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Photodynamic therapy with fullerenes *in vivo*: reality or a dream?

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Abstract

Photodynamic therapy (PDT) employs the combination of nontoxic photosensitizers and visible light that is absorbed by the chromophore to produce long-lived triplet states that can carry out photochemistry in the presence of oxygen to kill cells. The closed carbon-cage structure found in fullerenes can act as a photosensitizer, especially when functionalized to impart water solubility. Although there are reports of the use of fullerenes to carry out light-mediated destruction of viruses, microorganisms and cancer cells *in vitro*, the use of fullerenes to mediate PDT of diseases such as cancer and infections in animal models is less well developed. It has recently been shown that fullerene PDT can be used to save the life of mice with wounds infected with pathogenic Gram-negative bacteria. Fullerene PDT has also been used to treat mouse models of various cancers including disseminated metastatic cancer in the peritoneal cavity. *In vivo* PDT with fullerenes represents a new application in nanomedicine.

Keywords

antimicrobial photosensitizers; functionalized fullerenes; hydroxyl radicals; invasive wound infection; mouse models; peritoneal carcinomatosis; photodynamic therapy; singlet oxygen

Photodynamic therapy

The fullerene molecule with its unique structure of 60 carbon atoms arranged in a soccer ball structure is a molecule of great potential for a variety of applications and has drawn the attention of lots of physicists, chemists and engineers. Recently these nanostructures have also been studied for their biological activities with a view towards using them for biomedical applications. One of the therapies for which fullerenes may have a medical application is the light-based therapy called photodynamic therapy (PDT) [1], which is a nonsurgical, minimally invasive approach that has been used in the treatment of solid tumors and many nonmalignant diseases [2]. PDT is a nonthermal photochemical reaction, which requires the simultaneous presence of a photosensitizing drug (photosensitizer [PS]), oxygen

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and visible light (Figure 1). It is a two-step procedure that involves the administration of a PS, followed by activation of the drug with the appropriate wavelength of light [3–5]. The photoactivation of the drug generates singlet oxygen and other reactive oxygen species (ROS), which cause a lethal oxidative stress and membrane damage in the treated cells and in the case of tumors, leads to cell death by direct cytotoxicity and a dramatic antivascular action that impairs blood supply to the area of light exposure [6]. It is known that depending on the parameters involved, *in vitro* PDT can kill cancer cells via apoptosis, necrosis or autophagy. The direct killing effect of PDT on malignant cancer cells that has been studied in detail *in vitro* also clearly applies *in vivo*, but in addition, two separate *in vivo* mechanisms leading to PDT-mediated tumor destruction have been described. They are the vascular shutdown effect mentioned above [7], and the PDT-induced activation of the host immune system [8]. In case of antimicrobial PDT Gram-positive bacteria are found to be more susceptible as compared with Gram-negative bacteria. This observation is explained by the difference in the structure of their cell walls (Figure 2) [9].

The most common chemical structures that have been employed as PS for PDT purposes are derived from the tetrapyrrole aromatic nucleus found in many naturally occurring pigments such as heme, chlorophyll and bacteriochlorophyll. Tetrapyrroles usually have a relatively large absorption band in the region of 400 nm known as the Soret band, and a set of progressively smaller absorption bands as the spectrum moves into the red wavelengths known as the Q-bands. Another broad class of potential PS includes completely synthetic, non-naturally occurring, conjugated pyrrolic ring systems. These comprise such structures as texaphyrins [10], porphycenes [11] and phthalocyanines [12]. Other compounds that have been studied as PS are nontetrapyrrole-derived dyes, which may be either naturally occurring or totally synthetic, and these compounds have often been used as antimicrobial PS. Examples of the first group (naturally occurring dyes) are hypericin [13] and from the second group (synthetic dyes) are toluidine blue O [14] and Rose Bengal [15].

Some of the characteristics that the ideal PS should possess are the presence of low levels of dark toxicity and the presence of absorption bands that should be in the so-called optical window (600–900 nm) for sufficient tissue penetration of light. They should have relatively high absorption bands (>20,000–30,000 $M^{-1}cm^{-1}$) to minimize the dose of PS needed to achieve the desired effect. PS should ideally have high triplet and singlet oxygen quantum yields. The usefulness of various PS proposed for antimicrobial PDT has to be judged on different criteria. One of the requirements is that an antimicrobial PS should be able to kill multiple classes of microbes at relatively low concentrations and low fluences of light. PS should be reasonably nontoxic in the dark and should show selectivity for microbial cells over mammalian cells. To date there is no perfect PS that meets all the characteristics of an ideal PS. The reason why fullerenes are seen as potential PDT agents is that they possess some the characteristics that render them well suited as a photosensitizing drug as detailed below.

Fullerenes as PS

Due to rapidly growing interest in the medical application of fullerenes in nanotechnology [16], these molecules have gained considerable attention as possible PS for mediating PDT [17] of various diseases. Pristine C_{60} is highly insoluble in water and biological media and forms nano-aggregates that prevent its efficient photoactivity [18]. However, when fullerenes are derivatized by chemists who attach some functional groups to these molecules to make them more soluble in water and biological solvents, their biological usefulness is markedly improved [19]. Different hydrophilic or amphiphilic side chains or fused ring structures have been attached to the spherical C_{60} core. This functionalization imparts a higher ability to produce singlet oxygen, hydroxyl radicals and superoxide anion upon

illumination, and these reactive species have been proposed as effective PDT mediators in several applications. Some of the advantages that fullerenes possess over the traditional PS used for PDT are:

- Fullerenes are comparatively more photostable and demonstrate less photobleaching compared with tetrapyrroles and synthetic dyes;
- Fullerenes show both kinds of photochemistry comprising type I (free radicals) and type II (singlet oxygen) while tetrapyrroles demonstrate largely type 2 photochemistry;
- Fullerenes can be chemically modified for tuning the drug's partition coefficient (Log P or partition coefficient for [drug in *n*-octanol]/ [drug in H_2O]) and pK_a values for the variation of *in vivo* lipophilicity and the prediction of their distribution in biological systems;
- To enhance the overall quantum yield and the ROS production and to extend their absorption spectrum further into the red wavelengths, a light-harvesting antennae can be chemically attached to C_{60};
- Molecular self-assembly of fullerene cages into vesicles allows improved drug delivery and can produce self-assembled nanoparticles that may have different tissue-targeting properties.

