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# Biological Safety of LipoFullerene composed of Squalane and Fullerene-C60 upon Mutagenesis, Photocytotoxicity, and Permeability into the Human Skin Tissue

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*Abstract:* Fullerene-C60 (C60) is mainly applied in the aqueous phase by wrapping with water-soluble polymer or by water-solublizing chemical-modification, whereas C60 dissolved in oil is scarcely applied; still less explicable is its toxicity. We dissolved C60 in squalane at near-saturated or higher concentrations (220–500 ppm), named LipoFullerene (LF-SQ), and examined its biological safety. LF-SQ was administered at doses of 0.49–1000 μg/ml to fibroblast cells Balb/3T3, and showed that cell viability was almost equal to that of the control regardless of the UVA- or sham-irradiation, indicating no phototoxicity. Reverse mutation by LF-SQ was examined on four histidine-demanding strains of *Salmonella typhimurium* and a tryptophan-demanding strain of *Escherichia coli*. As for the dosages of LF-SQ (313–5000 μg/plate), the dose-dependency of the number of reverse mutation colonies of each strain did not show a marked difference when compared with the negative control, regardless of the metabolic activation, in contrast to twice or more differences for five positive controls (sodium azide, N-ethyl-N′-nitro-N-nitrosoguanidine, 2-nitrofluorene, 9-aminoacridine, and 2-aminoanthracene). In human skin biopsy built in a diffusion chamber, C60 permeated into the epidermis at 33.6 nmol/g tissue (24.2 ppm), on administration with LF-SQ containing 223 ppm of C60, but not detected in the dermis even after 24 hrs, as analysed by HPLC. It is presumed that LF-SQ can permeate into the epidermis via the corneum but can not penetrate the basement membrane, and so can not reach into the dermis, suggesting no necessity for considering a toxicity of C60 due to systemic circulation via dermal veins. Thus, C60 dissolved in squalane may not give any significant biological toxic effects such as photocytotoxicity, bacterial reverse mutagenicity, and permeability into the human skin.

Squalane and fullerene are used as cosmetic materials including a skin emollient and an antioxidant, respectively [1,2]. These compounds are not easily oxidized and are more stable than squalene and L-ascorbic acid which have similar effects. Moreover, in terms of the function, polyvinylpyrrolidone-wrapped fullerene derivative (C60/PVP) was demonstrated for the stronger anti-melanogenic potential than naturally occurring whitening agents, arubutin and L-ascorbic acid [3]. Fullerene-C60 (C60) dissolved in squalane may have an efficacy as a skin emollient and a scavenger of reactive oxygen species generated in the skin. However, an influence of nanoparticles on the living body is not adequately elucidated [4–6], so it is necessary to confirm biological safety sufficiently about it. We dissolved C60 in squalane (LipoFullerene [LF-SQ], a content of C60: 220–500 ppm, which is as rich as near the upper limit of saturation solubility) and examined the photocytotoxicity, bacterial reverse mutagenicity and permeability into the human skin biopsy composed of epidermis and dermis built in a modified Bronaugh's diffusion chamber.

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## Materials and Methods

*LipoFullerene.* Highly purified and organic solvent-free fullerene, provided by Vitamin C60 BioResearch Inc., Tokyo, was dissolved in squalane from olive oil provided by Nisshin Oillio Group, Ltd., Tokyo, by warming at 40° during gentle sonication for 10 min. and subjected to ultrafiltration with a 0.1-μm-pore filter resulting in a scarce amount of insoluble residues. The C60 solution in squalane is designated as LF-SQ, and C60 content in LF-SQ was determined by HPLC analysis on a 5PBB column (Nacalai Tesque Inc., Japan) and found to be approximately 220–500 ppm.

