

Cationic Fullerenes Are Effective and Selective Antimicrobial Photosensitizers

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Summary

Fullerenes are soccer ball-shaped molecules composed of carbon atoms, and, when derivatized with functional groups, they become soluble and can act as photosensitizers. Antimicrobial photodynamic therapy combines a nontoxic photosensitizer with harmless visible light to generate reactive oxygen species that kill microbial cells. We have compared the antimicrobial activity of six functionalized C₆₀ compounds with one, two, or three hydrophilic or cationic groups in combination with white light against gram-positive bacteria, gram-negative bacteria, and fungi. After a 10 min incubation, the bis- and tris-cationic fullerenes were highly active in killing all tested microbes (4–6 logs) under conditions in which mammalian cells were comparatively unharmed. These compounds performed significantly better than a widely used antimicrobial photosensitizer, toluidine blue O. The high selectivity and efficacy exhibited by these photosensitizers encourage further testing for antimicrobial applications.

Introduction

Fullerenes (originally buckminsterfullerenes) are a new class of all-carbon molecules; the first example was discovered by Kroto et al. in 1985 [1] and is composed of 60 carbon atoms arranged in a soccer ball-shaped structure. The condensed aromatic rings present in the compound lead to an extended π conjugation of molecular orbitals, and this arrangement therefore causes significant absorption of visible light. In recent years, there has been much interest in studying the possible biological activities of fullerenes (and other nanostructures produced in the nanotechnology revolution) with the

aim of using them in the field of medicine [2–5]. An important issue when dealing with unmodified fullerenes is their insolubility in biologically compatible solvents, limiting their use in biological applications. Therefore, fullerenes have to be chemically modified or functionalized by the introduction of addends in order to acquire aqueous solubility [6–8].

Interesting biological properties have been reported for various functionalized fullerenes (reviewed in [2, 5]). Quarternary functionalized derivatives were found to inhibit the protease of HIV1 virus [9], while amide-derivatized fullerenes acted as antiretroviral drugs in cells [10]. There was a report that a hexakis-carboxylic acid fullerene derivative prevented death from a group A *Streptococcus* infection in mice [11]. Polyhydroxylated fullerenes have been proposed as antioxidants and free radical scavengers [12–14]. Japanese workers have studied the antibacterial properties of cationic fullerenes as inhibitors of bacterial respiration [15–17] (see following sections).

The combination of absorption of visible light by fullerenes, referred to above, and a long-lived triplet state allows fullerenes to act as photosensitizers (PS). In a similar fashion to the tetrapyrrole PS used for photodynamic therapy (PDT) [18], illumination of solubilized fullerenes in the presence of oxygen leads to the generation of reactive oxygen species (ROS) via energy transfer from the excited triplet state of the fullerene to molecular oxygen. A recent report [19] has shown that in polar solvents, especially those containing reducing agents (such as NADH, found in cells), illumination will generate the reduced oxygen species, superoxide anion and hydroxyl radical, while in nonpolar solvents, singlet oxygen is the main product. These different pathways are analogous to the Type II and Type I photochemical mechanisms frequently discussed in PDT with tetrapyrroles [18]. Fullerenes have been used to carry out in vitro PDT, leading to cleavage of DNA strands [20], photoinactivation of viruses [21], a demonstration of mutagenicity in *Salmonella* species [22], and photo-induced killing of mammalian cells in tissue culture [23]. There has also been a report of fullerene-mediated PDT resulting in cures in a murine subcutaneous tumor model [24].

Although PDT was originally discovered over 100 years ago by its effect on killing microorganisms [25], it was largely developed as a cancer therapy [26] and recently has achieved great success as a treatment for age-related macular degeneration [27]. The possible use of PDT as a treatment for localized infections due to its antimicrobial potential is only just being seriously investigated [28–30]. It has been known for some time that PS molecules that bear one or more net cationic charges perform best as antimicrobial PS compared to neutral or anionic structures [31–33]. The positive charges allow the PS to bind to the negative charges borne by most microbial cells, and, in the case of gram-negative bacteria, the cationic charges weaken the permeability barrier of the outer membrane structure, allowing the PS to penetrate to more sensitive intracellular locations [30, 34].

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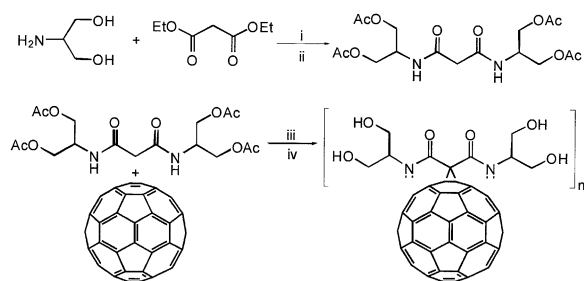


Figure 1. Synthesis of BF1–3

(i) 200°C, 45 min. (ii) Acetic anhydride, pyridine, 18 hr. (iii) CBr₄, DBU, acetone, toluene, 4.5 hr. (iv) K₂CO₃, methanol, H₂O. Et, ethyl; Ac, acetyl; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene.

In this study, we investigated the broad-spectrum antimicrobial photodynamic activities of two series of functionalized C₆₀; a first series with one, two, or three polar diserinol groups (BF1–3), and a second series with one, two, or three quarternary pyrrolidinium groups (BF4–6). Gram-positive bacteria, gram-negative bacteria, and fungi were tested, and the photodynamic killing was compared with that mediated by a widely used antimicrobial PS, toluidine blue O (TBO). Finally, the extent to which the photoactivated C₆₀ derivatives allowed selective killing of microbes while sparing mammalian cells was assessed.

