Pure Appl. Chem., Vol. 71, No. 11, pp. 2047–2053, 1999.
Printed in Great Britain.

Biological functions of fullerene*

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Abstract: Fullerene (C_{60}) is known to efficiently generate singlet oxygen when irradiated with light. In spite of such an unique photodynamic nature, little has been studied on biological functions of C_{60}. This paper describes a tumor-therapeutic trial of C_{60} based on the photo-induced generation of active oxygen. To achieve preferential accumulation of C_{60} in the tumor tissue, this water-insoluble drug was chemically conjugated with poly(ethylene glycol) (PEG) not only to make it soluble in water but also to make the molecular size larger. When intravenously injected to tumor-bearing mice, the large-sized, as expected, water-soluble C_{60}-PEG conjugate was accumulated to a greater extent and retained for a longer time period in the tumor tissue than in the normal issue. Following intravenous injection of the C_{60}-PEG conjugate, local irradiation of visible light to the tumor site induced tumor necrosis, in contrast to the conjugate injection alone. When the C_{60} dose was 8.48 μg/mouse, the tumor mass completely disappeared at an irradiation power of 107 J/cm^2, indicating a high potentiality of the C_{60}-PEG conjugate for photodynamic tumor therapy.

INTRODUCTION

While much attention has been paid to fullerenes on chemical and physical properties, few papers have been reported on their biological functions. One of the reasons may be the poor solubility of fullerene (C_{60}) in water. This water insolubility will prevent C_{60} from directly interacting with biological substances and cells, resulting in no natural performance of biological functions. A mixed aqueous solution of C_{60} and poly(vinyl pyrrolidone) is reported to induce differentiation of chondrocytes [1]. Moreover, it has been found that water-miscible C_{60} derivatives inhibit HIV-1 protease activity [2] and proliferation of Hela S3 cells by visible-light irradiation [3].

Photodynamic therapy on tumor is based on photo-induced generation of active oxygen. The conceptual illustration is shown in Fig. 1. In advance, a photosensitizer with an affinity for the tumor tissue is intravenously administered into the body. After tumor accumulation of the photosensitizer has become maximum, only the tumor site is selectively light-irradiated. At that time, the photo-chemical reaction shown in Fig. 1b occurs in the tumor tissue irradiated, resulting in destruction of the tumor tissue. The photosensitizer accumulated generates singlet oxygen from oxygen present in the tissue by light irradiation. The generated singlet oxygen is so reactive that acts as an effective cytotoxic agent [4], destroying the surrounding tumor tissue.

C_{60} is known to efficiently generate singlet oxygen when exposed to visible light [4]. We have demonstrated that light irradiation in the presence of C_{60} gave cells a strong cytocidal effect in vitro [5]. Thus, if preferential accumulation of C_{60} at a tumor tissue is achieved, it is possible that local irradiation of visible light to the tumor tissue results in selective tumor necrosis and C_{60} functions as an effective photosensitizer for tumor photodynamic therapy. However, it is difficult to inject the water-insoluble C_{60} into the body without solubilization in water, much less to expect its tumor targeting.

There are anatomical differences between the tumor and normal tissues (Fig. 2). The substance

* Lecture presented at the 4th International Symposium on Functional Dyes—Science and Technology of Functional π-Electron Systems, Osaka, Japan, 31 May–4 June 1999, pp. 2009–2160.
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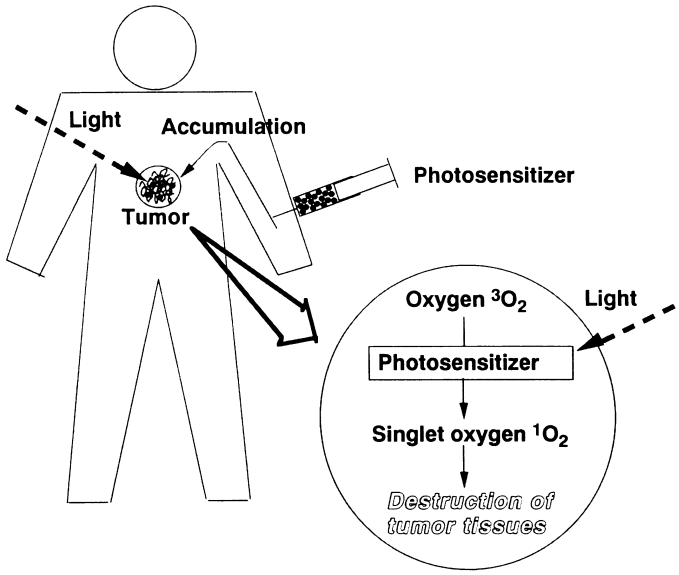

Fig. 1 Conceptual illustration of photodynamic therapy on tumor.