*Photocytoxicity test.* It was executed for LF-SQ in twelve dose-points (0.49–1000 μg/ml) under UVA-irradiation or sham-irradiation using Balb/3T3 fibroblast cell. Cell suspension was prepared by treatment with 0.25% trypsin and pipetting to form a single cell, and each well was added with 100 μl of cell suspension containing culture medium. The blank well received culture medium of 100 μl containing no cell. It was then incubated for 24 hrs, aspirated and rinsed with phosphate buffered saline [PBS(-)]. The positive control well received (chlorpromazine hydrochloride) solution of 100 μl. LF-SQ was dissolved in EtOH diluted with PBS(-) and added by 100 μl to each well at an LF-SQ concentration of 1 v/v%. No deposition from the solution was confirmed and then added to each well. Multi-well plates were then settled for 60 min. in a $CO_2$ incubator and irradiated with UVA at room temperature for 50 min. The sham-irradiation plate was similarly manipulated except settling in the dark. Chlorpromazine hydrochloride or LF-SQ solution was aspirated and washed twice with PBS(-). Culture medium of 100 μl was then added and incubated until the next day. Neutral red of 100 μl was prepared with 50 μg/ml of serum-free culture medium

*Table 1.*

Integrity of human skin biopsies by tritiated water before application of LipoFullerene (LF-SQ), which were built in a Bronaugh's diffusion chamber, originated from the abdomens of three Caucasian women, two of them were 60 years old (#1, #2) and one of them was 32 years old (#3).

| Donor | Age | Flux (dpm/cm$^2$/hr) | Kp (cm/hr) |
|---|---|---|---|
| #1 | 60 | 108,453 | 0.00121 |
| #2 | 60 | 129,756 | 0.00144 |
| #3 | 32 | 44,777 | 0.00050 |

and was added to each well in multi-well plates, which were put in a $CO_2$ incubator for 3 hrs. After cultivation, neutral red solution was removed and rinsed with PBS(-). The mixed solution of 150 µl of 50% EtOH-1% acetic acid was added and stirred gently with a microtiter plate shaker for 10 min. The extract of 50% EtOH-1% acetic acid from the cell layer was measured for absorbance at 550 nm with a micro plate reader (Corona Electric Co., Ltd., type MTP-32).

*Bacterial reverse mutagenicity.* Bacterial reverse mutagenicity by LF-SQ was examined on TA98, TA100, TA1535, and TA1537 which were histidine-demanding strains of *Salmonella typhimurium*, and WP2uvrA (pKM101) which was a tryptophan-demanding strain of *Escherichia coli* (Molecular Toxicology, Inc., USA). LF-SQ of 100 µl was administered by five dosages (313, 625, 1250, 2500, and 5000 µg/plate), and added to 500 µl of S9 mix for the metabolism activation, or 0.1 mol/l sodium phosphoric acid buffer solution (pH 7.4) for the non-metabolic activation. S9 mix was prepared by mixing commercial S9 (Oriental Yeast Co., Ltd., Japan) and co-factor A (Oriental Yeast Co., Ltd.) in 1 : 9 v/v ratio at the time of use. Furthermore, bacterial suspension of 100 µl was added and incubated by shaking the incubator at 37° for 20 min. Then, top agar of 2 ml was added and stirred, and the mixture was multi-layered on minimal glucose agar plate medium. After solidification of top agar, minimal glucose agar plate culture media was inverted and incubated at 37° for 48 hrs. Growth inhibition and deposition of LF-SQ were not found in any doses of LF-SQ. After incubation, the number of reverse mutation colony was counted automatically with a colony counter (ProtoCOL, Synoptics Ltd., UK).

