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Original article

Synthesis, properties and photodynamic inactivation of *Escherichia coli* by novel cationic fullerene C_{60} derivatives

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Abstract

A novel *N*,*N*-dimethyl-2-(4′-*N*,*N*,*N*-trimethylaminophenyl)fulleropyrrolidinium iodide (DTC_{60}^{2+}) has been synthesized by 1,3-dipolar cycloaddition using 4-(*N*,*N*-dimethylamino) benzaldehyde, *N*-methylglycine and fullerene C_{60}. This approach produced an *N*-methyl-2-(4′-*N*,*N*-dimethylaminophenyl)fulleropyrrolidine with 38% yield. Exhaustive methylation of this fullerene derivative with methyl iodide yielded 95% of dicationic DTC_{60}^{2+}. The spectroscopic and photodynamic properties of the DTC_{60}^{2+} were compared with a non-charged *N*-methyl-2-(4′-acetamidophenyl)fulleropyrrolidine (MAC_{60}) and a monocationic *N*,*N*-dimethyl-2-(4′-acetamidophenyl)fulleropyrrolidinium iodide (DAC_{60}^{+}). The dicationic DTC_{60}^{2+} is essentially aggregated in solution of different solvents and it is partially dissolved as monomer in benzene/benzyl-*n*-hexadecyldimethyl ammonium chloride (BHDC) 0.1 M/water ($W_0 = 10$) reverse micelles. The singlet molecular oxygen, O_2 ($^1\Delta_g$), production was evaluated using 1,3-diphenylisobenzofuran. The photodynamic effect was strongly dependent on the medium, diminishes when the sensitizer is aggregated and increases in an appropriately surrounded microenvironment. The photodynamic inactivation produced by these fullerene derivatives was investigated *in vitro* on a typical Gram-negative bacterium, *Escherichia coli*. Photosensitized inactivation of *E. coli* cellular suspensions by DTC_{60}^{2+} exhibits a ~3.5 log decrease of cell survival (99.97% of cellular inactivation), when the cultures are treated with 1 μM of sensitizer and irradiated for 30 min. This photosensitized inactivation remains high even after one washing step. Also, the photodynamic activity was confirmed by growth delay of *E. coli* cultures. The growth was arrested when *E. coli* was exposed to 2 μM of cationic fullerene and irradiated, whereas a negligible effect was found for the non-charged MAC_{60}. These studies indicate that dicationic DTC_{60}^{2+} is an interesting agent with potential applications in photodynamic inactivation of bacteria.

Keywords: Fullerene; Photosensitizer; Photodynamic inactivation; Bacteria; *Escherichia coli*

1. Introduction

Chemical and physical features of fullerene C_{60}, together with its spherical shape, have aroused the hope of successful use in many different fields either in biological or material chemistry [1,2]. The condensed aromatic rings present in the compound lead to an extended π conjugation of molecular orbitals causing significant absorption of visible light. The facile electron acceptability of up to six electrons makes them good candidates as electron acceptors [3,4]. Also, fullerene compounds have avid reactivity with free radicals. Potential biological activities of fullerenes have been investigated with the aim of using it in the field of medicine [5–7]. An important inconvenience for this application is the low solubility of fullerenes in polar solvents and the consequent formation of aggregates in aqueous solutions [1]. However, the development of covalent chemistry of C_{60} has opened the possibility to attach this spherical structure with several groups, which allows increment in the biological activity [1,3,8].

The emergence of antibiotic resistance amongst pathogenic bacteria has led to a major research effort to find alternative antibacterial therapies [9]. Bacterial photodynamic inactivation

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(PDI) is based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. The subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms [10,11]. Different oxidative mechanisms can occur after photoactivation of the photosensitizer. In the type I photochemical reaction, the photosensitizer interacts with a biomolecule to produce free radicals, while in the type II mechanism, singlet molecular oxygen, $O_2(^1\Delta_g)$, is produced as the main species responsible for cell inactivation [12,13]. Photoexcited fullerenes react with various electron donors to give the C_{60} radical anion ($C_{60}^{\bullet-}$) via type I electron-transfer pathway. Also, reduced active oxygen species, such as superoxide ($O_2^{\bullet-}$) can be generated by electron transfer from $C_{60}^{\bullet-}$ to molecular oxygen [14]. A variety of photosensitizers have shown to be effective to inactivate Gram-positive bacteria. However, Gram-negative bacteria exhibit a remarkable resistance to negatively charged or neutral agents [15,16]. This resistance has been ascribed to the presence of highly organized outer membrane, which hinders the interaction of the photosensitizer with the cytoplasmic membrane and intercepts the photogenerated reactive species [17–20]. In general, photosensitizers containing cationic groups produce direct photoinactivation of Gram-negative bacteria even in the absence of additives [21–27].

