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Decacationic [70]Fullerene Approach for Efficient Photokilling of Infectious Bacteria and Cancer Cells

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Abstract

Photodynamic inactivation of pathogenic bacteria and cancer cells by novel water-soluble decacationic fullerene monoadducts, C₆₀[>M(C₃N₆⁺C₃)₂] and C₇₀[>M(C₃N₆⁺C₃)₂], were investigated. In the presence of a high number of electron-donating iodide anions as parts of quaternary ammonium salts in the arm region, we found that C₇₀[>M(C₃N₆⁺C₃)₂] produced more highly reactive HO[•] radical than C₆₀[>M(C₃N₆⁺C₃)₂], in addition to singlet oxygen (¹O₂). This finding offers an explanation of the preferential killing of Gram-positive and Gram-negative bacteria by C₆₀[>M(C₃N₆⁺C₃)₂] and C₇₀[>M(C₃N₆⁺C₃)₂], respectively. The hypothesis is that ¹O₂ can diffuse more easily into porous cell walls of Gram-positive bacteria to reach sensitive sites, while the less permeable Gram-negative bacterial cell wall needs the more reactive HO[•] to cause real damage.

Introduction

Broad-spectrum one-photon based photodynamic therapy (1 γ -PDT) mediated killing of pathogenic Gram-positive (*e.g.* *Staphylococcus aureus*) and Gram-negative (*e.g.* *Escherichia coli*) bacterial targets using a conventionally accessible light source is an emerging medical approach to treat infectious diseases, especially, those caused by multi-antibiotic-resistant bacteria (1–5). The efficacy of 1 γ -PDT depends on several parameters including the photophysical characteristics of the photosensitizer, the ability of the photosensitizer to target bacteria, the method of administration, and the availability of an appropriate light source. Fullerenes are highly photostable molecules suitable for single-dose multiple-treatments applications. Nearly quantitative efficiency of intersystem crossing from the excited singlet state of [60]fullerene (¹C₆₀^{*}) to its triplet excited state (³C₆₀^{*}) readily allows intermolecular triplet energy transfer from ³C₆₀^{*} to molecular oxygen leading to the production of singlet oxygen (¹O₂) (6,7), which is highly reactive toward biological

substrates producing subsequent cell damage. This photochemical mechanism serves as the basis of photodynamic cytotoxicity against pathogenic microorganisms, including multiply antibiotic-resistant bacteria. However, chemical functionalization of C_{60} is necessary to enhance its solubility in water. In general, attachment of multiple hydroxyl, carboxylic acid, and glycolic oxide addend groups may serve the purpose. Water-solubility of these derivatives increases as the number of hydrophilic groups increases, whether these functional groups are located in either the same addend group or different addend moieties. The latter case leads to the synthesis of fullereryl multiadducts that may change significantly the molecular orbital configuration of the fullerene cage and, thus, its HOMO-LUMO energy gap level and effectiveness in the production of 1O_2 . The 1γ -PDT efficiency can be optimized by performing only a limited number of addition reactions to the fullereryl olefinic bonds to preserve the low HOMO-LUMO energy gap level of the cage. This restriction suggests that [60]fullerene monoadducts using hydrophilic or amphiphilic groups would be suitable candidates. However, since only one addend group is able to be attached to the cage in a monoadducts, sufficient hydrophilicity is required to allow compatibility of the resulting derivative with water.

It has been demonstrated that polycationic photosensitizers exhibited high activity as 1γ -PDT agents for targeting and photokilling against both Gram-positive and Gram-negative bacterial species (8,9). These reported findings revealed the importance of cell surface interactions between multicationic drug molecules and anionic peptide residues in the cell wall. Specifically, several factors including differences in physiology, cell wall, and cytoplasmic membrane structures between Gram-positive and Gram-negative bacteria (10–12) affect the properties of particular functional groups to be attached on the fullerene cage to allow effective targeting selectivity, drug-delivery, and photodynamic inactivation.

