Antimicrobial Photodynamic Therapy with Functionalized Fullerenes:
Quantitative Structure-activity Relationships

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Abstract
Photosensitive dyes or photo sensitizers (PS) in combination with visible light and oxygen produce reactive oxygen species that kill cells in the process known as photodynamic therapy (PDT). Antimicrobial PDT employs PS that is selective for microbial cells and is a new treatment for infections. Most antimicrobial PS is based on tetrapyrrole or phenothiazinium structures that have been synthesized to carry quaternary cationic charges or basic amino groups. However we recently showed that cationic-substituted fullerene derivative were highly effective in killing a broad spectrum of microbial cells after illumination with white light. In the present report we compared a new group of synthetic fullerene derivatives that possessed either basic or quaternary amino groups as antimicrobial PS against Gram-positive (Staphylococcus aureus), Gram-negative bacteria (Escherichia coli) and fungi (Candida albicans). Quantitative structure-function relationships were derived with LogP and hydrophilic lipophilic balance parameters. Compounds with non-quaternary amino groups tended to form nanoeaggregates in water and were only effective against S. aureus. The most important determinant of effectiveness was an increased number of quaternary cationic groups that were widely dispersed around the fullerene cage to minimize aggregation. S. aureus was most susceptible; E. coli was intermediate, while C. albicans was the most resistant species tested. The high effectiveness of antimicrobial PDT with quaternized fullerenes suggest they may have applications in treatment of superficial infections (for instance in wounds and burns) where light penetration into tissue is not problematic.

Keywords: Photodynamic therapy; Quantitative structure-function relationship; Antimicrobial photosensitizer; Cationic charge; Hydrophilic lipophilic balance


Introduction
The relentless worldwide increase in antibiotic resistance amongst bacteria and other infectious microorganisms has led to predictions of untreatable infections caused by “super-bugs” [1] and forecasts of “the end of the antibiotic era” [2]. Many researchers are therefore investigating alternative strategies for destroying microorganisms that are infecting tissue, among which is antimicrobial photodynamic therapy (PDT) [3]. PDT is based upon the concept that photosensitive dyes called photosensitizers (PS) can be excited by visible light of the correct wavelength to a long-lived triplet state that can interact with molecular oxygen by energy transfer (Type II) or by electron transfer (Type I) processes. In the former case the reactive molecule, singlet oxygen is produced and in the latter case, superoxide anion is formed that can then go on to form more reactive oxygen species (ROS) such as hydroxyl radicals [4]. These ROS are highly reactive and are not able to travel far from where they are formed. The goal in antimicrobial PDT is therefore to selectively target the PS to microbial cells rather than to host mammalian cells. It has been found that this can be accomplished by taking into account two findings. Firstly it was discovered that PS with cationic substituents such as quaternary ammonium groups bound to and penetrated the cell walls of prokaryotic Gram-positive and Gram-negative bacteria and also eukaryotic fungal cells [5]. Secondly it was found that this microbial uptake process was rapid compared to the uptake of these PS by host mammalian cells which occurred slowly over time [6]. Therefore if light is delivered soon after applying the PS to the infected area, microbial cells can be killed without causing undue harm to host tissue [7, 8].

It has been known for some years that fullerenes (and in particular soluble functionalized fullerenes) have a high triplet yield and long triplet lifetime and are therefore able to act as PS in PDT [9]. In contrast to porphyrins and other tetrapyrrole based PS that are largely thought to produce singlet oxygen as the chief photoxic species, water soluble fullerenes in the presence of biological reducing agents efficiently produce superoxide [10, 11]. There is some evidence in the literature that this Type I photochemical process is relatively more cytotoxic.

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towards microbial cells than it is toward mammalian cells [12]. We showed that fullerenes that had been functionalized to carry either one, two, or three N-methylpyrrolidinium cationic groups were effective broad-spectrum antimicrobial PS, with the effectiveness increasing as the number of cationic groups increased [13]. In a second publication [14] we investigated six more cationic functionalized fullerenes as antimicrobial photosensitizers and derived quantitative structure-function relationships that showed the most effective compounds had more cationic charges and a lower log P value. In the present report we test a further set of five new functionalized fullerenes for their efficacy as antimicrobial PS and compare them with the most active compound from our previous studies (BB6) [13].