Results

Synthesis and Characterization of Functionalized Fullerenes

BF1–3 and BF4–6 were synthesized according to modifications of established literature procedures [35–38] as shown in Figures 1 and 2. There are 8 possible regioisomers of the bis-substituted fullerenes and 46 possible regioisomers of the tris-substituted fullerenes, and it was not practical to separate these mixtures of regioisomers into individual pure compounds; therefore, BF2, BF3, BF5, and BF6 were studied as mixtures of regioisomers. The identity of the compounds, however, was confirmed by mass spectrometry, giving molecular ions identical to those calculated. The proton and C13 NMR spectra of the immediate precursors of BF1 and BF4 have been previously reported [35, 37]. The absorption spectra of BF4–6 and TBO, all at the same concentration of 10 μM in DMSO:water (1:9), are shown in Figure 3. The overall extinction coefficients of the fullerenes were in the following order: BF4 > BF5 > BF6. The shoulder in the UVA range moved from 340 nm for BF4 to 310 nm for BF5 and disappeared altogether for BF6.

Initial Screening of Functionalized Fullerenes as Antimicrobial PS

We initially screened the fullerenes BF1–6 to assess their potential to mediate photodynamic inactivation (PDI) against the gram-positive *S. aureus* after 10 min incubations with 100 μM concentrations and no wash. Compounds BF5 and BF6 were completely dark toxic to *S. aureus* and gave zero colonies or >99.9999% killing regardless of the amount of light delivered (data

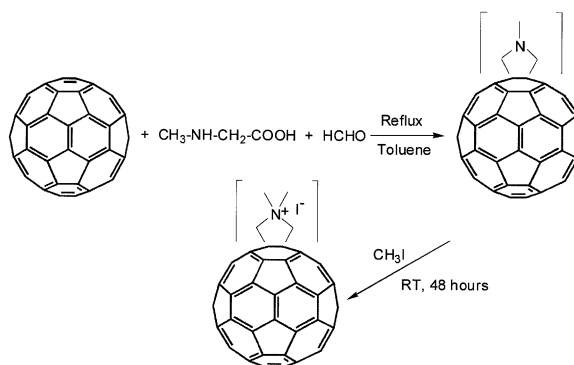


Figure 2. Synthesis of BF4–6

not shown). Compound BF4 showed significant dark toxicity (99%; see first point of curve with squares in Figure 5). Compounds BF1–3, however, showed only minor dark toxicity toward *S. aureus* (60%–80%; see Figure 4A). When relatively large fluences of broad-band white light were delivered to bacterial suspensions still containing the fullerenes, a fluence-dependent loss of viability of *S. aureus* ranging from 2–4 logs of killing, as shown in Figure 4A (closed symbols), was observed. BF1–3 displayed significant differences in effectiveness between members of the series. Their effectiveness was BF3 > BF2 > BF1, and the differences in the survival fraction were significant ($p < 0.05$) at the two highest fluences (80 and 120 J/cm²).

In order to test whether the fullerenes actually bound to the bacterial cells, we compared PDI with and without a wash (centrifugation of the suspension containing the fullerene and resuspension in fresh PBS). As can be seen by comparing curves with open and closed symbols in Figure 4A, there was no difference in killing with and without a wash, showing that the fullerenes bound to the bacteria and could not easily be washed out.

We tested BF1–3 under the same conditions (100 μM incubation for 10 min and no wash) with the gram-negative *E. coli*. As shown in Figure 4B, there was no dark toxicity and only a very small amount of light-mediated killing (less than 90%). BF1 was significantly less effective than BF2–3 ($p < 0.05$).

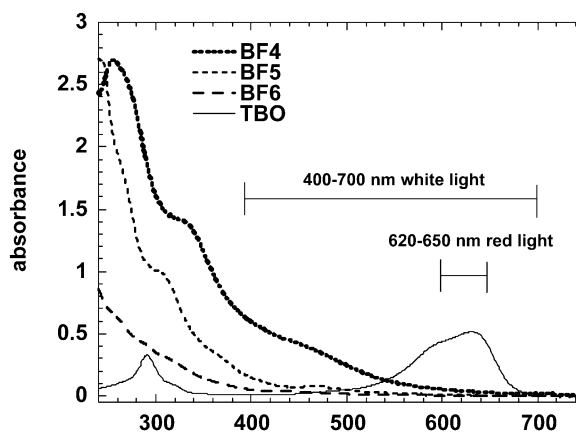


Figure 3. UV-Visible Absorption Spectra of BF4–6 and TBO at 10 μM in 1:9 DMSO:Water