*Permeability into the human skin biopsy.* Three human skin biopsies originating from the abdomen of two 60-year-old and one 32-year-old Caucasian women were examined. Informed consent for these human skin biopsies was obtained. Tritiated water permeability coefficients (Kp values) of human skin biopsies from three donors were 0.00050–0.00144 cm/hr so it was confirmed that there was no breakdown (table 1). LF-SQ was applied at three doses (C60: 223, 22.3, and 2.23 ppm, diluted in squalane) by 17.7 µl (10 µl/cm$^2$) on skin biopsies for 24 hrs. Then, the skin biopsies fixed in the modified Bronaugh's diffusion chamber were cut out and separated into pieces of the epidermis and the dermis. Chloroform of 500 µl was added in each skin sample which underwent an elution of C60 with a pestle and was centrifuged at 880 ×*g* for 10 min. The supernatant was analysed by HPLC. Chloroform of 500 µl was added to a receptor solution of 500 µl and centrifuged (880 ×*g*, 10 min.), and then the chloroform layer was analysed by HPLC. A regression equation was pursued from a calibration curve by Buckminster fullerene (99.5% C60, Sigma-aldrich Inc., USA), and C60 concentration of each sample was calculated.

*HPLC analytical condition.* Column: Cosmosil 5PBB-packed column, 4.6 mm I.D. × 250 mm (Nacalai Tesque Inc., Japan); temperature: room temperature; mobile phase: toluene; flow rate: 1.0 ml/min; detector: UV 285 nm; injection volume: 10 µl; monitoring time: 20 min.

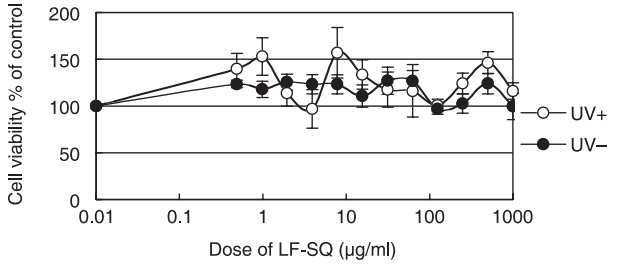


Fig. 1. Cell viability curves of LipoFullerene (LF-SQ)-administered Balb/3T3 fibroblast cells in culture. It was executed for LF-SQ at 12 graded doses of 0.49–1000 µg/ml under the condition of UV-irradiation [UV(+)] or sham-irradiation [UV(–)], mean ± SD, n = 4.

*Statistical analysis.* Results of photocytotoxicity are expressed as mean ± SD, for n = 4. The comparison of the results between the UVA-irradiation group and their corresponding sham-irradiation negative control was made using one-way ANOVA. The differences were considered to be significant when $P \leq 0.05$.

## Results

*Photocytotoxicity.*

Cell viability at each dose of LF-SQ was calculated and was compared with that on an UVA-irradiation group or a sham-irradiation negative control group. Cell viability by LF-SQ was 96.5–156.0% for the UVA group and 96.9–127.0% for the sham group and it did not decrease dose-dependently (fig. 1). Significant differences between the UVA group and the sham group were not detected at a level of $P \leq 0.05$. Based on a cell viability of the negative control of 100%, the $IC_{50}$ value of the positive control chlorpromazine hydrochloride was 2.8 µg/ml for the UVA group and 24 µg/ml for the sham group, hence, photo-irradiation factor = $IC_{50}$ [UV(–)]/$IC_{50}$ [UV(+)] is calculated to be 8.57 (fig. 2). It was judged that this test showed a reasonable reaction. Cell viability by LF-SQ was almost equal to the negative-control group. The $IC_{50}$ value could not be calculated.

*Bacterial reverse mutagenicity.*

As for the dosage of LF-SQ (313–5000 µg/plate), the number of reverse mutation colonies of each strain showed neither the difference twice or more versus the negative control nor a different dose-dependency, regardless of the metabolic

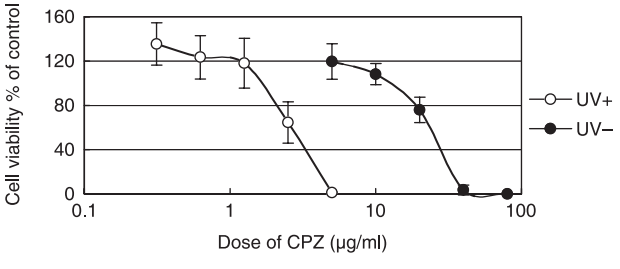


Fig. 2. Cell viability curves of chlorpromazine hydrochloride (CPZ)-administered Balb/3T3 fibroblast cells in culture under the condition of UVA-irradiation [UV(+)] or sham-irradiation [UV(–)], mean ± SD, n = 4.