Besides these advantages, fullerenes show some disadvantages although they can be overcome by applying special strategies. One concern for the use of fullerenes is the question concerning biodegradability, as nanostructures such as fullerenes may conceivably accumulate in the environment [20]. However, studies have concluded that C_{60} itself is remarkably nontoxic [21]. Another concern was their extreme hydrophobicity and their innate tendency to aggregate, which renders them less promising for application as drugs in biomedicine. However, there have been many strategies applied for solubilization and drug delivery of fullerenes (e.g., liposomes; Figure 3B) [22–24], micelles (Figure 3A) [25,26], dendrimers (Figure 3e) [27,28], PEGylation (Figure 3F) [29–32], cyclodextrin encapsulation (Figure 3D) [33,34] and self-nanoemulsifying systems (Figure 3C) [35–38] to overcome this shortcoming of fullerenes. Figure 3 illustrates some of the strategies that have been used to solubilize fullerenes. Besides these disadvantages, the main optical absorption band of fullerenes is in the blue and green regions, whereas the absorption spectra of tetrapyrrole PS other than porphyrins (e.g., chlorins, bacteriochlorins and phthalocyanines) have been designed to have substantial absorption peaks in the red or far-red regions of the spectrum. For PS to be useful *in vivo* it is considered that the light used to excite them should be in the red/near-infrared spectral region where scattering and absorption of light by tissue is minimized. This unfavorable absorption spectrum of fullerenes can be overcome by various strategies such as covalent attachment of light-harvesting antennae to fullerenes [39–43] by using optical clearing agents [44–48] or by applying two-photon excitation where two near-infrared photons are simultaneously delivered to be equivalent to one photon of twice the energy (and half the wavelength, so that two 800 nm photons are equivalent to one 400 nm photon) [49–53].

Photophysics & photochemistry of fullerenes

On irradiation with visible light C_{60} is excited from the S_0 ground state to a short-lived (~1.3 ns) S_1 excited state. The S_1 state rapidly decays to a lower lying triplet state T_1 with a long lifetime of 50–100 μs (Equation 1). The $S_1 \rightarrow T_1$ decay is formally a spin-forbidden intersystem crossing, but is driven by an efficient spin-orbit coupling. In the presence of dissolved molecular oxygen ($^3O_2$), which exists as a triplet in its ground state, the fullerene T_1 state is quenched (as a consequence of the quenching, its lifetime is reduced to ~330 ns)

to generate singlet oxygen ($^1O_1$*) by energy transfer at a rate of 2×10^9 M $-1s^{-1}$ (Equation 2). The singlet oxygen quantum yield, $\phi\Delta$, for this process (at 532 nm excitation) has been reported to be near theoretical maximum, 1.0 [54].

It is being increasingly realized that as compared with the standard Type II ROS, (singlet oxygen) the ROS produced during PDT with fullerenes are inclined towards Type 1 photochemical products (superoxide, hydroxyl radical, lipid hydroperoxides, hydrogen peroxide). It is known that both pristine and functionalized C_{60} fullerenes are able to catalyze the formation of ROS after illumination [55]. However fullerenes dissolved in organic solvents in the presence of oxygen seem to preferentially produce reactive singlet oxygen (Equation 1) [54]. By contrast in polar solvents, especially those containing reducing agents (such as NADH at concentrations found in cells (Equation 2), illumination of various fullerenes will generate different Type 1 ROS, such as superoxide anion and hydroxyl radical [56].

$$C_{60}+h\nu \rightarrow {}^1C_{60}{}^* \rightarrow {}^3C_{60}{}^* + {}^3O_2 \rightarrow {}^1C_{60} + {}^1O_2{}^* \quad (1)$$

$$C_{60}{}^* + NADH \rightarrow 2\,C_{60}{}^{\bullet} + NAD^+ + H^+ \quad (2)$$

$$C_{60}{}^{-\bullet} + O_2 \rightarrow C_{60} + O_2{}^{-\bullet} \quad (3)$$

$$2\,O_2{}^{-\bullet} + 2\,H^+ \rightarrow O_2 + H_2O_2 \quad (4)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \quad (5)$$

$$Fe^{3+} + O_2{}^{-\bullet} \rightarrow Fe^{2+} + O_2 \quad (6)$$

Due to their triply degenerate low lying lowest unoccupied molecular orbital fullerenes are known to be excellent electron acceptors. They are capable of accepting as many as six electrons [57]. There is some evidence that fullerene excited states (in particular the triplet) are even better electron acceptors than the ground state [58,59]. It is thought that the reduced fullerene triplet or radical anion can transfer an electron to molecular oxygen forming superoxide anion radical $O_2^{-\cdot}$ (Equation 3). It has been shown that whereas $^1O_2$ was generated effectively by photoexcited C_{60} in nonpolar solvents such as benzene and benzonitrile, while $O_2^{-\cdot}$ and OH^- were produced in polar solvents such as water. Hydrogen peroxide is formed by dismutation of the superoxide anion (Equation 4). Fenton chemistry (using small amounts of ferrous iron found in cells) is able to produce hydroxyl radicals from hydrogen peroxide (Equation 5), while the Haber-Weiss reaction is able to reduce ferric iron back to ferrous iron in order to continue the cycle by regenerating the active divalent ferrous iron (Equation 6).

