Free Radical Scavenging Activity of Water-soluble Fullerenols

Long Y. Chiang,*[a] Fung-Jou Lu[b] and Jaw-Town Lin[c]

[a] *Center for Condensed Matter Sciences, National Taiwan University, Taipei, Taiwan*
[b] *Department of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan*
[c] *Department of Internal Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan*

Water-soluble polyhydroxylated fullerene derivatives (fullerenols) show excellent efficiency in eliminating superoxide radicals ($O_2^{-\cdot}$), generated by xanthine and xanthine oxidase, thus revealing the potential use of these compounds as novel potent free radical scavengers in biological systems.

Caged [60]fullerene and analogues exhibit high reactivity towards organic radical addition.[1,2] This reactivity is, presumably, correlated to the intrinsically large electronegativity of C_{60} molecules. Upon chemical functionalization of C_{60} with multiple electron-releasing groups, the electronegativity of the resulting derivatives decreases significantly. The decrease corresponds to the reduction of their chemical response toward radical additions, *e.g.* as shown with polyhydroxylated fullerene derivatives (fullerenols **1**, Fig. 1).[3–7] The reduced chemical reactivity of the remaining, conjugated double bonded moieties in **1** certainly reduces their inherent biological toxicity to less than that of the parent C_{60}. Combination of the moderate electron affinity and the allylic hydroxy functional groups of fullerenols makes them an appropriate candidate for applications such as a free-radical remover or a water-soluble antioxidant in biological systems. Our early efforts to study this hypothesis have shown promising results, *e.g.* in reducing the concentration of free radicals in diseased blood and in inhibiting the growth of abnormal or ailing cells.[8] In this communication, we demonstrate the utilization of water-soluble fullerenols as a free-radical scavenger for the absorption of superoxide radicals ($O_2^{-\cdot}$), generated by *in vitro* xanthine and xanthine oxidase in aqueous solution. These results suggest potential uses of polyhydroxylated fullerene derivatives in the biochemical or pharmaceutically related investigations in addition to those reported previously.[9,10]

Water-soluble fullerenols **1** used in this study were synthesized by a sequence of reactions involving the electrophilic attack of nitronium tetrafluoroborate on fullerenes in the presence of organocarboxylic acids as the key-step as reported previously.[11] [60]Fullerenols **1** were structurally characterized by various spectroscopic methods and the subsequent chemical derivatization to fullerene oxide with 18–20 hydroxy groups on average.[12] Aside from the desirable water solubility, conjugated diols and *vic*-diols in **1** are potentially good ligands for coordination and binding to enzyme surfaces. To evaluate and determine the influence of **1** on the enzymatic activity of xanthine oxidase, a concentration dependence study of fullerenol on the production of uric acid from xanthine, catalysed by xanthine oxidase, was performed. The conversion of xanthine **2** to uric acid **3** (Scheme 1) can be detected by the specific optical absorption of uric acid at 290 nm. Experimentally, xanthine oxidase (0.03 unit) and fullerenols (0–0.15 mg) were suspended and stirred in a buffer solution (3.0 ml, 50 mmol dm^{-3} of KH_2PO_4–K_2HPO_4, pH = 7.5) for 9 min at ambient temperature. Xanthine (0.38 μmol) was added and the mixture was stirred for an additional 1 min. The optical absorbance, at 290 nm, of uric acid produced in the solution was then measured. As shown in Fig. 2, the relative intensity of optical absorbance of uric acid remained nearly constant with increasing fullerenol concentration, indicating that the presence of fullerenols had no effect on the amount of uric acid generated. This indicated no enzymatic inhibition of the xanthine oxidase by fullerenols.

To monitor the trace amount of unstable superoxide radicals in aqueous solution, a chemiluminescence (CL) technique in connection with a highly sensitive photon detector was applied. It utilized lucigenin (bis-*N*-methylacridinium nitrate) **4** as a chemiluminigenic probe for enhancing the detectability of superoxide.[13–15] In this procedure, the dicationic nitrate salt of lucigenin was first reduced electronically by an enzyme to

