Published in final edited form as:

J Nanomed Nanotechnol. 2011 April 1; 2(2): 1–9. doi:10.4172/2157-7439.1000109.

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

Kazue Mizuno^{1,2}, Timur Zhiyentayev^{1,3}, Liyi Huang^{1,4,5}, Sarwat Khalil^{1,6}, Faria Nasim^{1,6}, George P Tegos^{1,4}, Hariprasad Gali⁷, Ashlee Jahnke⁷, Tim Wharton⁷, and Michael R Hamblin^{1,4,6,*}

¹Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA 02114, USA

²Departments of Nuclear Engineering and Management, Graduate School of Engineering, University of Tokyo, Japan

³Chemistry Department, Massachusetts Institute of Technology, Cambridge, MA, USA

⁴Departments of Dermatology, Harvard Medical School, Boston, MA, USA

⁵Department of Infectious Diseases, First Affiliated College & Hospital, Guangxi Medical University, Nanning, China

⁶Aga Khan Medical School, Karachi, Pakistan

⁷Lynntech Inc, College Station, TX, USA

⁸Harvard-MIT Divisions of Health Sciences and Technology, Cambridge, MA, USA

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 nanoaggregates 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.

Copyright: © 2011 Mizuno K, et al.

*Corresponding author: Michael R Hamblin, BAR414, 40 Blossom Street, Massachusetts General Hospital, Boston, MA02114, USA, Fax: 617-726-8566; Hamblin@helix.mgh.harvard.edu.

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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 phototoxic 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 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 logP 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 ^{13}C 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 calcd. For $C_{13}H_{28}O_2N_4$ 272.4, found 273.2 (M + H)⁺. C₆₀ (721 mg) was dissolved in 700 mL toluene. The diamide (138 mg) was dissolved in 30 mL 2:1 acetone: MeOH and added to the fullerene. The solution was degassed under nitrogen and stirred at ambient temperature for 18 h. The precipitated product was collected by centrifugation and washed with excess toluene to give 184 mg. FT-IR (KBr) ν (cm⁻¹) 1705, 1642, 525.

BB8—In a similar manner dimethyl malonate (13.2 g, 10.0 mmol) was condensed with N, N-dimethylethylenediamine (19.0 g, 21.6 mmol) in 40 mL methanol at reflux for 3 h. Removal of the solvent and recrystallization at 20 °C from ethyl acetate/hexanes gave ~10 g of the diamide product as colorless crystals. EI MS calcd. For $C_{11}H_{24}O_2N_4$ 244.3, found 245.2 (M + H)⁺. The Bingel-Hirsch method was used for the modification of C₆₀ [15, 16]. To a solution of 140 mg C₆₀ in 100 mL toluene were added 50.0 mg CBr₄ and 38.6 mg of the diamide compound in 10 mL THF. The solution was degassed under N₂ and stirred at ambient temperature for 18 h. The precipitated product was collected by centrifugation and washed with excess toluene. FT-IR (KBr) ν (cm⁻¹) 2934, 1714, 1647, 526

BB9—C₆₀ (100 mg, 0.14 mmol) and 1, 4, 8, 11-tetraazacyclotetradecane-5, 7-dione (31.7 mg, 0.14 mmol) were dissolved in 100 mL toluene. Carbon tetrabromide (46.0 mg, 0.14 mmol) and 1, 8-diazabicyclo [5.4.0] undec-7-ene (32.2 mg, 0.21 mmol) were added and the reaction mixture was stirred for 20 h at ambient temperature. The precipitated product was collected by centrifugation, rinsed with toluene (2× 50 mL), CH₃OH (30 mL) and CH₂Cl₂ (30 mL) and dried under vacuum. Product yield: 23 mg (17%) brown powder. FT-IR (KBr) ν (cm⁻¹) 2929, 1646, 526.

BB21—C₆₀ (100 mg) was dissolved in 40 mL chlorobenzene. DMSO (10.0 mL) was added followed by bubbling with O₂ for 5 min. 1-methylpiperazine (93 μ L) was added and the solution stirred for 72 h at ambient temperature under one atmosphere of O₂. The chlorobenzene was removed and the orange precipitate in DMSO was collected by centrifugation and washed with acetone. ¹H NMR (400 MHz, CDCl₃) and MS spectra were consistent with those previously reported [17, 18]. The material was methylated in 15 mL CHCl₃ with a large excess (2 mL) of CH₃I for 24 h at ambient temperature. The precipitate was collected by centrifugation and washed 3x with CHCl₃. The product was fully water soluble to give an orange solution. ¹H NMR was consistent with previously published data [17, 18].

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 K_{OW} = -1.721. MS (H₂O) calculated for C₈₄H₅₆N₈O 298.4 (M)⁴⁺, found 298.1.