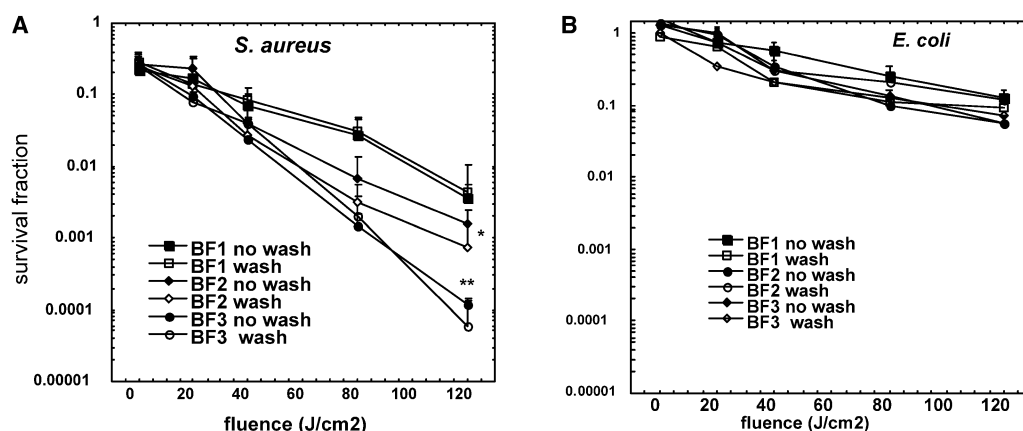


Figure 4. PDI of Bacteria with BF1–3

(A) *S. aureus* (10^8 cells per ml) were incubated for 10 min with BF1–3 at a 100 μ M concentration in PBS, followed, or not, by a wash (centrifugation and resuspension) and illumination with 400–700 nm light at an irradiance of 200 mW/cm². Aliquots were removed from the suspensions after the various fluences of light had been delivered and the CFU had been determined. Values are means of six independent experiments, and bars are SEM. * $p < 0.05$; ** $p < 0.01$; two-tailed unpaired t test.

(B) *E. coli* (10^8 cells per ml) was treated the same as *S. aureus*.

Cationic Fullerenes Mediate Photodynamic Inactivation of Three Microbial Classes

Compound BF4 showed significant dark toxicity toward *S. aureus* at 100 μ M, and we therefore decreased the concentration of BF4 in the incubation mixture in a step-wise manner to 50, 25, 10, and 1 μ M. These experiments were carried out with a wash. As shown in Figure 5, the dark toxicity decreased as the concentration was decreased until, at 10 and 1 μ M, it was nonexistent. When PDI experiments were carried out after incubation of *S. aureus* with these concentrations of BF4, a fluence-dependent loss of viability was observed in all

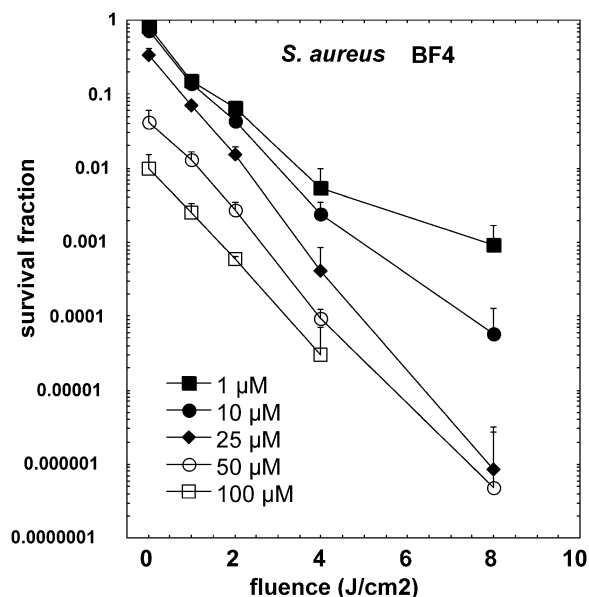


Figure 5. PDI of *S. aureus* with BF4

S. aureus at 10^8 cells per ml was incubated with the specified concentrations of BF4 for 10 min, followed by a wash and illumination with white light. Values are means of six independent experiments, and bars are SEM.

cases with comparatively low doses of light (4–8 J/cm²). Remarkably, the PDI killing curves were not significantly different (compare the slopes of curves in Figure 5) among the different concentrations. The difference between the curves was solely in the survival fraction at 0 J/cm², i.e., the dark toxicity.

Since the initial screening experiment had suggested that the bis- and tris-cationic fullerenes BF5 and BF6 would be more potent than BF4 (higher dark toxicity), we tested them against *S. aureus* at 1 μ M with a wash. As shown in Figure 6A, compounds BF5 and BF6 were highly active, with 2 and 1 J/cm² of light being sufficient to kill 4–5 logs, respectively; all three killing curves were significantly different ($p < 0.01$).

As it is well known that gram-positive bacteria are much more susceptible to PDI than gram-negative bacteria or fungal species [39, 40], we decided to test the cationic fullerenes BF4–6 at 10 μ M with a wash against the other microorganisms. Figure 6B shows the light-mediated killing of gram-negative *E. coli* with the three cationic fullerenes. BF5 and BF6 were highly effective, with 2 J/cm² giving 4 and 6 logs of killing, respectively. BF4 was much less potent, needing 8 J/cm² to give 3 logs of killing ($p < 0.001$). There was only minimal dark toxicity. Similar results were obtained with the yeast *C. albicans* (Figure 6C), in which BF6 was slightly more effective than BF5 and both were much better than BF4 ($p < 0.001$). The gram-negative bacterium *P. aeruginosa* was more resistant than the other organisms tested (Figure 6D). We doubled the maximum light dose delivered to 16 J/cm² in order to obtain more killing, but this had only a minimal effect. BF5 and BF6 were able to kill 3–5 logs, while BF4 only gave 2 logs of killing of *P. aeruginosa*.