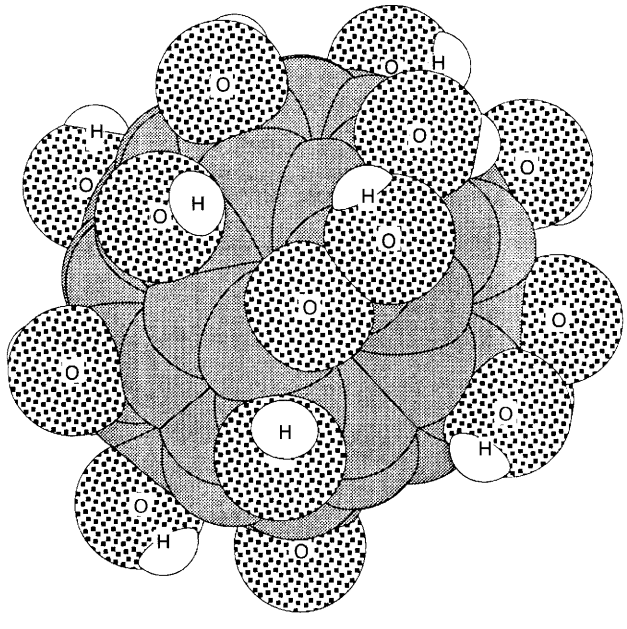
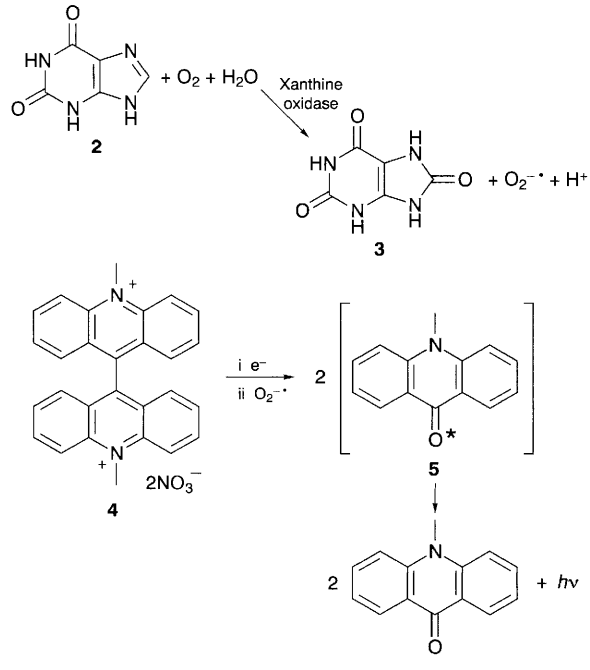


Fig. 1 Schematic structure representation: an averaged structure of fullerenols **1** containing polyhydroxy functions on the C_{60} cage



Scheme 1

afford the corresponding cation radical of lucigenin. The subsequent reaction of the lucigeninic cation radical with superoxide radical yielded *N*-methylacridone **5** in an electronically excited state, which relaxed back to its ground state resulting in the emission of a photon (Scheme 1).[13,14] Therefore, the intensity of lucigenin-derived chemiluminescence detected can be correlated precisely to the relative quantity of superoxide radical in the solution. Xanthine oxidase (45 mu), xanthine (0.45 μmol) and fullerenols (0–0.15 mg) were suspended and stirred in a buffer solution (3.0 ml, 50 mmol dm^{-3} KH_2PO_4–K_2HPO_4, pH = 7.5) at 37 °C for 100–200 s. The photon emission level of this aqueous solution was measured as the dark average of background intensity. Lucigenin (0.009 μmol) was then added and the chemiluminigenic emission from the resulting mixture was counted at intervals of 10 s and accumulated continuously for 400 s.[16,17] The total intensity of chemiluminescence, obtained in the presence of a variable concentration of fullerenols is depicted in Fig. 3. Consequently, we observed a systematic decrease in intensity of chemiluminescence upon the addition of increasing concentrations of fullerenols. These data verify the excellent efficiency of fullerenols in eliminating superoxide radical species generated by xanthine–xanthine oxidase. At an applied fullerenol concentration of 50 μg ml^{-1} in the final solution, a radical scavenging efficiency of approximatedly 80% was achieved.

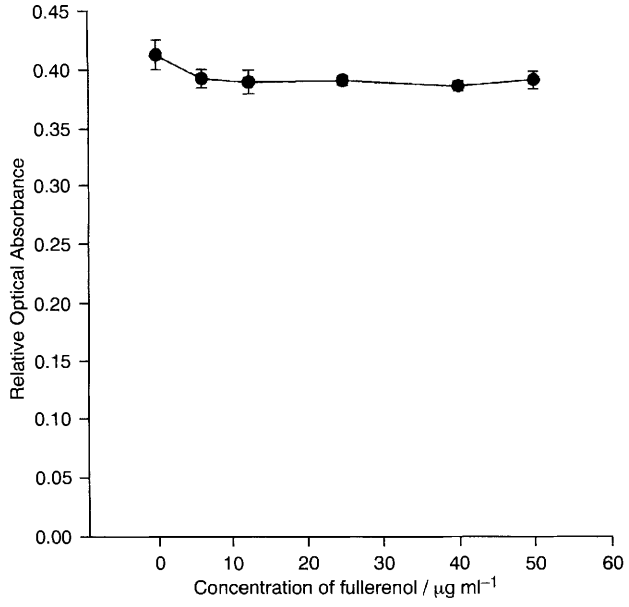


Fig. 2 The fullerenol concentration dependence of the optical absorbance of uric acid at λ = 290 nm in an aqueous solution of xanthine and xanthine oxidase