In this paper, we describe a rational linkage of water-soluble quaternary alkylammonium multi-salts and ester-amide functional groups into a well-defined decacationic malonate together with an efficient synthetic method for its attachment on either a C_{60} or C_{70} nanocage. The synthesis led to the preparation of new amphiphilic $C_{60}[>M(C_3N_6^+C_3)_2]$ (**1**) or $C_{70}[>M(C_3N_6^+C_3)_2]$ (**2**) nano-photosensitizers, respectively. In these structures, arm moieties each bearing a high number of cationic charges and an amide moiety, capable of inducing the H-bonding in the vicinity of the fullerene cage, are designed for cell-targeting. Accordingly, they may allow us to study specifically the feasibility as nano-PDT agents toward both Gram-positive and Gram-negative bacterial species. The approach also gives a systematic structure–function relationship of fullerene monoadducts on the influence of their biological activity, aiming to achieve the optimized photodynamic activation and enhancement of the PDT efficiency.

Results and Discussion

Surface binding interactions of fullerene derivatives with $-D$ -Ala- D -Ala residues of the bacteria cell wall may be increased by the incorporation of polar moieties on C_{60}/C_{70} capable of inducing multiple H-bondings and static charge interactions with amino acid functional residues. Based on this consideration, we applied a malonate precursor arm, as that shown in the structure of $C_{60}[>M(C_3N_6^+C_3)_2]$ (**1**) and $C_{70}[>M(C_3N_6^+C_3)_2]$ (**2**) (Figure 1), to include two esters and two amide moieties for a sufficient number of carbonyl and $-NH$ groups in a short length of ~ 20 Å for making effective multi-binding sites. More importantly, the use of a well-defined water-soluble pentacationic moiety $N_6^+C_3$ at each side of the arm may increase the crucial select-targeting specificity on the bacterial cell wall. Thus, $N_6^+C_3$ become a common synthon for the structural modification of PDT nanomedicines. It can be derived from the quarternization of N,N',N,N,N,N -hexapropyl-hexa(aminoethyl)amine precursor N_6C_3 . Attachment of $N_6^+C_3$ to two ester moieties of a

malonate forms two extended bipolar pentacationic side-arms capable of forming strong static-charge binding interactions to anionic carboxylate end-groups of the terminal *D*-Ala-*D*-Ala dipeptide of the bacterial cell wall.

The light-induced PDT efficiency can be correlated partially to optical absorption extinction coefficients of the PS in the wavelength range applied. Accordingly, we studied UV-vis spectra of $C_{60}[>M(C_3N_6^+C_3)_2]$ and $C_{70}[>M(C_3N_6^+C_3)_2]$ in DMA at the concentration of 1.0×10^{-5} M, as shown in Figure 2. It displayed a higher total visible absorption of **2** than that of the C_{60} analogue **1** in a ratio of 1.99 ($\varepsilon_2/\varepsilon_1$) over 400–700 nm. However, an opposite relationship was found in the UVA region (320–400 nm) giving a $\varepsilon_2/\varepsilon_1$ ratio of 0.79, indicating a stronger optical absorption of C_{60} monoadduct cage in these wavelength range. To correlate the light absorption efficiency of **2** under PDT experimental conditions, we compared its visible spectrum profile taken in DMAc-H₂O (1:19) to that in DMA and found a nearly identical ε value over the range of 400–700 nm. Photostability of $C_{60}[>M(C_3N_6^+C_3)_2]$ and $C_{70}[>M(C_3N_6^+C_3)_2]$ in aqueous solution was evaluated under a constant irradiation using a LED light source equipped with a fiber optical lens (200 mW/cm² at 400 ± 5 nm) over a period of 5.0 h. Consequently, high retention of a UV-vis absorption profile of fullerene derivatives was observed.