Comparison of BF4–6 and Toluidine Blue O-Mediated PDI of *E. coli* in the Presence of Serum

In order to obtain an objective measure of how cationic fullerenes performed as antimicrobial photosensitizers, we compared them with a widely used phenothiazinium

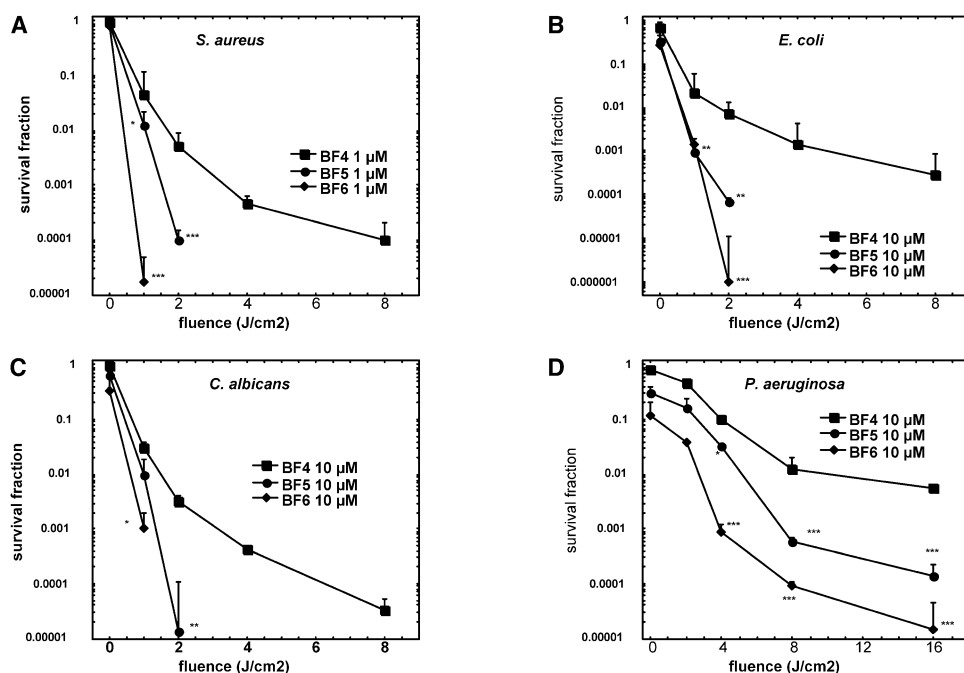


Figure 6. PDI of Bacteria and Yeast with BF4–6

(A–D) (A) *S. aureus* was incubated with a 1 μM concentration of BF4–6, and (B) *E. coli*, (C) *C. albicans*, and (D) *P. aeruginosa*, all at 10^8 cells per ml, were incubated with BF4–6 at 10 μM concentrations for 10 min, followed by a wash and illumination with white light. Values are means of six independent experiments, and bars are SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed unpaired t test.

dye. However, in order to be able to directly compare the PDI-mediated killing of bacteria with PDT killing of mammalian cells, we needed to add 10% serum to the bacterial suspension, as this is the standard growth condition for fibroblasts. It has previously been shown that the addition of serum to bacterial PS incubations significantly reduces the effectiveness, probably because the PS binds to serum proteins, thus reducing the effective concentration available to bind to bacteria [41, 42]. We therefore tested the PDI of *E. coli* by using BF4–6 and TBO under the same conditions (10 μM , 10 min incubation in the presence of 10% FBS and a wash). As shown in Figure 7A, TBO was almost ineffective in mediating PDI. When the BF4–6-mediated killing was compared with that shown in Figure 6B (no serum), it can be seen that the effectiveness of BF4 was unchanged, while the killing mediated by BF5 and BF6 was reduced by about 1 log in the presence of serum. TBO at the same concentration and fluence as was used for fullerenes (1 μM for *S. aureus* and 10 μM for both *P. aeruginosa* and *C. albicans*) produced less than 1 log of killing in the presence or absence of serum (data not shown).

Cationic Fullerenes Show Selectivity for Microbes over Mammalian Cells

In order to assess the selectivity of light-mediated killing for microbes over mammalian cells, we incubated mouse L929 fibroblasts with BF4–6 and with TBO under the same conditions (10 μM concentration for 10 min in 10% FBS and a wash), followed by delivery of white or red light, respectively, up to 120 J/cm². BF4–6 did show some dark toxicity (20%–60% killing) and some

additional phototoxicity (20%–30%) toward L929 cells, as shown in Figure 7. However, TBO displayed a different shape of killing curve with little dark toxicity, but a pronounced light-dependent toxicity, until the limit of the viability assay was reached at 80 J/cm².

Discussion

The effectiveness of various PS proposed for antimicrobial PDT can be judged on several criteria. These PS should be able to kill multiple classes of microbes at relatively low PS concentrations and low fluences of light. PS should be reasonably nontoxic in the dark and should demonstrate selectivity for microbial cells over mammalian cells. PS should ideally have large extinction coefficients in the red part of the spectrum and demonstrate high triplet and singlet oxygen quantum yields. In this report, we have shown that cationic fullerenes fulfill many (but not all) of these criteria. The main disadvantage of using fullerenes as antimicrobial PS lies in their absorption spectrum. As shown in Figure 3, the fullerenes have broad absorption in the UV range, with a tail that extends well into the visible spectrum (to 550 nm in the case of BF4). The UV absorption decreased as the number of substituents on the fullerene increased, and, consequently, the degree of π conjugation decreased. TBO, however, like many other PS used for PDT and PDI, has an absorption peak in the red at 635 nm. Many reports show that PDT in vivo is more effective with red light and near infrared light, as both the absorption and scattering of light by tissue decrease as the wavelength increases [43]. We used a broad-band pass filter that gives an output of the entire visible