One apparent contradiction that arises in this area is the observation that fullerenes can act as antioxidants, and that C_{60} and derivatives can act as scavengers of ROS. These antioxidant effects of C_{60} have generally been studied in the absence of light [21,60–63]. How then can we reconcile the established ability of fullerenes to scavenge ROS and act as antioxidants, with the demonstrated ability of fullerenes to act as efficient producers of ROS under illumination with the correct light parameters? It was assumed that the double bonds of the fullerene cage reacted with ROS forming covalent bonds, thereby removing the ability of the ROS to react with sensitive biomolecules similar to those damaged during PDT. If this

hypothesis were true it would be difficult to explain how fullerenes could act as efficient generators of ROS during PDT. A hypothesis that may explain this seeming contradiction was reported in 2009 by Andrievsky *et al.* [64]. They showed that the main mechanism by which hydrated C_{60} can inactivate the highly reactive ROS, hydroxyl radical, is not by covalently scavenging the radicals, but rather by action of the coat of `ordered water' that was associated with the fullerene nanoparticle [65]. Andrievsky *et al.* claimed that the ordered water coat could slow down or trap the hydroxyl radicals for sufficient time for two of the hydroxyl radicals to react with each other thus producing the less reactive ROS, hydrogen peroxide.

In vitro PDT with fullerenes

Phototoxicity using fullerenes combined with illumination to kill various cells *in vitro* has been demonstrated in many studies. It was Tokuyama *et al.* who first revealed the phototoxic potential of carboxylic acid functionalized fullerenes in HeLa (human cervical carcimoma) cells [66]. Burlaka *et al.* used pristine C_{60} at 10 μM with visible light from a mercury lamp to produce some phototoxicity in Ehrlich carcinoma cells or rat thymocytes [67]. In another study it was shown that *tris*-malonic acid fullerene derivative was found to be more phototoxic than a dendritic derivative in killing Jurkat cells when irradiated with UVA or UVB light [68]. Three C_{60} derivatives with two to four malonic acid groups (DMA C_{60}, TMA C_{60} and QMA C_{60}) were tested for their relative efficacy in HeLa cells and the results showed the following order for their efficacy DMA C_{60} > TMA C_{60} > QMA C_{60}[69].

In our laboratory we have tested a group of six functionalized fullerenes that were prepared in two groups of three compounds [70]. We demonstrated that the C_{60} molecule mono-substituted with a single pyrrolidinium group (BF4; Figure 4a) was a very efficient PS which efficiently killed off a panel of mouse cancer cells at the low concentration of 2 μM on exposure to white light. Chiang *et al.* reported the synthesis of two new photoresponsive diphenylaminofluorene nanostructures and investigated their intramolecular photoinduced energy and electron transfer behavior [71]. They demonstrated that the large light-harvesting enhancement provided by the cyano-containing CPAF-OMe moiety led to a more efficient triplet state generator than the keto-containing DPAF-OMe. C_{60}(>CPAF-OMe) was also significantly better than C_{60}(>DPAF-OMe) at light-mediated killing of human cancer cells.

Photodynamic reactions induced by photoactivated fullerenes have also been shown by many workers to inactivate enveloped viruses [72–75]. We have shown, in a series of reported experiments, that cationic fullerenes fulfill many of the desirable characteristics for efficient photoinactivation of bacteria and other pathogens. Our laboratory was the first to demonstrate that the soluble functionalized fullerenes, especially the *tris*-cationic compound BF6 (Figure 4B), were efficient and selective broad spectrum antimicrobial PS, and could mediate photodynamic inactivation of various classes of microbial cells [76]. In a study by Spesia *et al.* it was reported that a novel fulleropyrrolidinium iodide (DT-C_{60}^{2+} produced photodynamic inactivation *in vitro* of *Escherichia coli* [77]. Lee *et al.* showed that C_{60} derivatives were efficient in inactivating *E. coli* and MS-2 bacteriophage [75]. Recently we have demonstrated the use of innovative cationic fullerenes as broad-spectrum light-activated antimicrobials and carried out quantitative structure–function relationships to determine the optimal chemical structure of the fullerene derivatization [78]. The most effective compound overall against the various classes of microbial cells had the hexacationic structure illustrated as BF24 in Figure 4C. Another recent paper from our laboratory [79] looked at a further series of functionalized cationic fullerenes as antimicrobial PS and found a highly effective structure was the previously reported [80] tetrakis-cationic compound BF21 (Figure 4D).

Fullerenes have also been tested for their photodynamic efficacy by employing some of the delivery vehicles illustrated in Figure 3. Ikeda and coworkers used a series of liposomal preparations of C_{60} containing cationic or anionic lipids together. Illumination with 136 J/cm^2 350–500 nm light killed 85% of cells in the case of cationic liposomes and apoptosis was demonstrated [81]. Akiyama *et al.* solubilized unmodified C_{60} with high stability using various types of poly(ethylene glycol) (PEG)-based block copolymer micelles, which showed cytotoxicity under photoirradiation in HeLa cells [26]. Another study demonstrated direct and short-time uptake of the fullerine into the cell membrane within 10 min using an exchange reaction from a fullerene-γ-cyclodextrin complex (Figure 3D) and PDT activity against cancer cells occurred [82]. Doi *et al.* compared the PDT activity of C_{60} and C_{70} encapsulated in dimyristoylphosphatidylcho-line liposomes (Figure 3B) against HeLa cells and found C_{70} was five times more active than C_{60} and attributed the difference to an improved absorption spectrum in the latter case [24]. In another publication [83] this group compared delivery of C_{70} encapsulated in surface cross-linked lipid vesicles called cerasomes with the above mentioned liposomes. They found additional stability of cerasomes coupled with equivalent PDT activity against HeLa cells suggesting the fullerene could mediate cell killing without being released from its delivery vehicle.