Fullerenes have a long lifetime of triplet excited state to produce efficiently $O_2(^1\Delta_g)$ [28]. Therefore, they are interesting candidates for use in photosensitization processes, especially in situations where absorption in the red part of the spectra is not required. According to its photochemical properties, fullerene has been proposed for PDI of viruses in biological fluids [29]. Recently, cationic fullerene derivatives have shown interesting applications against the HIV strains [30]. Furthermore, cationic fullerenes bearing quaternary pyrrolidinium groups have been evaluated as effective antimicrobial photosensitizers [31].

In previous works, we have investigated the photodynamic activity of porphyrins with different number of cationic charges *in vitro* as agents to eradicate Gram-negative bacteria [32,33]. Amphiphilic porphyrin derivatives showed to be active photosensitizers to inactivate *Escherichia coli* cells. Also, we have recently used for the first time a porphyrin–fullerene dyad for the photodynamic inactivation of tumoral cells [34,35]. The results showed that molecular dyads, which can form photoinduced charge-separated state, offer a promising molecular architecture for photosensitizing agents that can produce cellular inactivation still under anoxic condition.

In the present work, we have synthesized a novel *N*,*N*-dimethyl-2-(4′-*N*,*N*,*N*-trimethylaminophenyl)fulleropyrrolidinium iodide (DTC_{60}^{2+}) bearing two cationic groups. The spectroscopic and photodynamic properties of DTC_{60}^{2+} were compared with a non-charged *N*-methyl-2-(4′-acetamidophenyl)fulleropyrrolidine (MAC_{60}) and a monocationic *N*,*N*-dimethyl-2-(4′-acetamidophenyl)fulleropyrrolidinium iodide (DAC_{60}^{+}) in different media and in a typical Gram-negative bacterium, *E. coli*. These studies show that DTC_{60}^{2+} is an interesting photosensitizer with potential applications in PDI of bacteria.

2. Materials and methods

2.1. General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on an FT-NMR Bruker Avance DPX400 multinuclear spectrometer at 400 MHz. FAB mass spectra were taken with a ZAB-SEQ Micromass equipment. Silica gel thin-layer chromatography (TLC) plates of 250 μm from Aldrich (Milwaukee, WI, USA) were used. All the chemicals from Aldrich were used without further purification. Benzyl-*n*-hexadecyldimethylammonium chloride (BHDC) from Sigma was recrystallized twice from ethyl acetate and dried under vacuum over P_2O_5. Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01. Semiempirical molecular orbital calculations (AM1) were carried out using HyperChem software.

2.1.1. Sensitizers

Fulleropyrrolidines were synthesized according to modifications of established procedures [36].

2.1.1.1. N,N-Dimethyl-2-(4′-N,N,N-trimethylaminophenyl)fulleropyrrolidinium iodide (DTC_{60}^{2+}). A solution of C_{60} (50 mg, 0.069 mmol), 4-*N*,*N*-dimethylaminobenzaldehyde (10 mg, 0.067 mmol) and *N*-methylglycine (6 mg, 0.069 mmol) in 52 mL of dry toluene was stirred at reflux in atmosphere of argon for 6 h. Then, the solvent was removed under vacuum. Flash column chromatography (silica gel) using toluene/heptane (50:50 to 80:20 gradient) as eluent afforded 22 mg (38%) of *N*-methyl-2-(4′-*N*,*N*-dimethylaminophenyl)fulleropyrrolidine (DC_{60}). TLC (silica gel, toluene/heptane, 1:1) analysis R_f 0.10. MS [*m*/*z*] 896 (M^+) (896.1313 calculated for $C_{71}H_{16}N_2$). Anal. calcd. C 95.08, H 1.80, N 3.12; found C 95.12, H 1.85, N 3.07. ^{1}H NMR ($CDCl_3$, TMS) δ [ppm] 2.88 (s, 3H), 3.10 (s, 6H), 4.34 (d, 1H, $J = 9.5$ Hz), 5.01 (s, 1H), 5.09 (d, 1H, $J = 9.5$ Hz), 6.99 (d, 2H, $J = 9.0$ Hz), 7.52 (d, 2H, $J = 9.0$ Hz). Then, a mixture of this fullerene derivative (18 mg, 0.020 mmol) and 2 mL of methyl iodide in 2 mL of *N*,*N*-dimethylformamide (DMF) was stirred for 72 h at 70 °C. The solvents were removed under vacuum. The solid was re-suspended in heptane/toluene (1:1) and filtered to yield 22 mg (95%) of DTC_{60}^{2+}. MS [*m*/*z*] 926 ($M^+ - 2I$) (926.1783 calculated for $C_{73}H_{22}N_2$). Anal. calcd. C 74.25, H 1.88, N 2.37; found C 74.33, H 1.84, N 2.42. λ_{max} (DMF) [nm] (ε, $M^{-1}\,cm^{-1}$) 429 (2100).