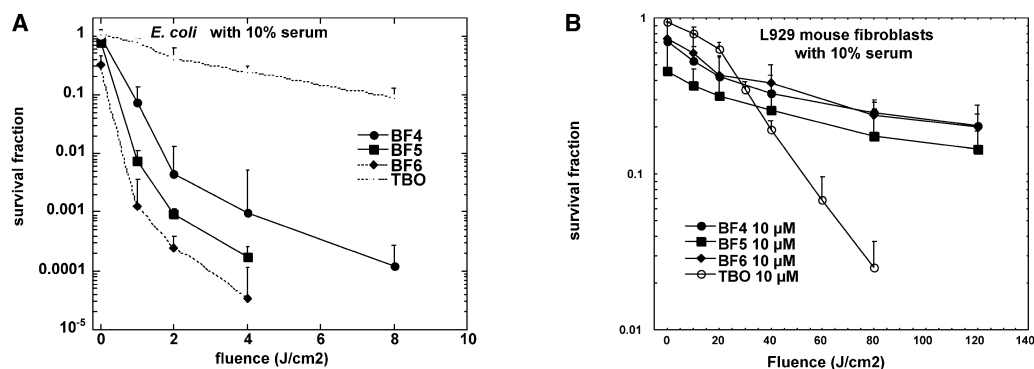


Figure 7. Comparison of PDI of *E. coli* and Fibroblasts with BF4–6 and TBO

(A) *E. coli* was incubated with a 10 μM concentration of BF4–6 or TBO in the presence of 10% FBS for 10 min, followed by a wash and illumination with white or red light, respectively.

(B) L929 murine fibroblasts at 80% confluence were incubated with BF4–6 or TBO, both at a 10 μM concentration, for 10 min in the presence of 10% FBS, followed by a wash and illumination with white or red light. The survival fraction was calculated by using the MMT viability assay. Values are means of six independent experiments, and bars are SEM.

spectrum (400–700 nm) to excite the fullerenes that maximized the absorption by the tail in the visible range. We did not use UV light to excite the fullerenes, as UV light is highly germicidal and can kill most microorganisms. As can be seen from Figure 3, the effective absorption of the delivered wavelength ranges was not very different between the fullerenes and TBO.

Our initial screening experiment carried out against *S. aureus* at a 100 μM concentration showed that the C₆₀ substituted with pyrrolidinium groups behaved very differently than the series substituted with di-serinol groups. The cationic fullerenes gave high levels of dark toxicity (except for BF4), while the di-serinol-functionalized C₆₀ showed a typical light dose-dependent loss of colony-forming ability. However, cationic fullerenes were highly effective PS at lower concentrations. This finding agrees with numerous reports in the literature that demonstrate that PS with one (or preferably more) cationic group are efficient antimicrobial PS [30, 31, 33, 44, 45]. Quarternary nitrogen-based groups are superior to primary, secondary, or tertiary amino groups, as the positive charge is less dependent on the pH of the surrounding media, or the pK_a of the molecules that the PS is interacting with. Microbial cells possess overall negative charges, and it is thought that cationic PS bind to these groups on the outer layers of the cell surface. Gram-positive and fungal cells have relatively permeable outer layers of peptidoglycan and lipoteichoic acid or β-glucan, respectively, although the mannan layer of *Candida* species can present a permeability barrier. This allows cationic, and to a lesser extent, noncationic PS to diffuse inward to the plasma membrane, where the generation of reactive oxygen species under illumination can damage the membrane structure, allowing for leakage of essential components and causing cell death. Our finding that BF1–3 were equally effective against the gram-positive *S. aureus* with and without a wash demonstrates that the neutrally charged fullerenes were indeed able to penetrate to a sufficient extent into the cell and that they could not easily be washed out. By contrast, gram-negative bacteria have a double membrane structure that presents

a barrier to diffusion of many PS. Cationic compounds are able to displace divalent cations (Ca²⁺ and Mg²⁺) that play a role in the attachment of lipopolysaccharide to the outer membrane [46]. This displacement weakens the structure of the outer permeability, allowing the PS to penetrate further in a process that has been termed “self-promoted uptake” [47]. The fact that this mechanism requires cationic compounds explains why BF1–3 were relatively ineffective against the gram-negative *E. coli*, and such findings have been reported with numerous other noncationic PS [30].

Because the neutral, alcohol-functionalized fullerenes (BF1–3) had only modest activity against *S. aureus*, we decided to concentrate our efforts on the cationic pyrrolidinium-functionalized fullerenes (BF4–6). These compounds (especially BF5 and BF6) demonstrated high levels of dark toxicity against *S. aureus*. This finding is in agreement with reports by Mashino et al. [15, 16, 48]. These workers proposed that cationic fullerenes could inhibit the growth of *E. coli* and *S. aureus* by interfering with the respiratory chain. There was a biphasic dose response, with the fullerenes inhibiting oxygen uptake at low concentrations and increasing oxygen consumption at high concentrations with concomitant production of hydrogen peroxide. However, we found that at concentrations that gave only low values of dark toxicity, BF4–6 were surprisingly effective in causing light-mediated killing of *S. aureus*. BF5 and BF6 needed only a 1 μM concentration and 1 or 2 J/cm² of white light to kill 4–5 logs. BF4 showed unusual behavior in mediating PDI of *S. aureus*. At 100 μM, there were 2 logs of dark toxicity and an additional 2 logs of light-mediated killing. As the concentration of BF4 was decreased, the dark toxicity decreased as expected. However, we were surprised to observe that the slope of the light-dependent killing curves were similar for the range of concentrations (although the slope for 1 μM BF4 was somewhat less; see Figure 5). It would have been expected that the slopes would decrease as the concentration of fullerene able to produce reactive oxygen upon illumination decreased. This finding suggests that the binding of BF4 is largely independent

of concentration, i.e., the cellular uptake is saturated at a fairly low **BF4** concentration. Alternatively, this phenomenon may be due to the relatively poor solubility of **BF4** that may cause the molecule to be aggregated in aqueous solvents at high concentrations and that would lead to its binding to bacteria being limited by aggregation.