Although fullerenes have been widely used for PDT, little attention has been given to studying their interaction with different intracellular organelles and determining the cellular site of their potential action. The main reason for this lack of attention is that in contrast to the vast majority of other PS that are highly fluorescent, fullerenes are nonfluorescent; thus it is not feasible to use the common technique of fluorescence (confocal) microscopy to examine the intracellular uptake and subcellular localization of fuller-enes. Overcoming this limitation, Scrivens *et al.* were the first to demonstrate its uptake by human keratinocytes in tissue culture by preparing a radiolabeled fullerene [84]. In serum-free medium they found a time- (up to 6 h) and concentration-dependent uptake so that 50% of added fullerene was taken up. One group used indirect immunofluorescence staining with antibodies that recognize fullerenes and other organelle probes to show that a dicarboxylic acid derivative was localized in mitochondria and other intracellular membranes [85]. A recent paper described the use of energy-filtered transmission electron microscopy and electron tomography to visualize the cellular uptake of pristine C_{60} nanoparticulate clusters. When human monocyte-derived macrophages were examined C_{60} was found in the plasma membrane, lysosomes and nucleus [86]. In another study a pristine C_{60} preparation was obtained by sonication in methanol (toluene is more commonly used) and gave uniformly sized particles with photoluminescence detection at 750 nm after excitation at 488 nm. This emission was used to demonstrate cell uptake in normal and malignant breast cells after culturing them on fullerene-coated dishes [87].

Following on from the above *in vitro* studies the question arises whether these fullerene derivatives could be used to mediate PDT for *in vivo* applications either for antimicrobial use in infections or for anticancer use in tumor models.

In vivo PDT with fullerenes

As the previous section on *in vitro* studies demonstrated, fullerenes have the potential to act as efficient PS able to kill numerous classes of microbial cells as well as cancer cells. Now the key question is whether this high degree of *in vitro* activity could be translated into an *in vivo* anti-cancer effect or an antimicrobial effect in an appropriate animal model. In 1997, Tabata *et al.* first reported the use of fullerenes to carry out PDT of actual tumors [88]. In this study the fullerenes were chemically modified to make the molecules soluble in water as well as to enlarge the molecular size by pegylating C_{60} (Figure 3F). On injecting the PS intravenously in mice carrying a subcutaneous tumor on their backs, the C_{60}–PEG conjugate

demonstrated a higher accumulation and more prolonged retention in the tumor tissue than in normal tissue. The conjugate was excreted without being accumulated in any specific organ. Following intravenous injection of either C_{60}–PEG conjugate or the Photofrin® (a recognized PS) to tumor-bearing mice, coupled with exposure of the tumor site to visible light, the volume increase of the tumor mass was suppressed and the C_{60} conjugate exhibited a stronger suppressive effect than Photofrin. Histological examination revealed that intravenous injection of the fullerene plus light irradiation strongly induced tumor necrosis without any damage to the overlying normal skin. The antitumor effect of the fullerene conjugate increased with increasing fluence and with increasing C_{60} dose. The antitumor effect was achieved by treatment with a dose of 424 μg/kg at a fluence of 107 J/cm^2.

A novel PS was prepared from fullerene C_{60} that possessed additional MRI activity for efficient PDT of tumor. After chemical conjugation of PEG to C_{60} to form C_{60}–PEG, diethylenetri diethylenetriaminepentaacetic acid (DTPA) was subsequently conjugated to the terminal group of PEG to form C_{60}–PEG–DTPA. C_{60}–PEG–DTPA was mixed with gadolinium acetate solution to obtain Gd^{3+}-chelated C_{60}–PEG–DTPA–Gd. Following intravenous injection of C_{60}–PEG–DTPA–Gd into tumor-bearing mice, the PDT antitumor effect and the MRI tumor imaging were evaluated. Similar generation of superoxide upon illumination was observed with or without Gd^{3+} chelation. When intravenously injected into tumor-bearing mice, C_{60}–PEG–DTPA–Gd maintained an enhanced MRI signal at the tumor tissue for a longer time period than Magnevist®. Injection of C_{60}–PEG–DTPA–Gd plus light irradiation showed significant tumor PDT effect, although the effect was dependent on the timing of light irradiation. The PDT efficacy of C_{60}–PEG–DTPA–Gd was observed at the time when the tumor accumulation was detected by the enhanced intensity of the MRI signal. This therapeutic and diagnostic hybrid system was found to be a promising tool to enhance the efficacy of PDT against tumors [31].

A preliminary *in vivo* study of PDT using hydrophilic nanospheres formed from hexa(sulfo-*n*-butyl)-C_{60} (FC_4S; Figure 4e) was performed by Chiang and his coworkers [89]. This study was performed in imprinting control region mice bearing sarcoma 180 subcutaneous tumors. They were given either intraperitoneal or intravenous injection of water-soluble FC_4S in PBS (5 mg/kg body weight). The tumor site was subsequently irradiated with an argon ion laser beam at a wavelength of 515 nm or an argon-pumped dye laser at 633 nm with the beam focused to a diameter of 7–8 mm with the total light dose adjusted to a level of 100 J/cm^2 in each experiment. Consistently, inhibition of the tumor growth was found to be more effective using a lower wavelength (i.e., the 515 nm laser was bett absorbed than the 633 nm laser). Administration of FC_4S to mice intraperitoneally demonstrated slightly better inhibition effectiveness than the intravenous method.