ROS Probe Experiments

Illumination of C_{60} or C_{70} monoadduct derivatives with visible or UVA light promotes its transition to a long-lived triplet excited state, via intersystem crossing processes, and subsequent intermolecular energy transfer to the molecular oxygen yielding a highly reactive singlet oxygen (1O_2) in Type-II reactions (6). In the presence of physiological concentration of reductants, such as NADH, more reactive ROS species, such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^\bullet$), may be produced in polar solvent and water (7). In this study, a comparative singlet oxygen production efficiency study of the compounds **1** and **2** in DMSO-H₂O (1:520, 1.0×10^{-6} M) was carried out by many incremental exposures of the sample to a UVitron visible-enhanced lamp (200 W, 250–600 nm with λ_{max} at 420 nm) in an exposure interval period of 5.0 sec (the light dose measured to be ~ 5.0 mJ/cm² per period) followed by the fluorescence emission measurement of the probe. For highly efficient *in situ* selective trapping of singlet oxygen, we applied water-compatible 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMA, 8.0×10^{-6} M) as a fluorescent probe which exhibits the emission maximum at 429 nm. The corresponding most effective excitation wavelengths were found to be 379 and 400 nm. Accordingly, the later one closer to the visible range was used. At this wavelength, the optical absorption extinction coefficient value of **2** is slightly higher than that of **1**. The chemical trapping of 1O_2 by highly fluorescent ABMA leads to the formation of non-fluorescent 9,10-endoperoxide product. The conversion allows us to follow the loss of fluorescence emission intensity at 429 nm for its correlation to the proportional quantity of 1O_2 produced due to the high kinetic rate constant of the trapping reaction in aqueous media (13), assuming the internal decay of 1O_2 in the solvent system is identical for both experiments of **1** and **2**. As a result, we observed a slightly higher 1O_2 production rate of $C_{60}[>M(C_3N_6^+C_3)_2]$, even though the difference is not statistically significant. The use of a lower irradiation dose for the similar measurement directly in the UV-vis-NIR spectrometer, operated at λ_{ex} 400 nm (single wavelength excitation) with the monochromator, gave a much slower production rate of 1O_2 for both cases that displayed a clearly higher rate for $C_{60}[>M(C_3N_6^+C_3)_2]$ at this excitation wavelength.

A similar trend was also confirmed by using 1O_2 probe singlet oxygen sensor green (SOG) reagent. Upon the trapping of 1O_2 by the anthracene moiety of SOG, the resulting 9,10-endoperoxide-linked fluorescein product SOG-EP is highly green fluorescent (excitation/emission maxima $\sim 504/525$ nm) that can be used for the correlation of relative 1O_2 quantity

produced. It is worthwhile to mention that the SOG probe chemistry is potentially more complicated than the statement above due to the fact that both SOG and SOG-EP are capable of sensitizing $^1\text{O}_2$ production and undergoing photobleaching processes themselves, resembling those of fluorescein derivatives (14). This complication can be lessened by using blue light for fullerene excitation that is not absorbed by the probe. However, if we assume the amount of the additional $^1\text{O}_2$ molecules derived from SOG and SOG-EP and the photobleaching rate being nearly identical among experiments under the same conditions, the fluorescence emission intensity of SOG-EP detected will still be valuable for the relative comparison purpose. Accordingly, Figure 3a showed the results from the SOG-based fluorescence probe assay experiments of **1** and **2** in 96-well black-sided plates under incremental irradiation with a blue LED light source (415 ± 15 nm) under the total fluence intensity range of 0–6.0 J/cm². It is evident that $\text{C}_{60}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ gave a significantly higher rate of $^1\text{O}_2$ detected than that of $\text{C}_{70}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$.

Indeed, under the similar experimental procedures relevant to PDT as those used for the data collection of Figure 3a, but with the application of the fluorescent probe of 3'-p-(hydroxyphenyl)-fluorescein (HPF) (15) instead, a reverse relationship was observed with higher fluorescence emission intensity of fluorescein for $\text{C}_{70}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ than that for $\text{C}_{60}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ under the total fluence intensity range of 0–5.0 J/cm², as shown in Figure 3b. Since the HPF probe is an essential tool for the selective detection of HO• and peroxynitrite, via quinone formation detached from the fluorescein moiety (excitation/emission maxima ~490/515 nm), with the detection sensitivity reported to be roughly 145- and 90-folds higher for HO• than $^1\text{O}_2$ and $\text{O}_2^{\cdot-}$, respectively (15), the measured fluorescence intensity can be correlated roughly to the yield of hydroxyl radicals produced. Besides, $\text{O}_2^{\cdot-}$ is the precursor species to the formation of HO•, directly monitoring the presence of HO• will also serve the confirmation purpose of superoxide radical formation, i.e. higher yield for $\text{C}_{70}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ than $\text{C}_{60}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$.