BF4–6 were tested against *E. coli* at 10 μM , and **BF5** and **BF6** showed similar high levels of activity, giving 4–6 logs of killing after 2 J/cm^2 . The fact that these levels of killing were obtained with a wash showed that the fullerenes bound firmly to the gram-negative cells, as has been found with other cationic PS [45]. **BF5** and **BF6** were equally effective against *C. albicans* at 10 μM with a wash. Although *C. albicans* does not have the permeability barriers associated with gram-negative bacteria, the eukaryotic cells are very much larger than bacteria [45]. This means that there is correspondingly more membrane and proteins per cell to react with the reactive oxygen species produced by illuminated fullerenes, and, consequently, a higher concentration is necessary to achieve killing compared to the smaller *S. aureus*. *P. aeruginosa* is known to have even more efficient permeability barriers than the majority of other gram-negative species [49, 50]. Although 10 μM **BF5** and **BF6** with a wash did give 4–5 logs of killing, it was necessary to use significantly more light (up to 16 J/cm^2) compared to what was used with *E. coli*. Although **BF6** was generally more effective than **BF5** in killing all species, the difference between these two compounds was much smaller than the difference between **BF4** and **BF5**. The addition of extra pyrrolidinium groups to the fullerene backbone decreases the absorption of light (see Figure 3) and hence reduces the efficiency of photoinactivation per photon delivered. Presumably, the addition of a third cationic group to the fullerene molecule increased the binding and/or penetration into the cells sufficiently to do more than compensate for the reduced light absorption.

In order to make comparisons between the effectiveness and selectivity of the cationic fullerenes with an established antimicrobial PS, we studied the phenothiazinium dye **TBO** under the same conditions. **TBO** has been widely used to kill multiple classes of microbes in vitro after illumination with red light [51–55]. It has also been tested in several animal models of localized infections. Wong et al. [56] used topical **TBO** and red light to cure an otherwise fatal wound infection with *Vibrio anguillarum* in mice, Komerik et al. [57] used **TBO** and light to treat a rat model of periodontal infection, and Teichert et al. [58] used the closely related phenothiazinium dye methylene blue combined with light to treat a mouse model of oral candidiasis. In our studies, **TBO** (under the same conditions as cationic fullerenes, i.e., 1 or 10 μM , 10 min incubation, and up to 16 J/cm^2 of red light) did not kill more than 90% of any of the microbial species. Therefore, **BF5** and **BF6** are many orders of magnitude more effective than **TBO**, a widely used antimicrobial PS.

If it is proposed to employ antimicrobial PS to treat localized infections in animals or patients, it is necessary to address the question of selectivity of the PS for microbial cells as compared to host mammalian cells. One reason why this selectivity may be relatively

easy to demonstrate is that antimicrobial PDI is often carried out with relatively short incubation times (minutes) before illumination. By contrast, mammalian cells in tissue culture are frequently incubated with PS for periods of hours (even 24 hr). Hence, if killing is compared between microbes and mammalian cells after a short incubation time, it is likely to favor microbial killing. Another difficulty in comparisons between killing microbes and mammalian cells depends on the difference in viability assays. The CFU assay for microorganisms can detect 6 logs of killing, while the MTT assay for mammalian cell viability has a maximum detection limit of 2 logs of killing. Nevertheless, it is clear from the data in the present report that the fullerenes show a greater level of selectivity for microbes over mammalian cells than is observed for **TBO** under the same conditions. Soukos et al. reported that 16 μM **TBO** and red light selectively killed *Streptococcus sanguis* compared to oral keratinocytes and fibroblasts after a 5 min incubation [53].

It is at present uncertain whether the mechanism of microbial inactivation with photoactivated fullerenes involves singlet oxygen (Type 2 mechanism) or superoxide and hydroxyl radicals (Type 1 mechanism). Yamakoshi et al. reported that, while fullerenes were efficient generators of singlet oxygen upon illumination in organic solvents, in biological systems that include reductants, such as NADH, the mechanism shifted to Type 1 [19]. Another consideration is the ability of fullerenes to act as antioxidants or scavengers of ROS in cells [59, 60]. If fullerenes are able both to generate ROS and to scavenge ROS at the same time, it could be asked which process is more important in antimicrobial PDI, since they appear to be competing against one another. Another consideration is the fact that the bis- and tris-functionalized fullerenes were used as mixtures of regioisomers. Hamono et al. [61] have shown that, not only the number of substituents on the fullerene, but also the relative positions of the substituents, may affect the photochemical production of reactive oxygen species. Future work should endeavor to determine which of the possible regioisomers of **BF5** and **BF6** are most active as antimicrobial PS. In conclusion, we have shown that bis- and tris-cationic fullerenes are highly active antimicrobial PS that mediate the destruction of a broad spectrum of microbial classes and show better selectivity for microbes over mammalian cells than **TBO**, a widely used antimicrobial PS. We believe that cationic fullerenes deserve further investigation as antimicrobial PS, particularly in those situations in which red light activation is not important for the light to penetrate deep into tissue.