2.1.1.2. N,N-Dimethyl-2-(4′-acetamidophenyl)fulleropyrrolidinium iodide (DAC_{60}^{+}). A mixture of C_{60} (30 mg, 0.042 mmol), 4-acetamidobenzaldehyde (14 mg, 0.086 mmol) and *N*-methylglycine (5 mg, 0.056 mmol) in 5 mL of dry toluene was stirred at reflux in atmosphere of argon for 7 h. The solvent was evaporated under vacuum and flash

column chromatography (silica gel) using toluene/ethyl acetate (100:0 to 80:10 gradient) as eluent to yield 32 mg (41%) of *N*-methyl-2-(4′-acetamidophenyl)fulleropyrrolidine (MAC_{60}). TLC (silica gel, toluene/ethyl acetate, 1:1) analysis R_f 0.25. MS [*m*/*z*] 910 (M^+) (910.1106 calculated for $C_{71}H_{14}N_2O$). Anal. calcd. C 93.62, H 1.55, N 3.08; found C 93.55, H 1.61, N 3.02. ^{1}H NMR ($CDCl_3$, TMS) δ [ppm] 2.37 (s, 3H), 2.86 (s, 3H), 4.31 (d, 1H, $J = 9.5$ Hz), 4.97 (s, 1H), 5.07 (d, 1H, $J = 9.5$ Hz), 7.03 (d, 2H, $J = 8.5$ Hz), 7.54 (d, 2H, $J = 8.5$ Hz), 7.71 (br s, 1H). λ_{max} (DMF) [nm] (ε, $M^{-1}\,cm^{-1}$) 430 (4000). Then, a mixture of MAC_{60} (17 mg, 0.020 mmol) and 2 mL of methyl iodide in 2 mL of *N*,*N*-dimethylformamide (DMF) was stirred for 72 h at 70 °C. The solvents were removed under vacuum. The solid was resuspended in heptane and filtered to yield 18 mg (96%) of DAC_{60}^+. MS [*m*/*z*] 925 ($M^+ - I$) (925.1341 calculated for $C_{72}H_{17}N_2O$). Anal. calcd. C 82.20, H 1.70, N 2.73; found C 82.14, H 1.63, N 2.66. λ_{max} (DMF) [nm] (ε, $M^{-1}\,cm^{-1}$) 429 (3500).

2.2. Spectroscopic studies

Spectra were recorded using 1-cm path length quartz cells at 25.0 ± 0.5 °C. The fluorescence quantum yield (ϕ_F) of fullerenes was calculated by comparison of the area below the corrected emission spectrum with that of C_{60} as a fluorescence standard, excited at $\lambda_{exc} = 450$ nm [37]. A value of $\phi_F = (2.3 \pm 0.1) \times 10^{-4}$ for C_{60} in DMF was calculated by comparison with the fluorescence spectrum in toluene using $\phi_F = 2.2 \times 10^{-4}$ and taking into account the refractive index of the solvents [28,37]. Studies in reverse micelles were performed using a stock solution of BHDC 0.1 M, which was prepared by weighing and dilution in benzene. The addition of water to the corresponding solution was performed using a calibrated microsyringe. The amount of water present in the system was expressed as the molar ratio between water and the BHDC present in the reverse micelle (W_0=[H_2O]/[BHDC]). In all experiments, $W_0 = 10$ was used. The mixtures were sonicated to obtain perfectly clear micellar system [38].

2.3. Steady-state photolysis

Solutions of 1,3-diphenylisobenzofuran (DPBF, 20 μM) and photosensitizer in different media were irradiated in 1-cm path length quartz cells (2 mL) with monochromatic light at $\lambda_{irr} = 470$ nm (sensitizer absorbance 0.1) from a 75 W high-pressure Xe lamp through a high intensity grating monochromator, Photon Technology Instrument [38]. The light intensity was determined as 0.55 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). The kinetics of photooxidation were studied by following the decrease in the absorbance (*A*) at $\lambda_{max} = 415$ nm for DPBF. Under these conditions, absorption due to fullerene derivatives at 415 nm is unchanged and therefore, the absorption changes observed in the presence of DPBF sensitized by fullerenes can be assigned to the oxidation of DPBF. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of ln A_0/A *vs* time. Photooxidation of DPBF was used to determine singlet molecular oxygen, $O_2(^1\Delta_g)$, production by the photosensitizers. Fullerene C_{60} was used as the standard ($\Phi_\Delta = 1$) [39,40]. Measurements of the sample and reference under the same conditions afforded Φ_Δ for sensitizers by direct comparison of the slopes in the linear region of the plots [41]. All the experiment were performed at 25.0 ± 0.5 °C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%.