 

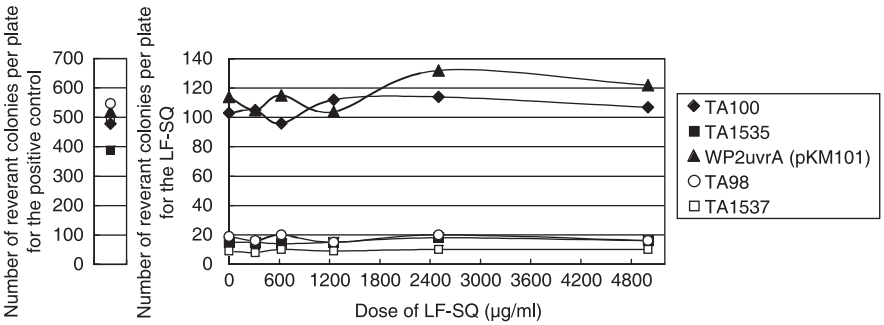


Fig. 3. Dose-response curves of revertant colonies by LipoFullerene (LF-SQ) of differential bacterial strains undergoing no metabolic activation in the bacterial reverse mutation test, mean, n = 2. Base-pair substitution type: TA100, TA1535, and WP2uvrA (pKM101). Frame-shift type: TA98 and TA1537. Positive control: sodium azide 1.5 μg/plate for TA100 and TA1535. N-ethyl-N′-nitro-N-nitrosoguanidine 1.0 μg/plate for WP2uvrA (pKM101), 2-nitrofluorene 5.0 μg/plate for TA98, 9-aminoacridine 80.0 μg/plate for TA1537.

activation, in contrast to marked differences for the positive controls (sodium azide, N-ethyl-N′-nitro-N-nitrosoguanidine, 2-nitrofluorene, 9-aminoacridine, and 2-aminoanthracene, figs 3 and 4). The growth inhibition of bacterial strains and the deposition of LF-SQ were not found. The number of bacterial reverse mutation colony by LF-SQ is within the background data by the positive and negative control.

*Permeability into the human skin biopsy.*

The permeability of LF-SQ into the human skin biopsy built in a modified Bronaugh's diffusion chamber for 24 hr was measured by HPLC analysis. C60 was not detected on both the epidermis and dermis for administration of LF-SQ at low concentrations (C60-eq.: 2.23 and 22.3 ppm). But upon administration with LF-SQ at a concentration as high as 223 ppm C60-eq., C60 was detected to permeate into the epidermis at 33.63 ± 3.51 nmol/g tissue (24.24 ± 2.53 μg), but not detected in the dermis of human skin biopsy (table 2).

## Discussion

Photocytotoxicity of LF-SQ was examined using Balb/3T3 fibroblastic cells. Cell viability by LF-SQ did not decrease dose-dependently regardless of whether UVA-irradiation or the sham-irradiation (fig. 1). These results show that LF-SQ does not possess photocytotoxicity to Balb/3T3 fibroblastic cells and suggest C60 dissolved in squalane may not exert a UVA-catalytic reactive oxygen species-increasing action. Correspondingly, C60/PVP has been reported to protect the keratinocytes from UVA-induced cell injuries without photocytotoxicity [7].