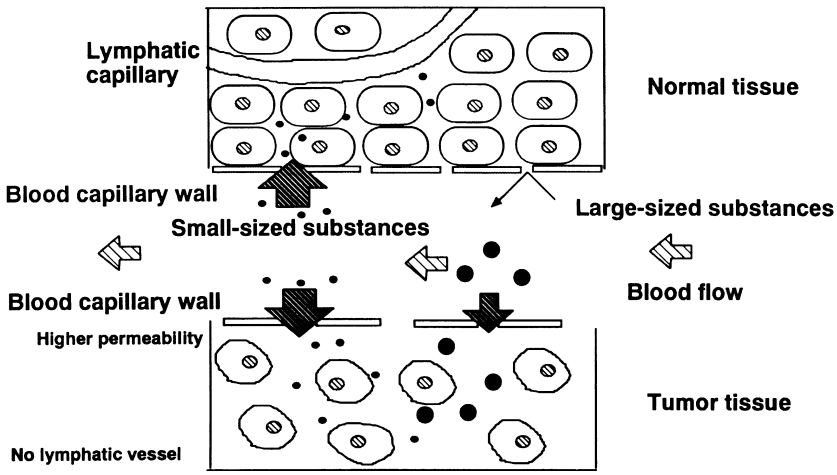

Fig. 2 Anatomical difference in distribution of large and small-sized substances between normal tumor tissues.

permeability of blood vessels newly formed in the tumor tissue is generally higher than that of normal blood vessels. In addition to the hyperpermeable vasculature, it has been reported that immatured lymph systems in the tumor tissue makes it difficult to excrete large-sized substances from the tissue, enabling them to accumulate to a greater extent and retain for a longer time period at the tumor tissue than at the normal tissue [6–13]. An increase in accumulation and retention of antitumor drugs at the tumor tissue could be achieved by increasing their apparent molecular size through chemical conjugation with polymers [7,8]. They are examples of passive drug targeting on the basis of the anatomical characteristics of tumor tissue.

The objective of this study is to passively target C_{60} to the tumor tissue. When water-soluble polymers were intravenously injected to the tumor-bearing mice, their accumulation in the tumor tissue was higher than that in the normal tissue, irrespective of the polymer type [14–19]. The retained period of accumulated polymers was long compared with that in the normal tissue. If the apparent molecular size of water-insoluble C_{60} is increased through conjugation with a water-soluble polymer, passive tumor targeting of C_{60} will be realized in addition to its solubilization in water. Poly(ethylene glycol) (PEG) was selected as the water-soluble polymer for C_{60} conjugation because it has been widely used for chemical modification of drugs due to simple conjugation chemistry [20,21]. It is likely that once accumulated in

the tumor tissue, the C_{60}-PEG conjugate remains for a long time period compared with that in the normal tissue. This pharmacodynamic difference, at a certain time period after injection, will enable C_{60} to increase the ratio of tumor/normal tissue concentration, achieving so-called passive tumor targeting of C_{60}. Then, only if the tumor site is light irradiated, it will be selectively destroyed (Fig. 3). As expected, PEG conjugation results in enhanced accumulation of C_{60} in the tumor tissue. In this paper, we describe feasibility of the C_{60}-PEG conjugate as a photosensitizer for tumor photodynamic therapy showing its tumor therapeutic effect on mice carrying a tumor mass on the back subcutis. To our knowledge, no large-sized, water-soluble C_{60} has been applied for photodynamic therapy and this paper is the first trial, although there have been reported some studies regarding polymerized C_{60} and its modification with polymers [22,23].