2.4. Bacterial strain and preparation of cultures

E. coli strain (EC7) recovered from clinical urogenital material was previously characterized and identified [32,33]. *E. coli* strain was grown aerobically at 37 °C in 30% w/v tryptic soy (TS) broth overnight. Aliquots (~40 μL) of this culture were aseptically transferred to 4 mL of fresh medium (30% w/v TS broth) and incubated at 37 °C to mid logarithmic phase (absorbance ~0.6 at 660 nm). Cells in the logarithmic phase of growth were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0). Then the cells were diluted 1/1000 in PBS, corresponding to ~10^6 colony forming units (CFU)/mL. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and the sensitizer was added from a stock solution of sensitizer (4.5×10^{-4} M) in DMF. Viable bacteria were monitored and their number was calculated by counting the number of colony forming units after appropriate dilution on TS agar plates [32]. Bacterial cultures grown under the same conditions with and without photosensitizers kept in the dark as well as illuminated cultures without sensitizer served as controls.

2.5. Photosensitized inactivation of bacteria cells

Cell suspensions of *E. coli* (2 mL, ~10^6 CFU/mL) in PBS were incubated with 1 μM of sensitizer for 30 min in the dark at 37 °C. After that, two protocols were followed: (a) irradiation of cultures without cell washing step and (b) washing the cells once with PBS prior to irradiation. In both cases, the cultures were exposed for different time intervals to visible light. The light source used was a Novamat 130 AF slide projector equipped with a 150 W lamp. The light was filtered through a 2.5-cm glass cuvette filled with water to absorb heat. A wavelength range between 350 and 800 nm was selected by optical filters [42]. The light intensity at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). Control and irradiated cell suspensions were serially diluted with PBS, each solution was plated in triplicate on TS agar and the number of colonies formed after 18–24 h incubation at 37 °C was counted.

2.6. Growth delay of E. coli cultures

Cultures of *E. coli* cells were grown overnight as described above. A portion (60 μL) of this culture was transfer to 20 mL of fresh TS broth (10%) medium. The suspension was homogenized and aliquots of 2 mL were incubated with 2 μM of sensitizer at 37 °C. The culture grown was measured by turbidity at 660 nm using a Tuner SP-830 spectrophotometer [27,32]. Then the flasks were irradiated with visible light at 37 °C, as described above.

In all cases, control experiments were carried out without illumination in the absence and in the presence of sensitizer. Each experiment was repeated separately three times.

3. Results and discussion

3.1. Synthesis of fullerene derivatives

Fulleropyrrolidine derivatives were synthesized by 1,3-dipolar cycloaddition of azomethine ylides to C_{60} (Schemes 1 and 2) [3]. A two-step method was used to synthesize DTC_{60}^{2+}. First, the reaction of C_{60} with *N*,*N*-dimethylaminobenzaldehyde and *N*-methylglycine ([1:1:1] molar relation) in refluxing toluene afforded *N*-methyl-2-(4′-*N*,*N*-dimethylaminophenyl)fulleropyrrolidine with 38% yield after purification by flash chromatography. This fullerene amino derivative was treated with an excess of methyl iodide at 70 °C for 72 h in DMF (Scheme 1). The exhaustive methylation produces DTC_{60}^{2+} with 95% yield.

A similar procedure of cycloaddition was used to obtain MAC_{60} from a mixture of C_{60}, 4-acetamidobenzaldehyde and *N*-methylglycine with 41% yield (Scheme 2). Methylation of MAC_{60} yields 96% of DAC_{60}^{+}.

The compound DTC_{60}^{2+} bears a hydrophobic carbon sphere substituted by two cationic groups forming one amphiphilic monoadduct. To evaluate the effect produced by the last groups upon the intramolecular polarity, the dipole moment of fullerene derivatives was estimated. Semiempirical method for molecular modeling (AM1) was used in structure geometry optimization and calculations giving values of 3.4, 17.3 and 46.0 D for MAC_{60}, DAC_{60}^{+} and DTC_{60}^{2+}, respectively. As expected, the presence of a dicationic moiety in the periphery of fullerene derivative considerably enhances the dipole moment with respect to the non-charged structures.