As cancer cells endocytose glucose more effectively than normal cells due to upregulation of glucose receptors, Otake and colleagues prepared a set of C_{60}–glucose conjugates that also served the purpose of solubilizing the fullerene [90]. The glycoconjugated-C_{60} compounds produced selective phototoxicity (after irradiation with UVA1) towards cancer cells compared with normal fibroblasts showing the importance of targeting glucose receptors. Inhibition by sodium azide showed the involvement of singlet oxygen in the cell killing. In order to investigate the effect of PDT *in vivo*, human-melanoma (COLO679)-xenograft bearing mice were injected intratumor-ally with C_{60}–(Glc)1 (Figure 4F; 0.1 or 0.2 mg/tumor) and irradiated with 10 J/cm^2 UVA1 after a 4 h drug–light interval. PDT with C_{60}–(Glc)1 was reported to suppress the tumor growth, with the higher dose performing better than the lower dose.

We have recently shown [91] that intraperitoneal PDT with a fullerene and white light was able to demonstrate significant therapeutic effects in a challenging mouse model of disseminated abdominal cancer. In humans this form of cancer is characterized as a thin covering of tumor nodules on the intestine and other abdominal organs, and responds poorly to standard treatment such as surgery or chemotherapy. In this study we formulated the monocationic BF4 (Figure 4A) in micelles composed of Cremophor EL (Figure 3A) to treat intraperitoneally disseminated colorectal cancer in a mouse model. We used a colon adenocarcinoma cell line (CT26) expressing firefly luciferase to allow monitoring of intraperitoneal tumor burden by noninvasive optical imaging. Intraperitoneal injection of a preparation of BF4 formulated in micelles (5 mg/kg), followed by white-light illumination (100 J/cm^2) delivered through the peritoneal wall (after creation of a skin flap; Figure 5A & 5B) produced a statistically significant reduction in bioluminescence (Figure 5C & 5D) and a survival advantage in mice (Figure 5F). White light was more effective than green light (Figure 5F), while red light produced unacceptable toxicity to the mice due to excessive tissue penetration of the light into abdominal organs. A drug–light interval of 24 h was more effective than a 3 h drug–light interval (compare Figure 5e & 5F) showing the importance of allowing enough time for the fullerene to be taken up into the cancer cells. The intraperitoneal tumors were destroyed by the process of necrosis rather than the more usual apoptosis

Due to the aforementioned limitation of being nonfluorescent, the *in vivo* phamacokinetic characteristics of fullerenes such as the biodistribution and the organ/target specific binding has been less studied. It is considerably challenging to collect good biodistribution data of fullerenes. Approaches that could be applied for studying the biodistribution of fullerenes include radiolabeling fullerene with I^{125} or C^{14}. In one study fullerenes were labeled with I^{125} and biodistribution studies were carried out. It was shown that after injection in to tumor-bearing mice the C_{60}–PEG conjugate disappeared gradually from the blood circulation and 78% was excreted from the body within 24 h. This conjugate did not show any marked accumulation in any of the organs although the accumulation in the liver increased up to 24 h but decreased with time and was undetectable at 144 h after injection. The fullerene accumulated in the carcass and the GI tract in the early period but was eliminated thereafter in the same manner as the liver. This fullerene accumulated in the tumor tissue to a significantly higher extent than in the skin and the muscles and was also retained in the tumor tissue for a longer period than the normal tissue [32]. Liu and others conjugated PEG to C_{60} (C_{60}–PEG), and DTPA was subsequently introduced to the terminal group of PEG to prepare C_{60}–PEG–DTPA that was mixed with gadolinium acetate solution to obtain Gd^{3+}-chelated C_{60}–PEG–DTPA–Gd. Following intravenous injection of C_{60}–PEG–DTPA–Gd into tumor-bearing mice, they observed tumor accumulation by enhanced intensity of the MRI signal [31].

Another study aimed to evaluate a fullerene as a therapeutic PS in the treatment of atherosclerosis. An atherosclerotic experimental rabbit model was prepared by causing intimal injury to bilateral external iliac arteries using balloon expansion. In four atherosclerotic rabbits and one normal rabbit, PEG-modified fullerene was infused into the left external iliac artery and illuminated by a light-emitting diode, while the right external iliac artery was only illuminated by light emitting diode. It was concluded that the infusion of a high concentration of fullerene–PEG followed by photoillumination did not result in suppression of atherosclerosis but rather in a progression of the atherosclerosis lesion. However, this intervention showed no adverse effects on the normal iliac artery [30].

In addition to the *in vivo* anticancer application of fullerene PDT, we devised a study to test whether the high degree of light-mediated antimicrobial activity of fullerenes *in vitro* could produce an *in vivo* therapeutic effect in a mouse model of bacterial infection [92]. We used

stable biolumi nescent bacteria and a low light imaging system to follow the progress of the infection noninvasively in real time in two potentially lethal mouse models of infected wounds. An excisional wound on the mouse back was contaminated with one of two bioluminescent Gram-negative species, *Proteus mirabilis* (2.5×10^7 cells) or *Pseudomonas aeruginosa* (5×10^6 cells). A solution of *tris*-cationic BF6 (Figure 4B) was placed into the wound followed by delivery of up to 180 J/cm^2 of broadband white light (400–700 nm). Our results showed that in both cases there was a light- and dose-dependent reduction of bioluminescence from the wound not observed in control groups (light alone or BF6 alone;Figure 6A & 6C). Fullerene-mediated PDT of mice infected with *P. mirabilis* led to 82% survival compared with 8% survival without treatment ($p < 0.001$)(Figure 6B). PDT of mice infected with highly virulent *P. aeruginosa* did not lead to survival, but when PDT was combined with a suboptimal dose of the antibiotic tobramycin (6 mg/kg for 1 day) there was a synergistic therapeutic effect with a survival of 60% compared with a survival of 20% with tobramycin alone ($p < 0.01$;Figure 6D). In conclusion these data suggest that cationic fullerenes have clinical potential as an antimicrobial PS for superficial infections where red light is not needed to penetrate tissue.