Significance

The relentless world-wide increase in antibiotic resistance among multiple classes of pathogens has led to a search for alternative antimicrobial therapies. There has coincidentally been a search for biological and medical applications of fullerenes since their discovery 20 years ago, and, more recently, there has been a major effort to uncover biological applications of nanotechnology. For the first time, to our knowledge, we have demonstrated that cationic fullerenes with one, two, or three pyrrolidinium groups, after a short

incubation followed by illumination with white light, have a broad-spectrum antimicrobial activity and can rapidly kill more than 99.99% of bacterial and fungal cells. Although the light absorbance decreases with increased cationic substitution, the increasing positive charge allows the fullerenes to bind to cells and overcome microbial permeability barriers. Cationic fullerenes perform better as antimicrobial photosensitizers than the widely employed antimicrobial photosensitizer toluidine blue O. Cationic fullerene-mediated photodynamic therapy may have a role to play in the treatment of localized infections in such areas as wounds, burns, skin, and mucus membranes.

Experimental Procedures

Synthesis and Characterization of Functionalized Fullerenes

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification unless stated otherwise.

Synthesis of BF1, BF2, and BF3

This synthesis was carried out by using methods previously described in the literature [35, 36], with some modifications, as shown in Figure 1. Serinol (2.05 equivalents) and diethylmalonate (1 equivalent) were reacted at 200°C for 45 min in an open tube. Then, acetic anhydride (4.1 equivalents) and pyridine (4.1 equivalents) were added, and the solution was stirred for 18 hr at room temperature. The product termed MSA thus obtained was recrystallized by using a mixture of hexanes and ethyl acetate.

C₆₀ (99.5% purity) was purchased from SES Research, Houston, TX. C₆₀ (220 mg, 0.31 mmol) was dissolved in toluene (250 ml) by sonicating for 2 hr. The solution was filtered, and nitrogen was purged for 30 min. Then, CBr₄ (46.1 mg, 0.14 mmol) as a solid, MSA (58.2 mg, 0.14 mmol) in acetone (3 ml), and 1,8-diazabicyclo[5.4.0]undec-7-ene (31.7 mg, 0.21 mmol) in toluene (5 ml) were added. The reaction mixture was stirred at room temperature for 4.5 hr under a nitrogen atmosphere. Solvents were removed under vacuum. The product was dissolved in a minimum amount of chloroform and loaded onto a silica gel column (1 in × 9 in) and eluted with dichloromethane containing 0%–2% methanol to collect pure -OH-protected BF1, BF2, and BF3 (order of elution is C₆₀-MSA, C₆₀-MSA₂, and, finally, C₆₀-MSA₃). The protected compounds were characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). C₆₀-MSA, calculated mass 1137.02 and observed mass 1137.56; C₆₀-MSA₂, calculated mass 1553.40 and observed mass 1553.77; C₆₀-MSA₃, calculated mass 1969.78 and observed mass 1970.26. NMR data were obtained for C₆₀(MSA)-protected BF1: ¹H NMR (400 MHz, CDCl₃, TMS ref.) δ (ppm) 2.10 (s, 12H, CH₃), 4.34–4.41 (m, 8H, CH₂), 4.68–4.72 (m, 2H, CH), 7.37 (br d, J = 6.4 Hz, 2H, NH).

Deprotection of -OH groups was achieved by treating C₆₀-MSA_{1–3} with excess amounts of potassium carbonate in methanol and deionized water at room temperature for 90 min. Potassium ions were removed by adding strong cation exchange resin (Biorad AG MP-50W, treated with 1 M HCl) to the reaction mixture until the pH reached 7. The solution was filtered, and solvents were removed to obtain pure BF1, BF2, and BF3.

Synthesis of BF4, BF5, and BF6

This synthesis was carried out by using methods previously described in the literature [37, 38], with some modifications, as shown in Figure 2. The pyrrolidinium group is referred to as NMP in this synthesis. To a C₆₀ solution (described above), sarcosine (50.8 mg, 0.57 mmol) and paraformaldehyde (40.9 mg, 1.36 mmol) for C₆₀-NMP, sarcosine (62.5 mg, 0.70 mmol) and paraformaldehyde (21.5 mg, 0.71 mmol) for C₆₀-NMP₂, or sarcosine (125.0 mg, 1.4 mmol) and paraformaldehyde (43.0 mg, 1.43 mmol) for C₆₀-NMP₃, as solids directly, were added. The reaction mixture was refluxed for 2 hr for C₆₀-NMP and overnight for C₆₀-NMP₂ and C₆₀-NMP₃. Solvents were reduced under vacuum. The solution was loaded onto a silica gel column (1 in × 9 in) packed with toluene and eluted with toluene

containing 0%–5% acetone to collect pure C₆₀-NMP, C₆₀-NMP₂, or C₆₀-NMP₃ with yields of 30%–40%. The purity of the compounds in terms of mono-, bis-, and tris-substitutions was confirmed by thin-layer chromatography (TLC). Methylation of C₆₀-NMP, C₆₀-NMP₂, or C₆₀-NMP₃ was carried out by dissolving the compounds in a large excess of methyl iodide (1 ml per 20 mg C₆₀-NMP_n) and stirring for 48 hr at room temperature (7 days in the case of C₆₀-NMP₃). Pure methylated product BF4, BF5, and BF6 were precipitated by adding hexanes, and the precipitates were collected, washed with toluene and dichloromethane, and dried. The compounds were characterized by electrospray mass spectrometry (ES-MS). BF4, calculated mass 792.08 and observed mass 792.04; BF5, calculated mass 864.16 and observed mass 432.05 (M²⁺); BF6, calculated mass 936.24 and observed mass 312.08 (M³⁺). NMR data were obtained for BF4: ¹H NMR (400 MHz, 2:3 CDCl₃:DMSO-d₆, TMS ref.) δ (ppm) 4.08 (s, 6H, CH₃), 5.72 (s, 4H, CH₂). UV-visible absorption spectra (Figure 3) of the compounds were recorded in 1:9 DMSO:water at a concentration of 10 μM.