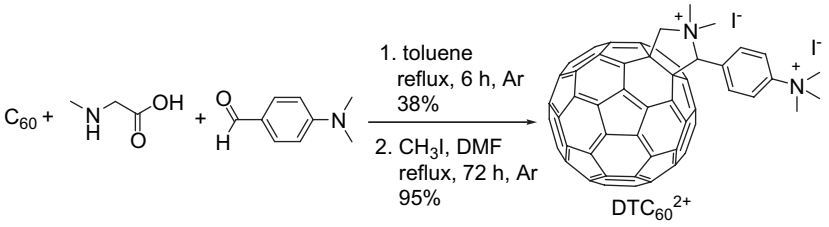
3.2. Spectroscopic studies

The UV–visible absorption spectra of MAC_{60}, DAC_{60}^{+} and DTC_{60}^{2+} were performed in homogeneous media and in a reverse micellar system formed by benzene/BHDC (0.1 M)/water ($W_0 = 10$). Representative results for DTC_{60}^{2+} are shown in Fig. 1. The spectra of these fullerene derivatives are typical of most C_{60} monoadducts [3]. Similarly to C_{60}, the electronic spectra of the pyrrolidine derivatives are dominated by strong absorptions mainly in the UV region. In the visible region, these fullerenes show a broader range of absorption up to almost 710 nm, with a characteristic sharp peak at 430 nm and broader band at around 700 nm. The absorption spectrum of MAC_{60} shows similar shape in DMF and in micelles indicating a moderate solubility as monomer in both media. On the other hand, a low intensity and a broadening of the bands are observed in the spectrum of DTC_{60}^{2+} in DMF, probably due to aggregation of dicationic fullerene in this medium (Fig. 1). Similar behavior was also found for DTC_{60}^{2+} in toluene and PBS. However, the spectrum intensity of DTC_{60}^{2+} increases in benzene/BHDC/water, indicating that this micellar system helps the solubilization of cationic fullerene as monomer.

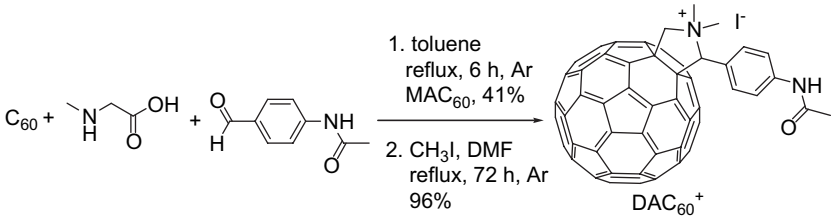
The steady-state fluorescence emission spectra of fullerenes in DMF are shown in Fig. 2. The spectra show a band centered at ~712 nm, which are characteristic for similar fulleropyrrolidines [43]. Taking into account the absorption spectrum, this band has been assigned to $0^* \rightarrow 0$ transition band [28]. By comparison with C_{60} as a reference, the values $\phi_F = (3.0 \pm 0.3) \times 10^{-4}$, $(2.0 \pm 0.2) \times 10^{-4}$ and $(5.4 \pm 0.3) \times 10^{-4}$ were obtained for MAC_{60}, DAC_{60}^{+} and DTC_{60}^{2+}, respectively. According to the photophysical properties of fulleropyrrolidines, low values of ϕ_F are expected [3,28,43]. In all cases, a small Stokes shift (~6 nm) was observed indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. Taking into account the energy of $0 \rightarrow 0$ electronic transitions, the energy levels of the singlet excited state (E_s) were calculated giving in the three cases a value of 1.77 eV. These results are in agreement with those previously reported for this family of photosensitizers [3,43].

3.3. Photodynamic activity

The photooxidation of 1,3-diphenylisobenzofurane (DBPF) sensitized by fullerene derivatives was investigated in homogenic DMF/water (10% v/v) medium and reverse micellar system formed by benzene/BHDC (0.1 M)/water ($W_0 = 10$). It is generally assumed that DBPF is decomposed only by $O_2(^1\Delta_g)$ generated by type II photoprocess to produce 1,2-dibenzoylbenzene. Moreover, it is known that DPBF quenches almost exclusively by chemical reaction, physical quenching being negligible [44,45]. Therefore, it



Scheme 1.