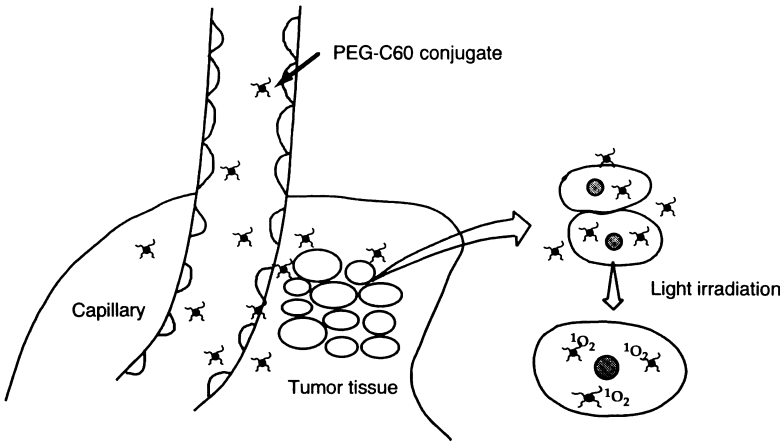

Fig. 3 Passive tumor targeting of PEG-C_{60} conjugate and generation of singlet oxygen by light irradiation.

PREPARATION OF C_{60}-PEG CONJUGATE

C_{60} (99.9%, MW = 720.66, Lot No. FHC03) was obtained from Tokyo Kasei Kogyo C. Ltd., Tokyo, Japan. Monomethoxy poly(ethylene glycol) with a primary amino group on the terminal was kindly supplied from Nippon Oil & Fats Company Ltd, Tokyo, Japan (PEG, MW = 5460). Given amounts of PEG were mixed with C_{60} in the benzene solution and coupling reaction was allowed to proceed under stirring at 25 °C for 24 h in the dark, followed by freeze-drying to obtain powdered C_{60}-PEG conjugates. Gel filtration chromatographic studies revealed that the peak of PEG itself disappeared, a new peak being detected at a shorter retention time with C_{60} conjugation. This clearly indicated that chemical coupling of PEG to C_{60} actually took place. When estimated in terms of water/benzene partition, the solubility of C_{60}-PEG conjugates in water increased with an increase in the PEG/C_{60} molar ratio. When the PEG/C_{60} ratio was 100, 100% of C_{60}-PEG conjugates prepared was partitioned to the water phase. This completely water-soluble C_{60}-PEG conjugate was used for the following *in vivo* experiments.

BODY DISTRIBUTION OF C_{60}-PEG CONJUGATE FOLLOWING INTRAVENOUS INJECTION

Radioiodination of the C_{60}-PEG conjugate was conducted according to a chloramine T method following their tyramine introduction [19]. Briefly, 2.7 mg of tyramine was added to a benzene solution containing 100 mg of C_{60}-PEG conjugate at a tyramine/C_{60} molar ratio of 100. After coupling reaction at 25 °C for 2 days, the resulting solution was dialyzed against water for 2 days to exclude non-coupled tyramine, followed by freeze-drying to obtain a tyramine-introduced C_{60}-PEG conjugate. The tyramine-introduced C_{60}-PEG conjugate was dissolved in 150 μL of 0.5 M potassium phosphate-buffered (KPB) solution (pH 7.5) to give a final concentration of 50 μg/mL. Then, 2 μL of $Na^{125}I$ solution and 100 μL of 0.05 M KPB solution (pH 7.2) containing 0.02 mg of chloramine T were added to the C_{60}-PEG conjugate solution. After agitation at 25 °C for 2 min, 100 μL of 0.01 M phosphate-buffered saline solution (PBS; pH 7.4) containing 0.4 mg of sodium pyrosulfite was added to stop the radioiodination. The resulting mixture was

allowed to pass through a column of Dowex resin to remove uncoupled, free ^{125}I molecules from the ^{125}I-labeled C_{60}-PEG conjugate.