Materials and Methods

Synthesis of fullerenes

The compounds were characterized by 1H and 13C NMR spectroscopy, 500 MHz (Innova500 spectrometer), FTIR (ATR), and mass spectrometry (MALDI-TOF).

BB6: The synthesis and characterization of BB6 has been fully described in a previous publication [13].

BB7: Dimethyl malonate (13.2 g, 10.0 mmol) was condensed with 3-(dimethylamino)-1-propylamine (22.5 g, 22.0 mmol) in 40 mL methanol at reflux for 3 h. Removal of the solvent gave pale yellow oil that crystallized to analytically pure product upon cooling at 4° C. The diamide material was waxy and extremely hygroscopic. MS calculated. For C60H6N6O4+ found 298.1. 1H NMR and 13C NMR (DMSO-d6, TMS ref.) δ = 173.60, 162.86, 154.81, 154.46, 144.30, 144.17, 143.59, 143.45, 143.29, 143.07, 142.91, 142.78, 142.67, 142.60, 142.48, 142.36, 142.27, 141.98, 139.41, 137.80, 81.215, 79.298, 72.050, 69.343, 61.678, 61.154, 58.197, 54.605, 54.605, 50.790, 44.616, 44.024, 43.242, 40.192, 39.981, 39.772, 39.563, 39.354, 39.146, 38.937, 35.884, 30.839, 21.161. Log Kow = -1.721. MS (H2O) calculated for C60H6N6O4 298.4 (M) +, found 298.1.

BB22: C80 (130 mg, 0.18 mmol) was dissolved in 150 mL toluene. Piperazine-2-carboxylic acid dihydrochloride (37.8 mg, 0.19 mmol) in ethanol (10 mL) and triethylamine (200 mL) was added followed by 4-pyridinecarboxaldehyde (34 μL, 0.36 mmol) and the reaction was refluxed for 2 h. The reaction mixture was loaded directly onto a silica gel column and eluted with toluene:acetone:ethanol (20:1:1). The products were characterized by 1H NMR (400 MHz, CDCl3), 13C NMR (DMSO-d6, TMS ref.) δ = 172.09, 162.35, 154.80, 154.45, 152.73, 151.89, 151.34, 148.86, 148.38, 148.06, 147.95, 147.87, 147.31, 147.18, 147.02, 146.82, 145.34, 145.21, 145.10, 144.93, 144.72, 144.55, 144.30, 144.17, 143.59, 143.45, 143.29, 143.07, 142.91, 142.78, 142.67, 142.60, 142.48, 142.36, 142.27, 141.98, 139.41, 137.80, 81.215, 79.298, 72.050, 69.343, 61.678, 61.154, 58.197, 54.605, 54.605, 50.790, 44.616, 44.024, 43.242, 40.192, 39.981, 39.772, 39.563, 39.354, 39.146, 38.937, 35.884, 30.839, 21.161. Log Kow = -1.721. MS (H2O) calculated for C80H8N8O2 298.4 (M) +, found 298.1.

Figure 1: Structures of the six fullerenes tested in the study.
triethylamine (39:10:1) to collect the pure product precursor. The brown material was dissolved in 12 mL CHCl₃ and treated with 5.0 mL CH₃I for 1 h followed by the addition of 10 mg K₂CO₃ and stirring at ambient temperature for 24 h. After filtration, the product was washed with CH₂Cl₂ and H₂O and dried in vacuo to give 16.8 mg of analytically pure compound.

\[ ^1H \text{ NMR (CDCl}_3, 400 MHz) \delta 2.47 (s, 6H), 4.03 (s, 3H), 4.35 (br m, 6H), 4.72 (s, 1H), 5.93 (s, 1H), 8.48 (d, 2H), 9.03 (br s, 2H); \]

\[ ^{13}C \text{ NMR DMSO-d}_6 \text{ 48.04, 48.76, 54.20, 57.27, 57.95, 63.23, 69.40, 71.84, 74.99, 127.8, 129.5, 135.7, 137.51, 137.67, 138.21, 139.93, 140.28, 140.42, 141.93, 142.07, 142.24, 142.47, 142.52, 142.66, 142.95, 143.07, 143.60, 144.68, 144.78, 145.14, 145.59, 145.66, 145.78, 146.05, 146.17, 146.41, 146.55, 146.77, 147.31, 147.53, 147.74, 149.73, 151.71, 152.04, 155.42, 155.83; MS calcd. For C\textsubscript{73}H\textsubscript{21}N\textsubscript{3} 470.0 (M)\textsuperscript{2+}, found 469.6. \]