Scheme 2.

was used in this work to evaluate the ability of the sensitizers to produce $O_2(^1\Delta_g)$. A time-dependent decrease in the DBPF concentration was observed by following a decrease in its absorbance at 415 nm (Fig. 3). From first-order kinetic plots the values of the observed rate constant (k_{obs}) were calculated for DBPF. The results are gathered in Table 1. In this condition, the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) was calculated comparing the slope for fulleropyrrolidines with the corresponding slope obtained for the reference, C_{60}. In DMF/water, non-charged MAC_{60} and C_{60}, photodecomposed DBPF with comparable rates, indicating that $O_2(^1\Delta_g)$ is efficiently produced by MAC_{60} in this medium. Values of $\Phi_\Delta = 1$ and 0.72 were calculated for C_{60} and MC_{60}, respectively. Similar results of singlet oxygen production were previously found for C_{60} and bis(ethoxycarbonyl)C_{61} measuring 1268 nm emissions in CS_2 [40]. Introduction of substituents on fullerene core produces a decrease in the photodynamic activity and it appears that this effect is not significantly dependent on the kind of addend.

On the other hand, DBPF reaction sensitized by DTC_{60}^{2+} was one order of magnitude lower than those of non-charged fullerene derivatives. This low $O_2(^1\Delta_g)$ production of DTC_{60}^{2+} is probably due to an incomplete monomerization of the cationic fullerene in the DMF/water medium.

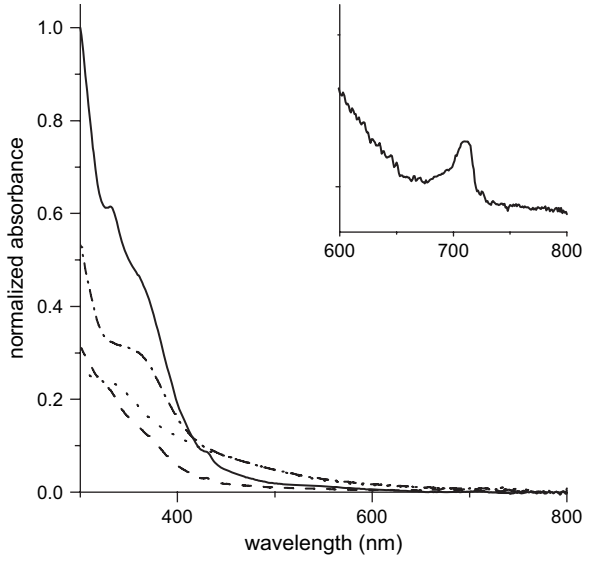


Fig. 1. Absorption spectra of DTC_{60}^{2+} in different media, DMF (dashed line), PBS (dotted line), toluene (dashed dotted line) and benzene/BHDC (0.1 M)/$W_0 = 10$ (solid line). Inset: DTC_{60}^{2+} spectrum enlargement in benzene/BHDC (0.1 M)/$W_0 = 10$.

Although, fullerene in micelles was not used to perform the biological experiments, these microheterogeneous systems are frequently used as an interesting model to mimic the water pockets that are often found in various bioaggregates such as proteins, enzymes and membranes [46]. Thus, water-soluble and water-insoluble compounds can be dissolved simultaneously in reverse micelles, which simulate a biomimetic microenvironment [47]. The rates of DBPF decomposition sensitized by fullerene derivatives in benzene/BHDC/water micelles are given in Table 1. As can be observed, in micellar system the value of k_{obs} sensitized by MAC_{60} is ~4 times higher than that obtained with DTC_{60}^{2+}, while in the homogenic medium this ratio reaches a value of ~15 times. This means that in the microheterogeneous media the photosensitizing activity of DTC_{60}^{2+} is more similar to that of the non-charged fullerene. This enhancement in reverse micellar system is probably facilitated for a better solubilization as monomer of DTC_{60}^{2+}, like it was previously shown by absorption spectroscopic studies. Thus, it can be noted that $O_2(^1\Delta_g)$ production is very dependent on the medium where the sensitizer is localized and considerably diminishes when the photosensitizer is aggregated.