Bacterial reverse mutagenicity by LF-SQ was examined on TA98, TA100, TA1535, and TA1537 which were histidine-demanding strains of *S. typhimurium* and on WP2uvrA (pKM101) which was a tryptophan-demanding strain of *E. coli*. *Salmonella typhimurium* and *E. coli* are sensitive to the mutation and generally used to estimate the mutagenicity for a microorganism in a comparatively short-term. The strains that were used to examine the mutation type of the base pair substitution were TA100, TA1535, and WP2uvrA (pKM101), and those of the frame shift were TA98 and TA1537. In any doses of LF-SQ (313–5000 μg/plate), the number of bacterial reverse mutation colonies of each strain did not show the twice or more increase to the negative control, and the dosage-dependency was not different regardless of the metabolic activation (figs 3 and 4). As a result, the bacterial reverse mutagenicity of LF-SQ was judged to be negative under the conditions of this test.

The permeability of LF-SQ into the human skin biopsy for 24 hrs was measured by HPLC analysis. On administration with LF-SQ (C60-eq: 223 ppm), C60 was detected to permeate into the epidermis at 33.63 ± 3.51 nmol/g tissue (24.24 ± 2.53 μg) but not detected in the dermis of human skin biopsy (table 2).

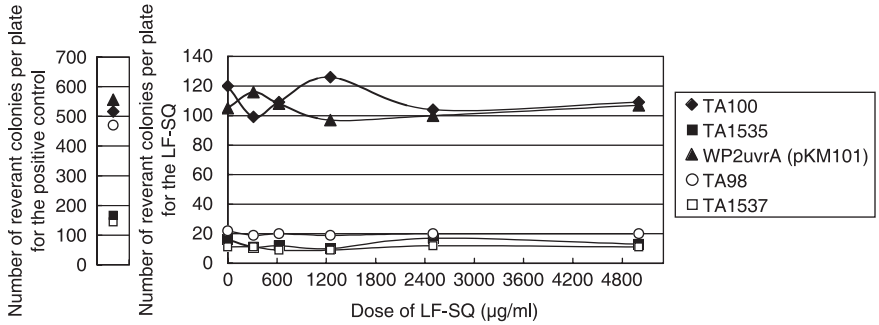


Fig. 4. Dose-response curves of revertant colonies by LipoFullerene (LF-SQ) of differential bacterial strains undergoing metabolic activation in the bacterial reverse mutation test, mean, n = 2. Base-pair substitution type: TA100, TA1535, and WP2uvrA (pKM101). Frame-shift type: TA98 and TA1537. Positive control: 2-aminoanthracene 1.0 μg/plate for TA100 and TA98; 2.0 μg/plate for TA1535, WP2uvrA (pKM101) and TA1537.

*Table 2.*

Permeability of LipoFullerene (LF-SQ) into human skin biopsies built in a Bronaugh's diffusion chamber originated from the abdomens of the same Caucasian women as in table 1.

| Dose of LF-SQ (C60-eq.) (ppm) | Skin/receptor solution | Donor | Weight (mg) | Permeation amount of C60 (nmol/g tissue) | | |
|---|---|---|---|---|---|---|
| | | | | Mean ± SD, n = 3 | N = 1 | N = 2 |
| 223 | Epidermis | #1 | 9.4 | | 35.7 | 31.3 |
| | | #2 | 11.4 | 33.63 ± 3.51 | 33.8 | 38.8 |
| | | #3 | 12.9 | | 28.6 | 33.6 |
| | Dermis | #1 | 41.2 | | n.d. | n.d. |
| | | #2 | 53.6 | n.d. | n.d. | n.d. |
| | | #3 | 59.2 | | n.d. | n.d. |
| | Receptor solution | | – | n.d. | n.d. | n.d. |
| 22.3 | Epidermis | #1 | 18.0 | | n.d. | n.d. |
| | | #2 | 14.5 | n.d. | n.d. | n.d. |
| | | #3 | 11.8 | | n.d. | n.d. |
| | Dermis | #1 | 62.0 | | n.d. | n.d. |
| | | #2 | 57.2 | n.d. | n.d. | n.d. |
| | | #3 | 57.7 | | n.d. | n.d. |
| | Receptor solution | | – | n.d. | n.d. | n.d. |
| 2.23 | Epidermis | #1 | 14.6 | | n.d. | n.d. |
| | | #2 | 8.4 | n.d. | n.d. | n.d. |
| | | #3 | 7.3 | | n.d. | n.d. |
| | Dermis | #1 | 43.7 | | n.d. | n.d. |
| | | #2 | 32.0 | n.d. | n.d. | n.d. |
| | | #3 | 25.5 | | n.d. | n.d. |
| | Receptor solution | | – | n.d. | n.d. | n.d. |