Future perspective

Fullerenes have been widely studied in recent years as potential PS that could mediate PDT of diverse diseases. Most of these reports have been confined to *in vitro* studies where viruses, bacteria, fungi or cancer cells have been incubated with a diverse array of functionalized or solubilized fullerene compounds followed by illumination with light that is usually UVA, blue, green or white because the absorption spectrum of fullerenes is biased towards lower wavelengths. Since *in vivo* PDT usually uses red light for its improved tissue-penetrating properties, it was unclear whether fullerenes would mediate effective PDT *in vivo*. This question can now be answered in the affirmative. In fact, the report in which mice suffered toxicity after fullerene PDT with red light but exhibited a beneficial therapeutic effect after white light illumination suggests that this supposed drawback may actually be an advantage instead. Future studies will include synthesis of new fullerene derivatives particularly those with light-harvesting antennae to broaden the range of activating light that can be used, hence increasing light penetration depth into tissue. More experiments should be designed to increase understanding of the mechanisms that govern the balance between type I and type II reactive oxygen species. These studies will establish whether fullerenes can compete with more traditional PS in clinical applications of PDT.

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Paper of special note have highlight as:

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Executive summary

Photodynamic therapy

- Photodynamic therapy (PDT) involves the combination of nontoxic photosensitizing dyes, harmless visible light and oxygen.
- The photosensitizing dyes absorb light to form long-lived triplet states that can mediate photochemistry leading to generation of either free radicals (type 1) or singlet oxygen (type 2).
- These reactive oxygen species can kill undesirable species that include cancer cells, pathogenic bacteria, fungi and viruses.
- PDT can destroy tumors by direct tumor cell killing, vascular shutdown and activation of the host immune system.

Fullerenes as photosensitizers

- Fullerenes have an absorption spectra that span the visible range, although they are biased towards the UVA and blue range.
- Fullerenes have high photostability and resistance to photobleaching.
- Fullerenes show both type I (free radicals) and type II (singlet oxygen) photochemistry.
- Fullerenes can be chemically modified for tuning the drug's physical and chemical properties.
- To extend their absorption spectrum further into the red wavelengths, they can be modified by attachment of light harvesting antennae.
- Molecular self-assembly of fullerene cages into vesicles allows improved drug delivery.

Photophysics & photochemistry of fullerenes

- Fullerenes efficiently generate singlet oxygen in organic solvents but switch to type 1 photochemistry in aqueous environments.
- Electron transfer to a triplet fullerene can give a radical anion that produces superoxide from oxygen.
- The highly toxic hydroxyl radical can be subsequently formed from superoxide.
- Despite the demonstrated ability of fullerenes to quench reactive oxygen species in some circumstances, PDT can be efficiently mediated by illuminated fullerenes.

***In vitro* PDT with fullerenes**

- Several studies have shown that cancer cells such as HeLa can be killed after incubation with various fullerenes both pristine and functionalized, and illumination with various wavelengths of light.
- Viruses can be inactivated and DNA breaks produced by photoactivated fullerenes.
- Gram-positive bacteria, Gram-negative bacteria and fungal cells can all be killed by PDT mediated by fullerenes with cationic charges.

- Determination of the subcellular localization of fullerenes in cancer cells is challenging due to a lack of fluorescence but some progress has been made.

***In vivo* PDT with fullerenes**

- There are reports that PDT with fullerenes can destroy or inhibit subcutaneous tumors growing in mice.
- One report shows increased survival in a challenging disseminated abdominal cancer model.
- An illuminated cationic fullerene can save the life of mice with an invasive bacterial wound infection.
- Biodistribution and imaging studies may be carried out if the fullerene has a suitable label such as gadolinium.

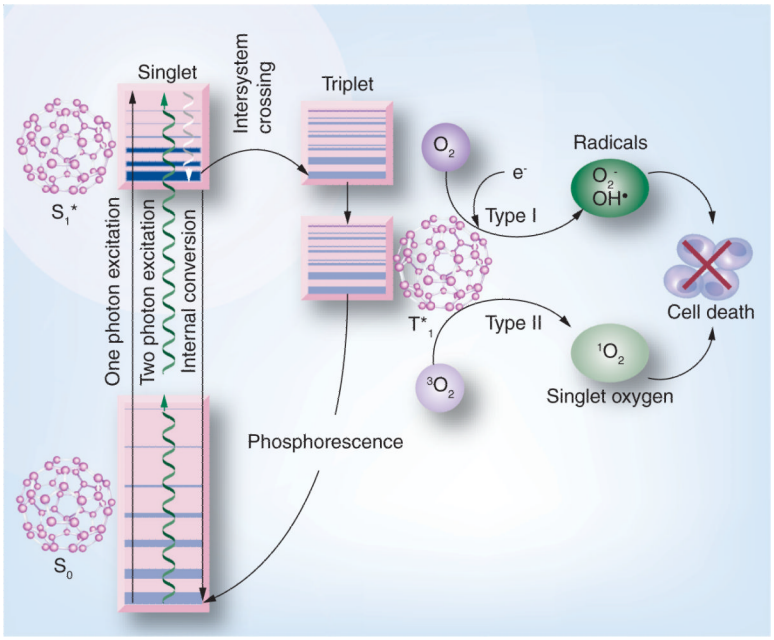

Figure 1. Jablonski diagram

Initial absorption of a photon (or two photons of twice the energy) by the ground state of the singlet fullerene, which gives rise to the short-lived excited singlet state. This can lose energy by fluorescence (negligible in the case of fullerenes), internal conversion to heat or by intersystem crossing to the long-lived triplet state. Fullerene triplet states are efficiently quenched by molecular oxygen (a triplet state) to give type II (singlet oxygen) and type I (superoxide and hydroxyl radical) reactive oxygen species. In the absence of oxygen fullerene triplet states lose energy by phosphorescence.