3.4. Studies in vitro on E. coli cells

Fullerene derivatives, MAC_{60}, DAC_{60}^{+} and DTC_{60}^{2+}, were evaluated as photodynamic agents *in vitro* using a typical

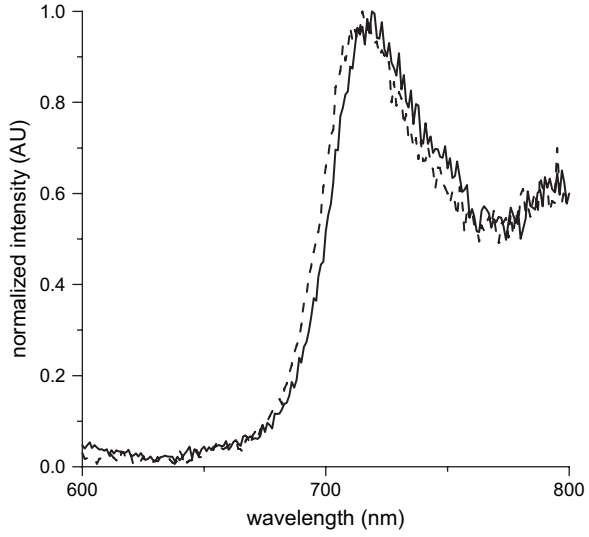


Fig. 2. Fluorescence emission spectra of MAC_{60} (solid line) and DTC_{60}^{2+} (dashed line) in DMF, $\lambda_{exc} = 450$ nm.

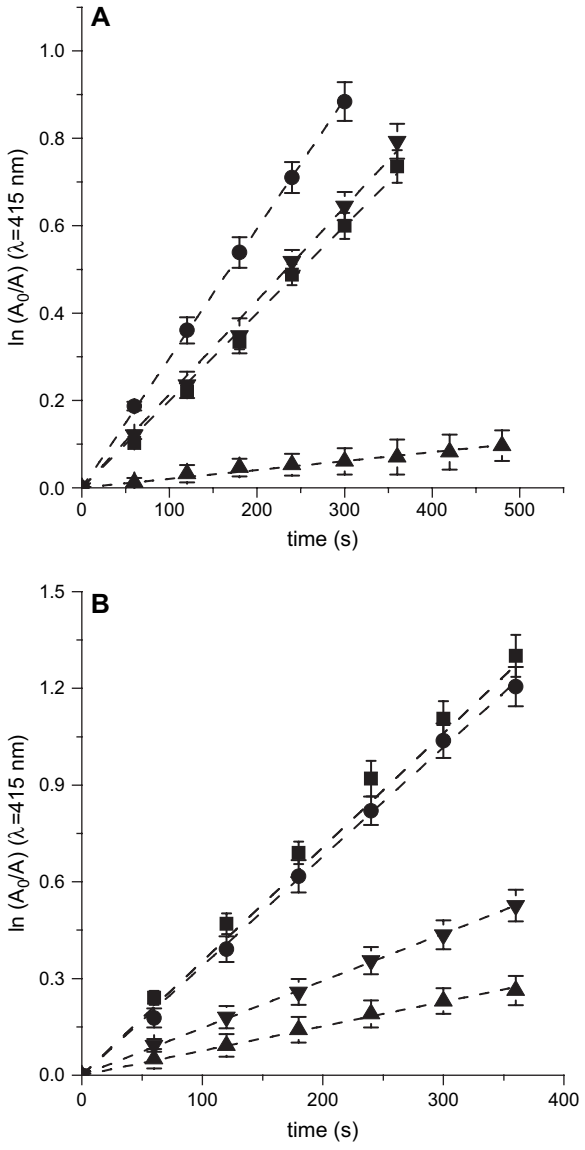


Fig. 3. First-order plots for the photooxidation of DPFB (20 μM) photosensitized by MAC_{60} (▼), DAC_{60}^{+} (■), DTC_{60}^{2+} (▲) and C_{60} (●) in (A) DMF/water (10%) and (B) benzene/BHDC (0.1 M)/$W_0 = 10$, $\lambda_{irr} = 470$ nm. Values represent mean ± standard deviation of three separate experiments.

Gram-negative bacterium, *E. coli*. First, the cell toxicity induced by these fullerenes was analyzed in the absence of light at different photosensitizer concentrations. When the cultures were treated with 5 μM of sensitizer for 30 min in dark, no toxicity was detected for MAC_{60}. However, compound DTC_{60}^{2+} was completely dark toxic to *E. coli* and formation of colonies was not detected. When 2.5 μM of sensitizer was used, compound DTC_{60}^{2+} showed only minor dark toxicity toward *E. coli* producing 23% of cellular inactivation. In previous studies, two isomers of C_{60}-bis(*N*,*N*-dimethylpyrrolidinium) iodide were used to study the bacteriostatic effects of fullerene derivatives on *E. coli* cells [48]. The inactivation of *E. coli* growth was attributed to the inhibition of energy metabolism by two opposite dose-dependent mechanisms. At low fullerene concentration the oxygen uptake is decreased, on the contrary at high concentration the oxygen uptake increases and oxygen is converted to H_2O_2. Besides, these fullerene concentrations inhibit the respiratory chain activity without photoirradiation. Finally in our case, no dark toxicity was found when cell cultures were treated with 1 μM of DTC_{60}^{2+} and therefore it was selected for photodynamic *in vitro* studies.