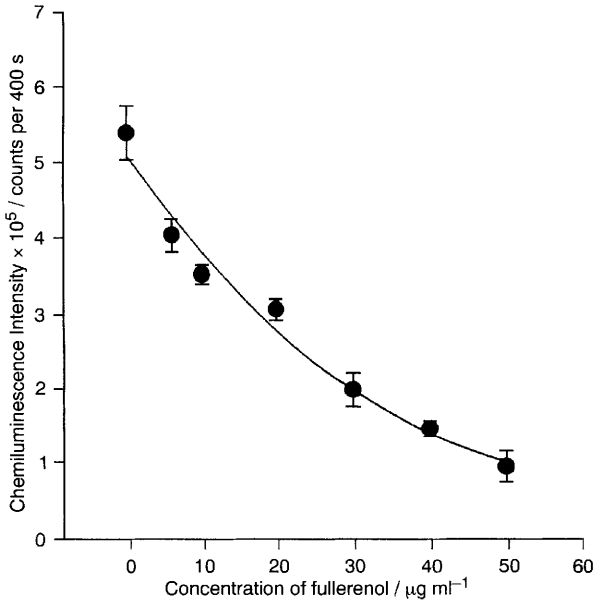


Fig. 3 The fullerenol concentration dependence of the total intensity of lucigenin-derived chemiluminescence, accumulated within a period of 400 s, in an aqueous solution of xanthine and xanthine oxidase

This work was supported by the National Science Council of Republic of China under Grant No. NSC 84-2113-M-002-028 and NSC 84-2621-B-002-014Z.

Received, 22nd February 1995; Com. 5/01089F

References

1 For review of fullerene functionalization chemistry see: R. Taylor and D. R. M. Walton, *Nature*, 1993, **363**, 685.
2 P. J. Krusic, E. Wasserman, P. N. Keizer, J. R. Morton and K. F. Preston, *Science*, 1991, **254**, 1183.
3 L. Y. Chiang, J. W. Swirczewski, C. S. Hsu, S. K. Chowdhury, S. Cameron and K. Creegan, *J. Chem. Soc., Chem. Commun.*, 1992, 1791.
4 L. Y. Chiang, L. Y. Wang, J. W. Swirczewski, S. Soled and S. Cameron, *J. Org. Chem.*, 1994, **59**, 3960.
5 L. Y. Chiang, R. B. Upasani and J. W. Swirczewski, *U.S. Pat.* 5,177,248, 1993; U.S. Patent 5 294 732, 1994.
6 N. S. Schneider, A. D. Darwish, H. W. Kroto, R. Taylor and D. R. M. Walton, *J. Chem. Soc., Chem. Commun.*, 1994, 15.
7 J. Li, A. Takeuchi, M. Ozawa, X. Li, K. Saigo and K. Kitazawa, *J. Chem. Soc., Chem. Commun.*, 1993, 1784.
8 L. Y. Chiang, F. J. Lu, H. C. Huang and J. T. Lin, *Symposium of Science and Technology of Fullerene Materials*, the Materials Research Society Fall Meeting, Boston, 1994; L. Y. Chiang, F. J. Lu and J. T. Lin, *Mater. Res. Soc. Symp. Proc.*, 1995, **359**, 327.
9 S. H. Friedman, D. L. DeCamp, R. P. Sijbesma, G. Srdanov, F. Wudl and G. L. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6506.
10 H. Tokuyama, S. Yamago and E. Nakamura, *J. Am. Chem. Soc.*, 1993, **115**, 7918.
11 L. Y. Chiang, R. B. Upasani and J. W. Swirczewski, *J. Am. Chem. Soc.*, 1992, **114**, 10154.
12 L. Y. Chiang, R. B. Upasani, J. W. Swirczewski and S. Soled, *J. Am. Chem. Soc.*, 1993, **115**, 5453.
13 R. C. Allen, *Methods in Enzymology*, 1986, **133**, 449.
14 *Bioluminescence and Chemiluminescence New Perspectives*, eds. J. Scholmerich, R. Andreesen, A. Kapp, M. Ernst and W. G. Woods, John Wiley & Sons, New York, 1987.
15 S. Rembish and M. A. Trush, *Free Radical Biology & Medicine*, 1994, **17**, 117.
16 F. J. Lu, J. T. Lin, H. P. Wang and W. C. Huang, *Experientia*, submitted.
17 J. S. Sun, Y. S. Hang, D. H. Huang and F. J. Lu, *Free Radical Biology & Medicine*, submitted.