n.d.: not detected.

That is, LF-SQ (C60-eq.: 223 ppm) was permeated by 6.9% of the administration amount and diluted to approximately eight times in the epidermis for 24 hr, indicating a considerable permeability of LF-SQ through the corneum assumedly owing to the molecular lipophilicity.

Subacute toxicity tests suggested that squalane did not give any significant toxic effects on dogs as well as rats, and orally administered squalane was gradually excreted through faeces and skin in dogs [8,9]. C60 is determined to be comparatively less toxic by a relative risk assessment for the industrial fabrication [10], and aqueous C60 suspensions have no acute or subacute toxicity in rodents [11]. Upon inhalation exposure of C60 in rats, no gross or microscopic lesions were observed [12].

Biological influences of C60 and its diverse derivatives have been carefully examined at the cellular level, and some water-soluble C60 derivatives exhibit cytoprotective effects by reducing the reactive oxygen species concentration [13,14]. But if exposed to visible light, C60 produces singlet oxygen and exhibits cytotoxicity through lipid peroxidation, whereas C60-united natural organic substrates in the environment are demonstrated as non-photocytotoxic [15]. DNA-damaging effects by C60 are scarcely exerted, but formation of reactive oxygen species may cause inflammation and genetic damage dose-dependently [16]. The bacterial reverse mutation assay (Ames test) and the chromosomal aberration test in cultured Chinese hamster lung cells revealed no genotoxicity of C60 [17]. Only at the high C60-concentration of 2.24 mg/ml, a slight genotoxic effect was observed on the cells of *Drosophila melanogaster*'s wing, but the hydroxylated-C60-derivative fullerol was demonstrated for no mutagenic effect at a concentration of 2.46 mg/ml [18]. These biological applications of C60 are mainly performed in a suspended form of the aqueous phase or in some water-soluble derivatives, but the research on the C60 dissolved in oil is scarcely reported.

We confirmed from the results, as for LF-SQ (C60 dissolved in squalane, a content of C60-eq: 500 ppm), that (i) photocytotoxicity was negative, (ii) bacterial reverse mutagenicity was negative, (iii) it was detected to permeate at a level of C60: 33.63 ± 3.51 nmol/g tissue (24.24 ± 2.53 μg) in the epidermis at a high dosage of LF-SQ (223 ppm of C60), but not in the dermis of human skin biopsy.

Attention should be paid to absorption, distribution and excretion of C60 in the living body with respect to the biological safety. Water-miscible fullerene ($^{14}$C-labelling C60-trimethylmethane derivative) was injected intravenously into rats, passed through the blood–brain barrier, and was accumulated at 0.84% in the brain after 6 hrs, and at 80.1% in the liver after 30 hrs [19]. In contrast, our results indicate that C60 in LF-SQ can permeate into the epidermis but not penetrate the basal lamina and thus not reach into the dermis. Thus, it is not necessary to consider a toxicity of C60 dissolved in squalane due to systemic circulation via dermal veins.

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## References


1 Parente ME, Gambaro A, Solana G. Study of sensory properties of emollients used in cosmetics and their correlation with physicochemical properties. J Cosmet Sci 2005;**56**:175–82.