Meth A fibrosarcoma cells, which had been passaged in the peritoneal cavity of Balb/c female mice aged 6 weeks (Japan SLC Inc., Shizuoka, Japan), were inoculated at a volume of 50 μL into the back subcutis of female CDF_1 mice aged 6 weeks (Japan SLC Inc., Shizuoka, Japan) (3×10^6 cells/mouse). When the tumor mass grew at the inoculation site to 7 mm in the average diameter, tumor-bearing mice received intravenous injection of 100 μL of PBS solution containing 0.02 wt% of ^{125}I-labeled C_{60}-PEG conjugates. At different time intervals, the radioactivity of blood samples, excised body tissues, and urine and faeces was measured with a gamma counter (Autowell gamma system Aloka ARC-301B, Aloka Company Ltd, Tokyo, Japan) for body distribution evaluation. Mouse skins ($1.5 \times 1.5\ cm^2$) with and without the tumor mass were taken out of tumor-bearing mice at their back subcutis and the radioactivity of both the skins was measured to calculate that of the tumor tissue itself from their difference. As normal controls, the skin and thigh muscle were taken to measure their radioactivity.

Table 1 shows the body distribution of C_{60}-PEG conjugates at different time intervals after intravenous injection. The C_{60}-PEG conjugates disappeared with time from the blood circulation and approximately 78% of conjugates injected was excreted from the body 24 h after injection. Liver accumulation of the conjugates tended to increase up to 24 h after injection, but thereafter decreased to an undetectable level at 144 h after injection (data not shown). The conjugates accumulated in the gastrointestinal tract and carcass were eliminated with time to disappear from the sites, similarly to the liver. There was no specific affinity of C_{60}-PEG conjugates for organs. It is possible that intravenously injected conjugates are finally excreted from the kidney because their molecular size is small enough to undergo the glomerular filtration. Liver accumulation of C_{60}-PEG conjugates during the initial 24 h after injection can be explained in terms of vascular permeability. Unlike other organs, liver vasculatures are composed of discontinuous vascular walls. The C_{60}-PEG conjugate may be distributed in the extravascular tissue after intravenous injection, but return to the blood circulation by diffusion because the conjugate concentration in the blood is always low due to quick elimination by the blood stream [24]. If the conjugate has an affinity for the liver, this phenomenon would not be observed.

Table 1 The time course of body distribution of C_{60}-PEG conjugates after intravenous injection to tumor-bearing mice

	Percentage radioactivity remaining after injection		
Organ	6 h	24 h	72 h
Blood	7.15 ± 1.05*	1.89 ± 0.14	0.83 ± 0.07
Heart	0.07 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
Lung	0.15 ± 0.05	0.08 ± 0.01	0.06 ± 0.01
Liver	5.23 ± 0.34	6.18 ± 0.13	2.13 ± 0.11
Spleen	0.14 ± 0.03	0.18 ± 0.01	0.06 ± 0.01
Kidney	2.81 ± 0.29	2.17 ± 0.09	1.28 ± 0.12
Gastrointestinal tract	5.15 ± 0.09	3.03 ± 0.89	5.23 ± 0.07
Carcass	12.0 ± 0.79	5.67 ± 0.06	5.24 ± 0.37
Excreted	64.5 ± 0.55	77.6 ± 1.30	79.0 ± 0.57

Mean ± SEM.

Table 2 shows the accumulation ratio of C_{60}-PEG conjugates at the tumor tissue to those at the normal tissue. C_{60}-PEG conjugates were accumulated in the tumor tissue to a significantly higher extent than in the normal skin and muscle. It should be noted that the conjugates were retained in the tumor tissue at a significantly higher amount and for a longer time period than in the normal tissue, although the accumulation amount at both the tissues tended to decrease with time. This is mainly ascribed to the anatomical feature of tumor tissues, e.g. the hyperpermeable vasculature and immatured lymph systems. Thus, it is likely that passive targeting of C_{60}-PEG conjugates to the tumor tissue is achievable based on

Table 2 Accumulation ratios of tumor/skin and tumor/muscle in tumor-bearing mice at various time periods after intravenous injection of C_{60}-PEG conjugates

Period after injection	Tumor/skin	Tumor/muscle
6 h	2.77 ± 0.15*	18.4 ± 2.02
24 h	2.51 ± 0.58	17.0 ± 2.50
72 h	1.21 ± 0.11	13.2 ± 1.56

Mean ± SEM.

this difference. Indeed, when measured at 24 h after intravenous injection, the conjugate accumulation ratios of the tumor to the normal skin and muscle were 2.5 and 17, respectively, which clearly indicates the C_{60} targeting to the tumor.