Cell lines and culture conditions

The following microbial strains were used: *Staphylococcus aureus* (strain 8325-4), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC MY A574). Bacteria were routinely grown on brain heart infusion (BHI) agar and BHI broth at 37 °C and yeasts were routinely grown in yeast extract-peptone-glucose (YPD) broth or YPD agar at 37 °C with shaking at 100 rpm. Bacterial cell numbers were estimated by measuring the OD at 600-nm (OD of 0.5 = 10\(^8\) cells/mL). Yeast cell numbers were assessed with a hematocytometer [19].

Photodynamic inactivation studies and CFU determination

Cells were grown overnight at 37°C. Cells were collected through centrifugation (1000g) for five min then suspended in PBS. A cell suspension consisting of 10\(^8\) cells/mL for bacteria and 10\(^7\) cells/mL for C. *albicans* was incubated with fullerene for 30 min at room temperature in the dark. Different cell densities were used for bacteria and yeast because yeast cells are approximately 10 times larger than bacterial cells [20]. 500 µl aliquots of cell suspension were transferred to a 48 well
plate and fullerenes were added from a DMSO stock solution prepared at 5mM. The highest concentration (for 100 µM fullerene) of DMSO used was 2% and this did not cause any toxicity to the cells. Cells were illuminated at room temperature with a white (400-700 nm) broadband light source (Lumacare, Newport Beach, CA) at 100 mW/cm² for 6.6 minutes to deliver 40 J/cm². At the completion of the illumination period 100 µL aliquots were removed from illuminated and non-illuminated wells (cells incubated with fullerene but kept in 48-well plates covered with aluminum foil at room temperature for the duration of the illumination) and serially diluted 10-fold in PBS to give dilutions of 10⁻¹ to 10⁻⁶ times the original concentrations and 10 µl aliquots of each of the dilutions were streaked horizontally on square BHI or (YPD for Candida) plates by the method of Jett and colleagues [16]. Plates were streaked in triplicate and incubated for 24 h at 37°C (or 30°C for 48 h for Candida) in the dark to allow colony formation.

Controls groups included cells that were not treated with fullerene or light, and cells treated with light but not with fullerene. Survival fractions (SF) were routinely expressed as ratios of CFU of microbial cells treated with light and fullerene or treated with fullerene in the dark, to CFU of microbes treated with neither.

**Fullerene uptake experiments**

Cell suspensions (10⁸/mL for bacteria and 10⁷/mL for yeast) were incubated with fullerene at 100 µM for 30 min at dark, and then centrifuged at 4000g for 5 min. The pellets were re-suspended in 1% sodium dodecyl sulfate (SDS) solution and allowed to completely dissolve over 24 hours. The absorbance spectra of supernatant and pellets were measured separately from 200-700-nm and compared with calibration of curves of solutions of the known concentrations of the particular fullerene alone.

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**Figure 3:** PDI of *E. coli*. Cell suspensions (10⁸/mL) were incubated with specified concentrations of (A) BB6; (B) BB7; (C) BB8; (D) BB9; (E) BB21 and (F) BB22 for 30 min followed by illumination or not with 40 J/cm² of white light.
Quantitative structure-function relationship studies

The software program ACD/Labs V11.01 (Toronto, Canada) was used to calculate the logarithm of the octanol:water partition coefficient (LogP) and the hydrophilic-lipophilic balance (HLB) parameter from the structural formulae. The HLB parameter was introduced by Griffin in 1949 [21, 22] and is defined as HLB = 20 x Mh/M where Mh is the molecular mass of the hydrophilic portion of the molecule, and M is the molecular mass of the whole molecule, giving a result on an arbitrary scale of 0 to 20. An HLB value of 0 corresponds to a completely hydrophobic molecule, and a value of 20 would correspond to a molecule made up completely of hydrophilic components. LD99 values (concentration of fullerene necessary to kill 2 logs of microbial cells after illumination with 40 J/cm² and MTD (concentration of fullerene at which light mediated toxicity began to be evident) were obtained from the PDI curves (Figures 2-4). Linear correlation coefficients (R values) were obtained with Kaleidagraph software (Synergy Software, Reading, PA).