It is well-recognized that $^1\text{O}_2$ can give rise to several other types of ROS, including $\text{O}_2^{\cdot-}$, H_2O_2 , and HO•, in a sequential reaction with iodide anion (I^-) (16). In this case, the yield of $\text{O}_2^{\cdot-}$ or HO• should be proportional to the quantity of $^1\text{O}_2$. Therefore, our observation of a higher $^1\text{O}_2$ production yield of $\text{C}_{60}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ in PBS solution (Figure 4a) giving a lower production yield of radical species (Fig. 4b) revealed no direct correlation of $^1\text{O}_2$ to $\text{O}_2^{\cdot-}$ or HO• yields, or the latter species may not derive directly from the former one in these experiments. Contrarily, they may be in a competitive production process to each other in either the triplet energy-transfer (Type-II) or electron-transfer (Type-I) mechanism involving the electron-donating iodide anion (I^-) and the electron-accepting fullerene cage. This mechanistic path can be realized by the facile photoexcitation of $\text{C}_{60}/\text{C}_{70}$ cages to their singlet excited state followed by the quantitative intersystem crossing to their corresponding excited triplet transient states, $^3\text{C}_{60}^*[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ and $^3\text{C}_{70}^*[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$. Apparently, the latter molecule exhibited a higher electron-accepting capability to allow electron-transfer from the iodide anion leading to the formation of $\text{C}_{70}^{\cdot-}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ prior to the further transfer this electron to O_2 that gave a higher yield of $\text{O}_2^{\cdot-}$ and, thus, HO•.

Antimicrobial Effect of Fullerene-mediated PDT

Two highly water-soluble decacationic fullerenes **1** and **2** were first applied for comparison in the PDT-killing of the Gram-positive *S. aureus*. In general, it is known that Gram-positive bacteria are more sensitive and susceptible to PDT destruction than Gram-negative bacteria. This allowed us to use a lower concentration (up to 10 μM) of fullerene derivatives for *S. aureus* killing than that (up to 100 μM) needed for effective *E. coli* killing. Our previous PDT experiments have indicated a fast binding process of PS to microbial cells (17). Thus, a

short incubation time of 30 min was applied for both species. As a result shown in Figure 4a, neither decacationic fullerene derivative **1** nor **2** gave any detectable toxicity to both bacteria in the dark in a concentration up to 10 μM . A pronounced PDT effect was observed after illumination on the **1/2**-incubated cells using a broad-band white light source with total fluence intensity of 100 J/cm^2 with a sharp cytotoxicity trend even at low fullerene concentration of 1.0 μM , giving 3 logs killing for $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ and 2 logs killing for $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$. As the administered dose increased to a concentration of 3.0 μM , a 5-log killing effectiveness was observed for the compound **1** and a corresponding 3-log for the compound **2**. Interestingly, $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ was able to eradicate the cells at higher concentrations than 3.0 μM . In the case of $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$, a 5-log effective killing was achieved at a concentration of 10 μM . Two killing curves of **1** and **2** were significantly different from each other ($p < 0.01$).

In the PDT experiments against *E. coli*, the need to use 8-times higher concentrations of fullerene drugs led to the observation of some dark toxicity (1–2 logs) at the higher concentrations of 60 and 80 μM . Surprisingly, the order of PDT effectiveness of these two fullerene drugs was opposite to that seen with *S. aureus*. In this case, $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ was most effective for *E. coli* killing with 3 logs at 40 μM , 5 logs at 60 μM , and eradicating the cells at 80 μM , as shown in Figure 4b. On the other hand, $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ only killed 1 log at 40 μM , 2 logs at 60 μM and 3 logs at 80 μM . These two curves were significantly different ($p < 0.01$).