PHOTODYNAMIC THERAPY OF C_{60}-PEG CONJUGATE ON TUMOR

For tumor therapeutic experiments, PBS solutions containing C_{60}-PEG conjugates with given amounts of C_{60} were intravenously injected to the tumor-bearing mice at an injection volume of 100 μL, and 24 h later, the tumor tissue was exposed to visible light (400–505 nm) from a light probe of 7 mm active diameter by Heliomat Multifunction Halogen-Light (Vivadent Co. Ltd, Liechtenstein). The light was delivered at a fluence of 89.2 mW/cm^2 for 20 min (107 J/cm^2). For Photofrin, the dose was 4 mg/kg body and light irradiation (610–800 nm) was conducted at a fluence of 72.5 mW/cm^2 (107 J/cm^2). The size of tumor grown was measured according to the method reported by Winn [25] and was expressed as the ratio of the tumor size to that before conjugate injection. As controls, the intravenous injection of C_{60}-free PEG with or without the following light irradiation or light irradiation without any injection was performed to estimate the time profile of tumor growth.

Histological examination revealed that treatment with conjugate injection coupled with light irradiation, strongly induced tumor necrosis whereas the overlaying normal skin was not damaged. On the other hand, conjugate injection alone did not induce any tissue necrosis. This finding demonstrates that light irradiation is essential to induce destruction of the tumor tissue of mice receiving conjugate injection. When the back skin of the conjugate-injected normal mice was exposed to light at 89.2 mW/cm^2 for 20 min, amounting to 107 J/cm^2, after 24 h of injection, no histological damage of the skin was observed (data not shown).

Figure 4 shows the photodynamic effect of C_{60}-PEG conjugates and on the *in vivo* growth of tumor cells together with that of a clinically used photosensitizer, Photofrin. The photodynamic effect of C_{60}-PEG conjugates on tumor greatly depended on the C_{60} dose and the power of light irradiation. The *in vivo* suppression of growth by the conjugate increased with an increase in the irradiation power (Fig. 4A). When the irradiation power was 107 J/cm^2, the tumor size ratio became less than 1.0 after 4 days of injection and observation of all the mice for a longer time period revealed that the tumor mass disappeared. An increase in the C_{60} dose of C_{60}-PEG conjugate enhanced its photodynamic effect on tumor (Fig. 4B). The size increase of the tumor mass was significantly suppressed by conjugate injection followed by light irradiation at the C_{60} dose more than 212 g/kg body. The treatment with the conjugate at the C_{60} dose of 424 μg/kg body decreased the tumor size ratio to less than 1.0 and finally, all the tumor-bearing mice were cured. The photodynamic effect of Photofrin on tumor was less efficient than that of the conjugate even at 10-times or more higher doses than the conjugate. Other studies demonstrated that the time profile of tumor growth of mice treated with C_{60}-free PEG was similar to that of PBS-injected, control mice, irrespective of light irradiation. Light irradiation alone did not affect the tumor growth at all (data not shown). These findings indicate that injection of C_{60} itself is a key to achieve light-induced tumor suppression.

Since hematoporphyrin derivatives are complex mixtures of several compounds, the different components may have different pharmacokinetic and photodynamic properties. Moreover, these substances are retained in the skin for at least 4–6 weeks, thereby causing severe skin sensitization to