Results

Synthesis of fullerenes

It is well generally well-known that electrophilic C₆₀ will react with nucleophilic amines, especially in neat amine¹ or at elevated temperatures [18, 23, 24]. The reaction mechanism typically involves ion radical intermediates and often results in unexpected products. Several fullerene derivatives that have amine groups present have been reported. However, in all cases, the amine is either separated from the fullerene core by a rigid linker [25, 26], or is only reported in the trifluoroacetate salt form [27, 28]. Notably missing from the literature is a report of a free basic amine attached to the fullerene core with a short, flexible linker. This is presumably due to an interaction between the fullerene and the close proximity amine that results in unexpected

Figure 4: PDI of C. albicans. Cell suspensions (10⁷/mL) were incubated with specified concentrations of (A) BB6; (B) BB7; (C) BB8; (D) BB9; (E) BB21 and (F) BB22 for 30 min followed by illumination or not with 40 J/cm² of white light.
products. In the present case we were unable to obtain satisfactory NMR or mass spectra of BB7, BB8, and BB9 presumably because of this alternative reaction mechanism.

**Antimicrobial PDI experiments**

Figures 2A-2F show the PDI graphs of Gram-positive *S. aureus* with and without illumination with fullerenes added at various concentrations: 0.001 to 1 µM for BB6 (Figure 2A), BB21 (Figure 2E) and BB22 (Figure 2F), and 0.01 to 100 µM for BB7 (Figure 2B), BB8 (Figure 2C) and BB9 (Figure 2D). BB6 and BB21 were clearly the most active compounds giving total elimination of the cells at the low concentration of 1 µM. BB22 also showed high activity with almost 5 logs of killing at 1 µM. BB7 and BB8 had weak to moderate activity with elimination and 6 logs of killing respectively obtained at 100 µM. BB9 was almost inactive with only 1-log of killing obtained at 100 µM. The dark toxicity was almost non-existent.

Figures 3A-3F show the PDI graphs of Gram-negative *E. coli*. Since Gram-negative species are more resistant to PDI than Gram-positive bacteria, we increased the concentration of fullerene compared to that used for gram positive *S. aureus*; to 10µM for BB6 (Figure 3A) and to 100µM for BB21 (Figure 3E) and BB22 (Figure 3F). The order of the effectiveness was BB6 > BB21 = BB22. BB9 (Figure 3D) showed marginal activity while BB7 (Figure 3B) and BB8 (Figure 3C) showed no activity. The biggest difference observed between Gram-positive and Gram-negative species was that BB7 and BB8 were effective against *S. aureus* but ineffective against *E. coli*.

Figures 4A-4F show the PDI graphs of the yeast *C. albicans*. BB6 (Figure 4A) again showed the highest activity eliminating the cells at 100 µM, BB22 (Figure 4F) was next most active and BB21 (Figure 4E) showed moderate activity, while in a pattern similar to that of the Gram-negative bacteria, BB7, BB8 and BB9 were ineffective.

**Fullerene uptake studies**

We initially expected that the active fullerenes would bind to the microbial cells while the inactive compounds would not bind to the cells. Contrary to our expectation we found that after centrifugation the less active fullerenes BB7, BB8 and BB9 were 100% in the pellet and 0% in the supernatant. Closer inspection however revealed that the pellet was in two parts: a colorless bacterial pellet and a deeply colored overlying layer of precipitated fullerene. This finding prompted us to centrifuge solutions of the fullerenes alone in PBS without any microbial cells. The three fullerenes (BB7, BB8 and BB9) completely precipitated after centrifugation at 4000g while the remaining fullerenes (BB6, BB21 and BB22) did not precipitate. It is impossible to say whether the results from the % of fullerenes (BB6, BB21 and BB22) present in the cells and shown in Figure 5 really do represent actual binding to the microbial cells rather than a mixture of cell binding and some precipitation caused by the presence of the microbial cells in the suspension.

