figures 1−3 as mentioned in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) A C−C single bond is 1.54 Å, and the bond angle of a C−C−C bond of a methylene chain is 104.5°. Therefore, the bond distance of a C−C bond along the methylene chain direction is 1.22 Å. The head-to-tail distance for the most extended conformation is 12.2 Å for $n = 9$ (10 C−C bonds) and 19.5 Å for $n = 15$ (16 C−C bonds). Assuming the distance, r, at these temperatures is ca. 90% and 85% of the r of the most extended conformation for $n = 9$ and 15, respectively, then the location of the fullerene cage of the C_3C_{60} can be obtained as between 11 and 16.6 Å away from the water−bilayer interface.
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