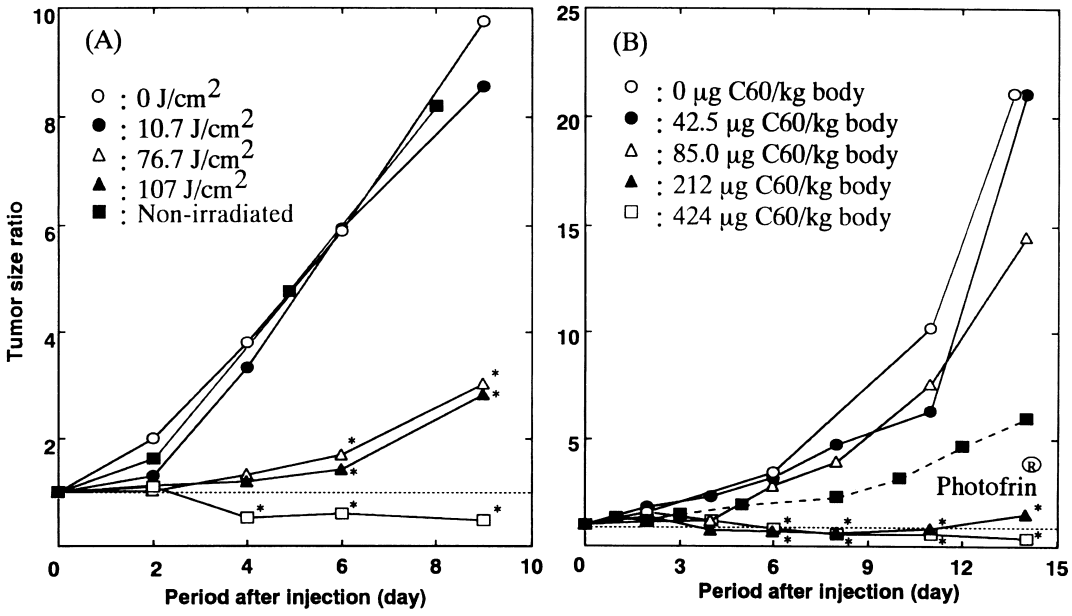

Fig. 4 Tumor photodynamic effect of C_{60}-PEG conjugates and Phoftorin. (A) Intravenous single injection of C_{60}-PEG conjugate (424 μg C_{60}/kg body) plus light irradiation. (B) Intravenous single injection of C_{60}-PEG conjugates and Photofrin plus light irradiation. $^*P < 0.05$, significant against untreated, control mice groups. A dotted line indicates the tumor size ratio of 1.0.

sunlight [26]. Contrary to this, C_{60}-PEG conjugates were excreted within 1 week by way of the kidney without such a long-term retention in normal tissues, implying their much less skin sensitization. This is because the conjugate, unlike hematoporphyrin derivatives, does not any specific affinity for cell receptors. However, compared with Photofrin, the conjugate has a small optical extinction coefficient in the wavelength region where light has its optimal penetration through tissue, i.e. in the 600–900 nm region [27].

TOXICITY CONCERN

The *in vivo* toxicity of C_{60}-PEG conjugates was evaluated in terms of the change of mouse body weight, histology, and blood examination of mice following their injection. When the time profile of body weight gain of normal mice intraperitoneally injected with C_{60}-PEG conjugates with C_{60} doses ranging from 3.6 μg/mouse to 36.0 mg/mouse was examined, no significant difference was observed in the body weight change between all the groups (data not shown). The time profile was similar to that of PBS-injected, control mice. Moreover, intravenous injection of the conjugates did not affect the time profile of normal growth of mice. Blood examinations demonstrated that the plasma concentration of GOT, GPT, and BUN of tumor-bearing mice was not changed by the conjugate injection, irrespective of light irradiation, and was similar to that of PBS-injected, normal mice. Moreover, the histological appearance of liver tissue of mice receiving the conjugate injection was similar to that of the normal mice, irrespective of light irradiation. These findings clearly indicate that conjugate injection followed by light irradiation neither exhibited systemic toxicity nor gave any damage to the liver and kidney.

CONCLUSION

Following intravenous injection of the PEG-C_{60} conjugate, light irradiation to the tumor site strongly induced tumor necrosis without any damage to the overlaying normal skin. The conjugate was found to suppress the in vitro growth of normal cells when accompanied with light irradiation but did not exhibit any suppressive effect of cell growth without light irradiation [5]. It is very unlikely that the C_{60}-PEG conjugate has cell selectivity for the cytotoxicity. Thus, this finding clearly indicates that the C_{60}-PEG conjugate was present in the tumor tissue to a greater extent than in the normal

tissue. As a result, the conjugates accumulated in the tumor tissue may be photodynamically activated by light irradiation to generate singlet oxygen, leading to the tumor necrosis without any damage to the normal tissue.

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