2 Takada H, Kokubo K, Matsubayashi K, Oshima T. Antioxidant activity of supramolecular water-soluble fullerenes evaluated by beta-carotene bleaching assay. Biosci Biotechnol Biochem 2006;**70**:3088–93.

3 Xiao L, Matsubayashi K, Miwa N. 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. Arch Darmatol Res 2007;**299**:245–57.

4 Kagan VE, Bavir H, Shvedova AA. Nanomedicine and nanotoxicology: two sides of thesame coin. Nanomedicine 2005;**1**:313–6.

5 Garnett MC, Kallinteri P. Nanomedicines and nanotoxicology: some physiological principles. Occup Med (Lond) 2006;**56**:307–11.

6 Lewinski N, Colvin V, Drezek R. Cytotoxicity of nanoparticles. Small 2007;**4**:26–49.

7 Xiao L, Takada H, Gan X, Miwa N. The water-soluble fullerene derivative 'Radical Sponge' exerts cytoprotective action against UVA irradiation but not visible-light-catalyzed cytotoxicity in human skin keratinocytes. Bioorg Med Chem Lett 2006;**16**:1590–5.

8 Kamimura H, Koga N, Oguri K, Yoshimura H, Inoue H, Sato K *et al*. Studies on distribution, excretion and subacute toxicity of sualane in dogs. Fukuoka Igaku Zasshi 1989;**80**:269–80.

9 Kamimura H, Fuchigami K, Inoue H, Kodama R, Yoshimura H. Studies on distribution and excretion of squalane in dogs administered for 2 weeks. Fukuoka Igaku Zasshi 1991;**82**:300–4.

10 Robichaud CO, Tanzil D, Welenmann U, Wiesner MR. Relative risk analysis of several manufactured nanomaterials: an insurance industry context. Environ Sci Technol 2005;**39**:8985–94.

11 Gharbi N, Pressac M, hadchouel M, Szwarc H, Wilson SR, Moussa F. Fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity. Nano Lett 2005;**5**:2578–85.

12 Baker GL, Gupta A, Clark ML, Valenzuela BR, Staska LM, Harbo SJ *et al*. Inhalation toxicity and lung toxicokinetics of C60 fullerene nanoparticles and microparticles. Toxicol Sci 2007;**101**:122–31.

13 Bosi S, Da Ros T, Spalluto G, Prato M. Fullerene derivatives: an attractive tool for biological applications. Eur J Med Chem 2003;**38**:913–23.

14 Krokosz A. Fullerenes in biology. Postepy Biochem 2007;**53**:91–6.

15 Lee J, Fortner JD, Hughes JB, Kim JH. Photochemical production of reactive oxygen species by C60 in the aqueous phase during UV irradiation. Environ Sci Technol 2007;**41**:2529–35.

16 Nielsen GD, Roursgaard M, Jensen KA, Paulsen SS, Larses ST. *In vivo* Biology and Toxicology of Fullerenes and Their Derivatives. Basic Clin Pharmacol Toxicol 2008;**103**: 197–208.

17 Mori T, Takada H, Ito S, Matsubayashi K, Miwa N, Sawaguchi T. Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. Toxicology 2006;**225**:48–54.

18 Zakharenko LP, Zakharov IK, Vasiunina EA, Karamysheva TV, Danielenko AM, Nikiforov AA. Determination of the genotoxicity of fullerene C60 and fullerol using the method of somatic mosaics on cells of Drosophila melanogaster wing and SOS-chromotest. Genetika 1997;**33**:405–9.

19 Yamago S, Tokuyama H, Nakamura E, Kikuchi K, Kananishi S, Sueki K *et al*. *In vivo* biological behavior of a water miscible fullerene: $^{14}C$ labeling, absorption, distribution, excretion, and acute toxicity. Chem Biol 1995;**2**:385–9.


 