Anti-cancer Effect of Fullerene-mediated PDT

A longer incubation period of 24 h was applied for the experiments with cancer cells owing to their relatively slow process to uptake fullerene derivatives. Furthermore as it appears that cancer cells are more sensitive to the dark toxicity effects of both fullerene derivatives and DMA organic solvent, we kept the concentration to a single low value of 2.0 μM . The irradiation was made by a white light source (400–700 nm,) delivered at the intensity of 100 mW/cm^2 with a variable fluence dose of 0, 10, 20, 40, and 80 J/cm^2 for giving the demonstration of a light-dose dependence of cell killing. As depicted in Figure 5, $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ was apparently more effective at cancer-cell killing than $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$, with 1 log of cells killing at 40 J/cm^2 and 2 logs at 80 J/cm^2 . Since the MTT assay cannot reliably measure more than 2 logs of killing, therefore, the latter light-dose effectiveness is equivalent to eradication. By contrast, the fullerene drug **1** killed less than 1 log at all fluences up to 80 J/cm^2 . These two curves were significantly different ($p < 0.05$ –0.001).

Conclusion

Chemical functionalization of C_{60} and C_{70} cages with the attachment of a well-defined decacationic malonate ester addend led to a series of novel water-soluble decaiodide monoadducts $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ and $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$. They were applied as a PS and potential nano-PDT agent against pathological bacteria and cancer cells. In the presence of a high number of electron-donating iodide anions as parts of quaternary ammonium salts in the arm region, we found that $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ produced more $\text{HO}^\bullet$ than $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$, in addition to $^1\text{O}_2$. Both ROS were detected using several fluorescence probes. This finding offers an explanation of the preferential killing of Gram-positive and Gram-negative bacteria by $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ and $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$, respectively. The data are consistent with the hypothesis that $^1\text{O}_2$ can diffuse more easily into porous cell walls of Gram-positive bacteria to reach sensitive sites, while the less permeable Gram-negative bacterial cell wall needs the more reactive $\text{HO}^\bullet$ to cause real damage. Since the production rate of $^1\text{O}_2$ was detected in a similar range for **1** and **2** in the absence of PBS, the

differences shown in the presence of PBS electrolyte salts led to our suggestion that the observed production of $\text{HO}^\bullet$ may arise partially from the photoinduced electron-transfer (Type-I) mechanism involving the electron-donating iodide anions (I^-) and the fullerene cages at their excited triplet transient states, $^3\text{C}_{60}^*[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ and $^3\text{C}_{70}^*[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ having different electron-accepting strength. The presence of electrolyte salts makes the ion exchange and I^- -dissociation possible and increases the efficiency of electron transport that forms the corresponding $\text{C}_{60}^{\bullet-}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ and $\text{C}_{70}^{\bullet-}[\text{>M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$, respectively, as the source for the $\text{O}_2^{\bullet-}$ and the derived ROS radicals generation.

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Figure 1.

The structure of decacationic C₆₀[>M(C₃N₆⁺C₃)₂] and C₇₀[>M(C₃N₆⁺C₃)₂] each showing two well-defined pentacationic moieties N₆⁺C₃ at each side of the water-soluble addend.

Figure 2. UV-vis spectra of (a) $C_{60}[>M(C_3N_6^+C_3)_2]$ and (b) $C_{70}[>M(C_3N_6^+C_3)_2]$ in DMA with a concentration of 1.0×10^{-5} M.

Figure 3.

Activation of fluorescence probes for detecting the ROS generation by **1** and **2** (5.0 μM in each well) using different fluorescent probes (a) SOG (5.0 μM) and (b) HPF (5.0 μM), followed by delivery of the stated fluence of blue light.

Figure 4. PDT killing of (a) Gram-positive bacteria, *S. aureus* and (b) Gram-negative bacteria, *E. coli*. Bacteria [$10(8)$ cells/mL] were incubated with stated concentration of $\text{C}_{60}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ **1** or $\text{C}_{70}[>\text{M}(\text{C}_3\text{N}_6^+\text{C}_3)_2]$ **2** for 30 min followed by delivery or not of 100 J/cm^2 of white light. ** $P < 0.01$.

Figure 5. PDT killing of human cancer cells. HeLa cells were incubated with 2.0 μ M of $C_{60}[>M(C_3N_6^+C_3)_2]$ **1** or $C_{70}[>M(C_3N_6^+C_3)_2]$ **2** for 24 h, followed by delivery of stated fluence of white light, returned to incubator for 24 h, and then carried out the MTT assay. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.