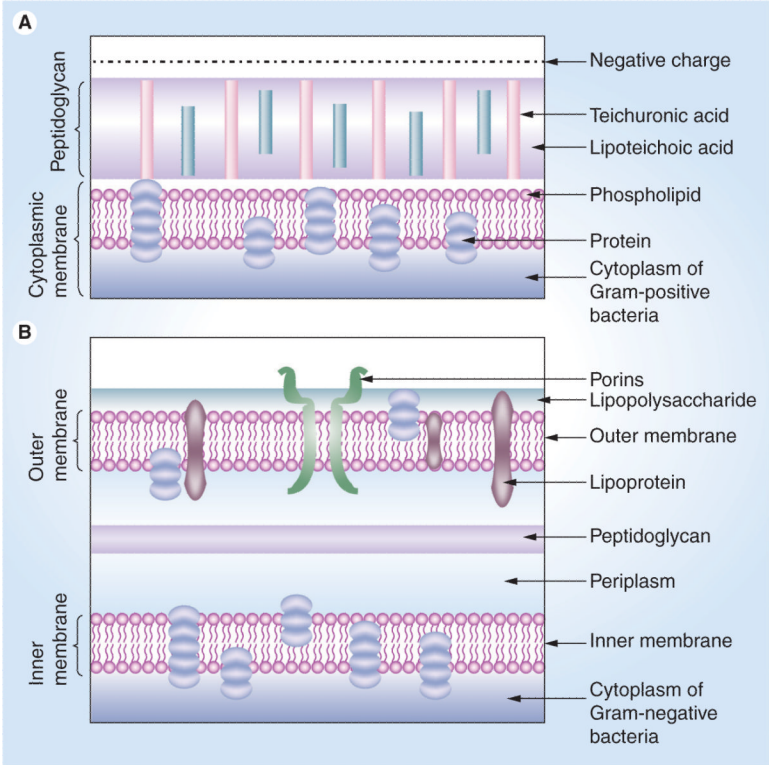

Figure 2. Structures of the cell walls of two different classes of bacteria

(A) Gram-positive bacterium showing a porous layer of peptidoglycan and single lipid bilayer. **(B)** Gram-negative bacterium showing a double lipid bilayer sandwiching the peptidoglycan layer and an outer layer of lipopolysaccharide.

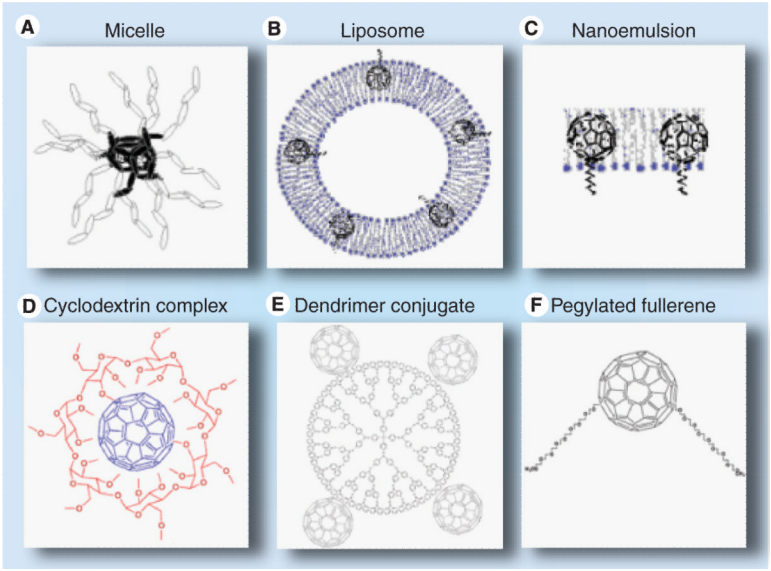

Figure 3. Drug delivery vehicles for solubilizing fullerenes

(A) Micelles; **(B)** liposomes; **(C)** nanoemulsion; **(D)** cyclodextrin complex; **(E)** dendrimer conjugate; and **(F)** pegylated fullerene.

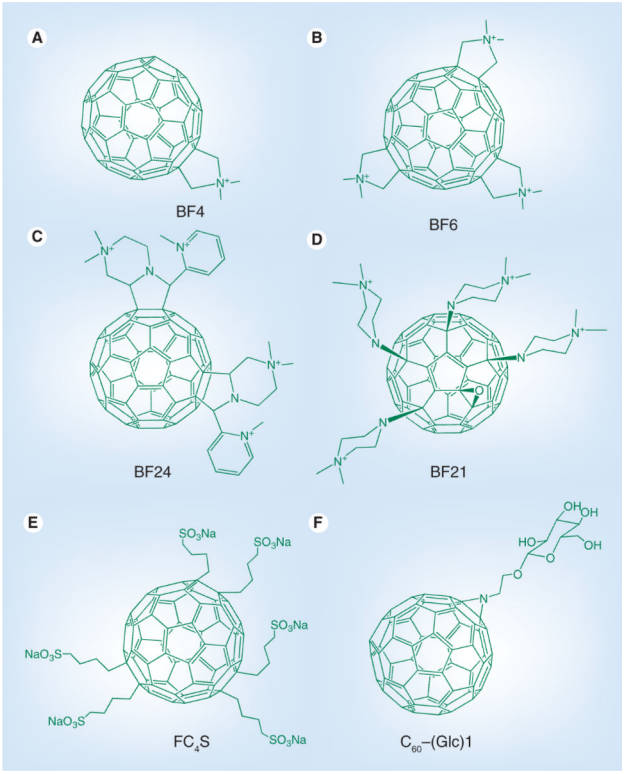

Figure 4. Chemical structures of functionalized fullerenes used for photodynamic therapy

(A) Mono-pyrrolidinium fullerene, BF4 [70]; **(B)** tris-pyrrolidinium fullerene, BF6 [76]; **(C)** tetrakis-cationic fullerene, BF24 [78]; **(D)** tetrakis-cationic fullerene, BF21 [79]; **(E)** hexakis-anionic fullerene, FC_4S [93]; and **(F)** mono-glycosylated fullerene C_{60}–(Glc)1 [90].