Microbial Strains and Culture Conditions

Staphylococcus aureus (ATCC #35556), *Escherichia coli* (ATCC #25922), and *Pseudomonas aeruginosa* (ATCC #BAA-47; PAO1) were cultured in brain-heart infusion (BHI) broth (Difco, BD Diagnostic Systems, Sparks, MD) at 37°C in aerobic conditions in a shaker at 150 rpm. *Candida albicans* (ATCC #18804) was grown in YM broth (Difco). Exponential cultures obtained by reculturing stationary overnight precultures were used for all experiments. *E. coli*, *P. aeruginosa*, and *S. aureus* were grown in fresh medium for approximately 1 hr to a density of 10⁸ cells/ml; the OD values at 650 nm were 0.6, 0.8, and 0.8, respectively. *C. albicans* was grown for approximately 4 hr to an approximate density of 10⁸ cells/ml, corresponding to an OD of 6 at 650 nm (10-fold dilution measured). Cells were used for experiments in the mid-log growth phase.

Photosensitizers and Light Source

Toluidine blue O was purchased from Sigma (St. Louis, MO) and was dissolved in water to give a 1 mM stock solution that was stored in the dark at 4°C for a maximum of 2 weeks. We used a noncoherent lamp with filtered liquid light guides (LumaCare LC122, MBG Technologies, Inc., Newport Beach, CA) to provide illumination. For illumination of fullerenes, we used a broad-band white light band pass filter (400–700 nm), and, for TBO, we used a band pass filter at 620–650 nm. The lamp was adjusted to give a uniform spot of 4 cm diameter with an irradiance of 200 mW/cm², as measured with a power meter (model DMM 199 with 201 Standard head, Coherent, Santa Clara, CA).

Photodynamic Inactivation Studies

We dissolved the fullerenes in DMSO to give 5 mM solutions (compound BF4 was poorly soluble; therefore, the concentration of the stock solution was 2.7 mM) and stored them in the dark at room temperature. In the initial screening experiments, we used suspensions of *S. aureus* cells (10⁸ per ml) incubated with fullerenes BF1–6 at a concentration of 100 μM in PBS at room temperature for 10 min. In subsequent experiments, the bacterial suspension was centrifuged (4000 × g for 10 min) after incubation and resuspended in fresh PBS before illumination; this procedure is referred to as a wash. *E. coli*, *P. aeruginosa*, and *C. albicans* were used at concentrations of 10⁸ cells per ml. Illumination was carried out from above with cell suspensions in wells of a 24-well plate, and aliquots were removed at times corresponding to the delivery of calculated fluences of light. These aliquots were serially diluted in PBS and streaked on square BHI or YM agar plates according to the method of Jett et al. [62]. Survival fractions were calculated with reference to cells incubated in PBS alone, and values on killing curves at 0 J/cm² represent the dark toxicity of the fullerenes. Cells treated with light and no photosensitizer did not show any loss of viability.

Mammalian Cell Culture Experiments

L929 murine fibroblasts (ATCC #CCL1) are a spontaneously transformed immortalized cell line established from the normal subcutaneous areolar and adipose tissue of a male C3H/An mouse [63]. The cells were cultured in Dulbecco's modified Eagle's medium (Sigma) at 37°C in a humidified atmosphere containing 5% CO₂. The medium

was modified by using 4 mM L-glutamine (containing 1.5 g/l sodium bicarbonate and 4.5 g/l glucose), 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were plated in 96-well cell culture plates, at a density of 300 cells/well, and were allowed to attach for 24 hr. Fullerenes or TBO were added at a concentration of 10 µM in 200 µl complete medium per well. After 10 min, fresh medium was added, followed by illumination with white light (for fullerenes) or red light (for TBO). At the completion of the illumination, cells were returned to the incubator for 24 hr. Cell viability was determined by using the MTT-microculture tetrazolium assay, a method of assessing cellular response to PDT [64]. This assay involves the reduction of a colorless substrate (3-[4,5-Dimethylthiazol-2-yl]-diphenyltetrazolium bromide; Sigma) to an insoluble dark-blue formazan product, which is formed in proportion to the amount of succinate dehydrogenase activity in the mitochondria of living cells. After incubation with MTT for periods ranging from 4 to 8 hr, the medium was aspirated off each well, and 100 µl DMSO was added; the absorbance at 570 nm was read by a microplate reader (Spectra Max 340 PC, Molecular Devices, Sunnyvale, CA). The fraction of cells surviving was calculated by dividing the mean absorbances of formazan produced from PDT-treated cells by the mean absorbances from dark controls incubated with PS and kept at room temperature for periods equal to irradiation times.

Statistics

Values are given as means and standard errors of at least six independent wells. Differences between killing curves were tested for significance at the highest comparable fluence by an unpaired two-tailed Student's *t* test, assuming equal or unequal variation in the standard deviations as appropriate. *P* values of less than 0.05 were considered significant.

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