**QSAR studies**

We initially plotted LogP against the LD99 values as shown in Figure 6A, 6C and 6D. Although the linear correlation between LogP and LD99 was excellent for *S. aureus* (Figure 6A, R=0.948), the respective linear correlations for *E. coli* (Figure 6C, R=0.189) and for *C. albicans* (Figure 6E, R=0.485) were poor. We then plotted LD99 values against a parameter calculated by subtracting 3X the number of cationic charges from the LogP values (LogP-3X (+/-)). The correlation coefficients thus obtained were much better than using LogP values alone; *S. aureus* gave R=0.978 (Figure 6B), while *E. coli* gave R=0.567 (Figure 6D) and *C. albicans* gave R=0.743 (Figure 6F).

Another parameter used in QSAR studies is HLB and in this case we plotted the parameter against the MTD values (maximal tolerated dose). Figure 7A shows the plot for *S. aureus*, Figure 7B shows the plot for *E. coli* and Figure 7C for *C. albicans*. Again it can be seen the correlation is much better for *S. aureus* than the other two microbial species.

**Discussion**

Fullerenes are becoming increasingly more studied as PS with potential applications in PDT [9]. Fullerenes have certain particular advantages over more traditional PS based on tetrapyrrole and phenothiazinium backbones. They have high absorption coefficients, have a high degree of photostability compared to other classes of PS, and exhibit a photochemical mechanism with a significant contribution of Type I reactive oxygen species especially hydroxyl radicals [10]. Their disadvantages include an absorption spectrum biased towards the blue and green wavelengths rather than the red wavelengths that have good tissue penetration and they tend to have difficulties in being made water-soluble and have a pronounced tendency to aggregate.
The present data provide further confirmation for the hypothesis that antimicrobial PS should have multiple cationic charges. In common with many other PS molecules the C_{60} fullerene molecule has not only a high degree of hydrophobicity but a very pronounced tendency to aggregate in aqueous media. Our previous studies [13, 14] also showed that the primary factor that governs the antimicrobial PDI activity of functionalized fullerenes was the overall number of cationic charges. In the present study the fact that much better correlations were obtained when 3X the number of cationic charges were subtracted from the LogP values emphasizes the importance of the number of cationic charges in determining the efficiency of the fullerenes as antimicrobial PS. Moreover it is apparent that the cationic charges should be distributed around the C_{60} cage for greatest activity. BB6 is composed of a number of unresolvable stereoisomers [13], but it can be assumed that isomers with the three pyrrolidinium groups widely dispersed from each other predominate on steric hindrance grounds. BB6 was more effective than BB22 with two cationic charges grouped closely together and also more effective than BB21 with four cationic charges grouped together around a single 6-membered ring (Figure 1). It is likely that even BB21 and BB22 display some degree of aggregation, although not enough to produce precipitation upon centrifugation.

It is known that fullerenes tend to form so-called nano-aggregates in aqueous solution. In fact there are several reported studies with

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**Figure 6**: QSAR studies with LogP parameter. LD99 values (from Figs 2-4) were plotted against LogP: (A) S. aureus; (C) E. coli; (E) C. albicans. In order to find better correlations LD99 values were then plotted against LogP-3X(#+): (B) S. aureus; (D) E. coli; (F) C. albicans.
pristine C₆₀ in this nano-aggregated form [29-32]. The solutions formed are apparently clear showing the particles have sizes below 200 nm. The fact that they were still able to mediate PDT killing of the Gram-positive S. aureus is interesting. We showed in a previous study [20] that the anionic dye Rose Bengal had no detectable binding to S. aureus, but was nevertheless highly efficient in mediating PDT killing.

In conclusion we have shown that the optimum molecular features necessary to produce broad-spectrum antimicrobial PS from functionalized fullerenes include maximizing the number of cationic charges and distributing these charges around the fullerene cage in order to minimize their tendency to aggregate.

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