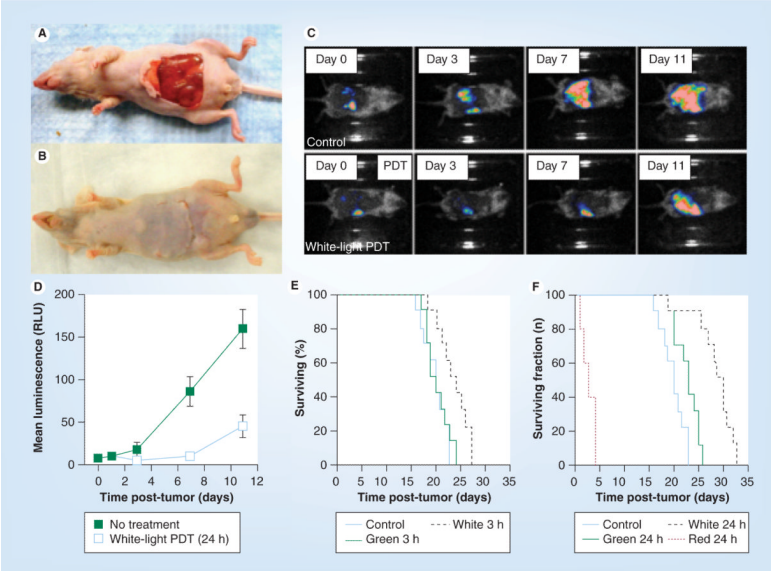

Figure 5. Fullerene photodynamic therapy for disseminated peritoneal cancer in mice

(A) Novel skin flap model to allow illumination of the abdominal cavity with white light. **(B)** Skin flap can be readily closed after illumination. **(C)** Noninvasive bioluminescence imaging of mouse colon carcinoma engineered to express luciferase after no treatment or after BF4 fullerene-mediated PDT. **(D)** Quantification of bioluminescence signals from **(C)**. **(E)** Kaplan–Meier survival plot of mice treated with 100 J/cm^2 of either green or white light or nothing 3 h after injection of 5 mg/kg BF4. **(F)** Similar to **(E)** but 24 h after injection and also including a red light treatment group.

PDT: Photodynamic therapy.

Data adapted with permission from [91].

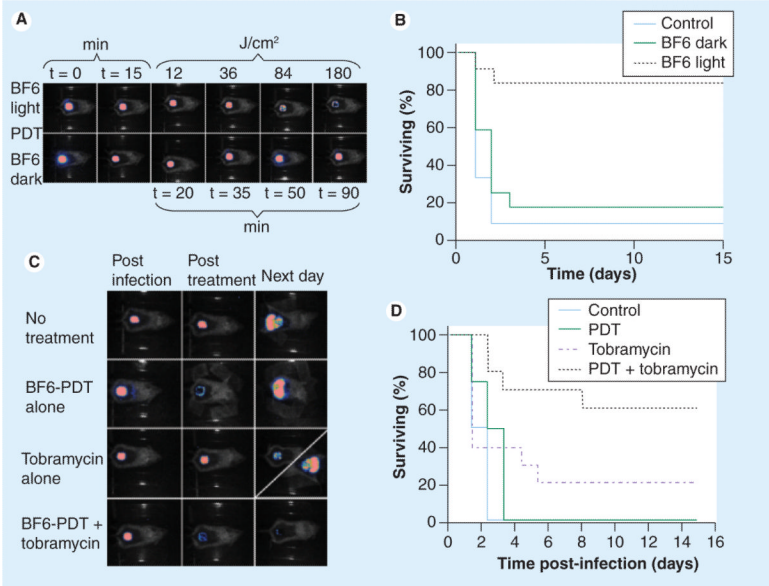

Figure 6. Fullerene-mediated photodynamic therapy for Gram-negative bacterial wound infections in mice

(A) Successive bioluminescence imaging of mice with wounds infected with bioluminescent *Proteus mirabilis*, treated with BF6 + light PDT (top row) or BF6 dark control (bottom row). **(B)** Kaplan–Meier survival curves for the groups of mice in **(A)**; no treatment control (n = 12); BF6 in dark (n = 12); BF6 + light (n = 11). **(C)** Representative bioluminescence images of *Pseudomonas aeruginosa* infected mice (captured immediately postinfection, immediately post-treatment, and 24 h post-treatment), receiving: no treatment (top row); treated with BF6-PDT alone (180 J/cm^2) (second row); treated with tobramycin alone (6 mg/kg for 1 day; third row, diagonal panel 24 h post-treatment shows two possible outcomes); treated with a combination of BF6-PDT and 1-day tobramycin (bottom row). **(D)** Kaplan–Meier survival curves for the groups of mice in Figure 6C; no treatment control (n = 10); PDT alone (n = 12); tobramycin alone (n = 12); PDT + tobramycin (n = 10). PDT: Photodynamic therapy.