BB22—C₆₀ (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 μ L, 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 followed by toluene:acetone:triethylamine (39:10:1) to collect the pure product precursor. The brown material was dissolved in 12 mL CHCl_3 and treated with 5.0 mL CH_3I for 1 h followed by the addition of 10 mg K_2CO_3 and stirring at ambient temperature for 24 h. After filtration, the product was washed with CH_2Cl_2 and H_2O and dried *in vacuo* to give 16.8 mg of analytically pure compound. ^1H NMR (CDCl_3 , 400 MHz) δ 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 NMR DMSO-d_6 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 $\text{C}_{73}\text{H}_{21}\text{N}_3$ 470.0 (M) $^{2+}$, 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 hemacytometer [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) broad-band light source (Lumacare, Newport Beach, CA) at 100 mW/cm^2 for 6.6 minutes to deliver 40 J/cm^2 . 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 (10^8 /mL for bacteria and 10^7 /mL for yeast) were incubated with fullerene at 100 μM for 30min 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.

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 $HLB = 20 \times M_h/M$ where M_h 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 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 μ M for BB6 (Figure 2A), BB21 (Figure 2E) and BB22 (Figure 2F), and 0.01 to 100 μ M 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 μ M. BB22 also showed high activity with almost 5 logs of killing at 1 μ M. BB7 and BB8 had weak to moderate activity with elimination and 6 logs of killing respectively obtained at 100 μ M. BB9 was almost inactive with only 1-log of killing obtained at 100 μ M. 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 10 μ M for BB6 (Figure 3A) and to 100 μ M for BB21 (Figure 3E) and BB22 (Figure 3F). The order of the effectiveness was BB6 > BB21 $\approx$ 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 μ M, 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, B21 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 ($\text{LogP}-3X(\#+)$). 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 C₆₀ 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 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 BB7, BB8 and BB9 precipitated after centrifugation, suggests that these compounds were present in aqueous as nano-aggregates. The fact that they were still able to mediate PDI 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 PDI 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.

Abbreviations

MS	Mass Spectrum
FT-IR	Fourier Transform Infrared Spectrum
HLB	Hydrophilic Lipophilic Balance
logP	Logarithm of Octanol: Water Partition Coefficient
NMR	Nuclear Magnetic Resonance Spectrum
PDI	Photodynamic Inactivation
PDT	Photodynamic Therapy
PS	Photosensitizer
ROS	Reactive Oxygen Species

Acknowledgments

This work was funded by the NIH (grants R44AI68400 to Lynntech Inc, and R01AI050875 to MRH).

References

1. Talan DA. MRSA: Deadly Super Bug or Just Another Staph? *Ann Emerg Med.* 2008; 51:299–302. [PubMed: 18222565]
2. Yoshikawa TT. Antimicrobial resistance and aging: beginning of the end of the antibiotic era? *J Am Geriatr Soc.* 2002; 50:226–229.
3. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobiol Sci.* 2004; 3:436–450.

4. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part one-- photosensitizers, photochemistry and cellular localization. *Photodiagn Photodyn Ther.* 2004; 1:279–293.
5. Merchat M, Spikes JD, Bertoloni G, Jori G. Studies on the mechanism of bacteria photosensitization by meso-substituted cationic porphyrins. *J Photochem Photobiol B.* 1996; 35:149–157. [PubMed: 8933721]
6. Soncin M, Fabris C, Buseti A, Dei D, Nistri D, et al. Approaches to selectivity in the Zn(II)-phthalocyanine-photosensitized inactivation of wild-type and antibiotic-resistant *Staphylococcus aureus*. *Photochem Photobiol Sci.* 2002; 1:815–819. [PubMed: 12656484]
7. Dai T, Huang YY, Hamblin MR. Photodynamic therapy for localized infections-State of the art. *Photodiagnosis Photodyn Ther.* 2009; 6:170–188. [PubMed: 19932449]
8. Hamblin MR, Dai T. Can surgical site infections be treated by photodynamic therapy? *Photodiagnosis Photodyn Ther.* 2010; 7:134–136. [PubMed: 20510311]
9. Mroz P, Tegos GP, Gali H, Wharton T, Sarna T, et al. Photodynamic therapy with fullerenes. *Photochem Photobiol Sci.* 2007; 6:1139–1149. [PubMed: 17973044]
10. Mroz P, Pawlak A, Satti M, Lee H, Wharton T, et al. Functionalized fullerenes mediate photodynamic killing of cancer cells: Type I versus Type II photochemical mechanism. *Free Radic Biol Med.* 2007; 43:711–719. [PubMed: 17664135]
11. Yamakoshi Y, Umezawa N, Ryu A, Arakane K, Miyata N, et al. Active oxygen species generated from photoexcited fullerene (C60) as potential medicines: O₂ versus 1O₂. *J Am Chem Soc.* 2003; 125:12803–12809. [PubMed: 14558828]
12. Martin JP, Logsdon N. Oxygen radicals are generated by dye-mediated intracellular photooxidations: a role for superoxide in photodynamic effects. *Arch Biochem Biophys.* 1987; 256:39–49. [PubMed: 3038028]
13. Tegos GP, Demidova TN, Arcila-Lopez D, Lee H, Wharton T, et al. Cationic fullerenes are effective and selective antimicrobial photosensitizers. *Chem Biol.* 2005; 12:1127–1135. [PubMed: 16242655]
14. Huang L, Terakawa M, Zhiyentayev T, Huang YY, Sawayama Y, et al. Innovative cationic fullerenes as broad-spectrum light-activated antimicrobials. *Nanomedicine.* 2009; 6:442–452. [PubMed: 19914400]
15. Wharton T, Wilson LJ. Highly-iodinated fullerene as a contrast agent for X-ray imaging. *Bioorg Med Chem.* 2002; 10:3545–3554. [PubMed: 12213469]
16. Camps X, Hirsch A. Efficient cyclopropanation of C-60 starting from malonates. *J. Chem Soc Perkin Trans I.* 1997:1595–1596.
17. Isobe H, Nakanishi W, Tomita N, Jinna S, Okayama H, et al. Gene Delivery by Aminofullerenes: Structural Requirements for Efficient Transfection. *Chem Asian J.* 2006; 1:167–175. [PubMed: 17441052]
18. Isobe H, Tomita N, Nakamura E. One-step Multiple Addition of Amine to [60]Fullerene. Synthesis of Tetra(amino)fullerene Epoxide under Photochemical Aerobic Conditions. *Org Lett.* 2000; 2:3663–3665. [PubMed: 11073670]
19. Sherman F. Getting started with yeast. *Methods Enzymol.* 1991; 194:3–21. [PubMed: 2005794]
20. Demidova TN, Hamblin MR. Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. *Antimicrob. Agents Chemother.* 2005; 49:2329–2335. [PubMed: 15917529]
21. Griffin WC. Classification of Surface-Active Agents by HLB. *J Soc Cosmetic Chem.* 1949; 1:311.
22. Griffin WC. Calculation of HLB Values of Non-Ionic Surfactants. *J Soc Cosmetic Chem.* 1954; 5:259.
23. Hirsch, A.; Brettreich, M. *Fullerenes: Chemistry and Reactions.* Weinheim, Germany: Wiley-VCH Verlag GmbH & Co KGaA; 2005.
24. Wang G, Chen X, Cheng X. Unexpected Reactions of [60]Fullerene Involving Tertiary Amines and Insight into the Reaction Mechanisms. *Chemistry.* 2006
25. Illescas BM, Martínez-Alvarez R, Fernández-Gadea J, Martín N. Synthesis of Water Soluble Fulleropyrrolidines Bearing Biologically Active Arylpiperazines. *Tetrahedron.* 2003; 59:6569–6577.

26. Li H, Haque SA, Kitaygorodskiy A, Meziana MJ, Torres-Castillo M, et al. Alternatively Modified Bingel Reacton for Efficient Syntheses of C₆₀ Hexakis-Adducts. *Org Lett*. 2006; 8:5641–5643. [PubMed: 17107092]
27. Garlaschelli L, Pasini D, Spiaggia F. Synthesis and Solubility Properties of Methanofullerenes Containing Primary Ammonium Ion Functionalities. *Eur J Org Chem*. 2005; 20:4322–4327.
28. Richardson CF, Schuster DI, Wilson SR. Synthesis and characterization of water-soluble amino fullerene derivatives. *Org Lett*. 2000; 2:1011–1014. [PubMed: 10804541]
29. Chen Z, Westerhoff P, Herckes P. Quantification of C₆₀ fullerene concentrations in water. *Environ Toxicol Chem*. 2008; 27:1852–1859. [PubMed: 19086313]
30. Fortner JD, Kim DI, Boyd AM, Falkner JC, Moran S, et al. Reaction of water-stable C₆₀ aggregates with ozone. *Environ Sci Technol*. 2007; 41:7497–7502. [PubMed: 18044532]
31. Ma XBouchard D. Formation of aqueous suspensions of fullerenes. *Environ Sci Technol*. 2009; 43:330–336. [PubMed: 19238960]
32. Zhang B, Cho M, Hughes JB, Kim JH. Translocation of C(60) from aqueous stable colloidal aggregates into surfactant micelles. *Environ Sci Technol*. 2009; 43:9124–9129. [PubMed: 19928758]

Figure 1.
Structures of the six fullerenes tested in the study.

Figure 2.

PDI of *S. aureus*. Cell suspensions ($10^8/\text{mL}$) 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 $40 \text{ J}/\text{cm}^2$ of white light.

Figure 3.

PDI of *E. coli*. Cell suspensions ($10^8/\text{mL}$) 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 $40 \text{ J}/\text{cm}^2$ of white light.

Figure 4.

PDI of *C. albicans*. Cell suspensions ($10^7/\text{mL}$) 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 $40 \text{ J}/\text{cm}^2$ of white light.

Figure 5.

Percentage of fullerenes in cell pellet. Cell suspensions ($10^8/\text{mL}$ for bacteria, $10^7/\text{mL}$ for yeast) were incubated with fullerene at $100\ \mu\text{M}$ for 30 min followed by centrifugation for 5 min at $4000g$. Pellets and supernatants were dissolved in SDS and absorption spectra compared with calibration curves.

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*.

Figure 7. QSAR studies with HLB parameter. MTD values (from Figs 2–4) were plotted against HLB: (A) *S. aureus*; (B) *E. coli*; (C) *C. albicans*.