The capacity of these photosensitizers to bind to bacterial cells was analyzed by fluorescence emission spectra according to the procedure previously described in the literature [32,33]. The *E. coli* cultures were incubated with 1 μM of fullerene for different times (5, 10 and 30 min) at 37 °C in the dark. However, the emission of fullerene in SDS (2%) solution was negligible due to the low fluorescence intensity of these compounds.

Suspensions of *E. coli* cells in PBS were treated with 1 μM of sensitizer for 30 min at 37 °C in dark and then the cultures were irradiated with visible light. Fig. 4 shows the survival of bacterial cells after different irradiation times. Control experiments showed that the viability of *E. coli* was unaffected by illumination alone or by dark incubation with 1 μM of the

Table 1
Kinetic parameters (k_{obs}) and quantum yield of O_2 ($^1\Delta_g$) production (Φ_Δ) of fullerene derivatives in different media

Fullerenes	k_{obs} (s^{-1}) DMF/water[a]	Φ_Δ[a]	k_{obs} (s^{-1}) micelles[c]
C_{60}	$(2.97 \pm 0.08) \times 10^{-3}$	1[b]	$(3.24 \pm 0.09) \times 10^{-3}$
MAC_{60}	$(2.14 \pm 0.07) \times 10^{-3}$	0.72 ± 0.05	$(1.60 \pm 0.08) \times 10^{-3}$
DAC_{60}^{+}	$(2.05 \pm 0.06) \times 10^{-3}$	0.69 ± 0.05	$(3.40 \pm 0.09) \times 10^{-3}$
DTC_{60}^{2+}	$(0.20 \pm 0.02) \times 10^{-3}$	0.07 ± 0.01	$(0.80 \pm 0.05) \times 10^{-3}$

[a] DMF/water (10%).
[b] Ref. [28].
[c] Benzene/BHDC (0.1 M)/water ($W_0 = 10$).

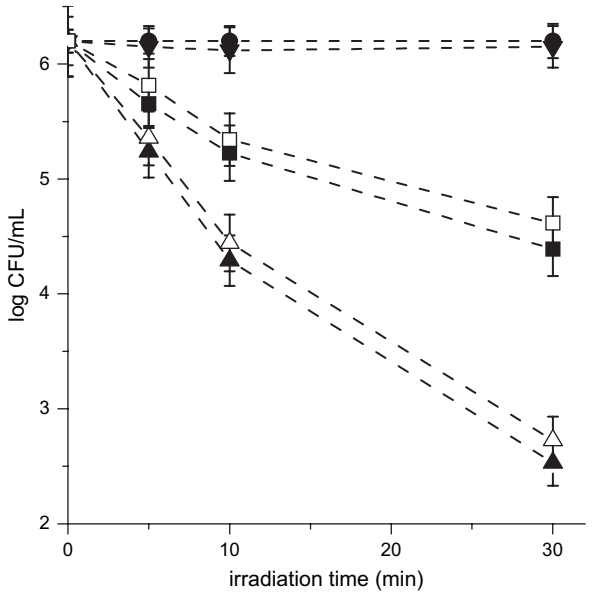


Fig. 4. Survival curves of *E. coli* cells (~10^6 CFU/mL) incubated with 1 μM of MAC_{60} (▼), DAC_{60}^{+} (■) and DTC_{60}^{2+} (▲) for 30 min at 37 °C in dark and exposed to visible light for different irradiation times and washing once the cells before illumination DAC_{60}^{+} (□) and DTC_{60}^{2+} (△). Control culture untreated irradiated (●). Values represent mean ± standard deviation of three separate experiments.

photosensitizer for 30 min, indicating that the cell mortality obtained after irradiation of the cultures treated with the fullerene is due to the photosensitization effect of the agent produced by visible light.

The viability of *E. coli* cells after irradiation was dependent upon both the fullerene derivatives used in the treatment and the light exposure level. As expected for non-charged photosensitizers, no inactivation effect was found for cultures treated with 1 μM of MAC_{60} still after 30 min of irradiation. Similar behavior was also found using 5 μM of MAC_{60}. This result is in agreement with that reported before for non-charged fullerene derivatives, indicating that these non-cationic sensitizers are unsuccessful sensitizers for Gram-negative bacteria under these conditions [31].

On the other hand, as it can be observed in Fig. 4 the *E. coli* cells are rapidly photoinactivated when the unwashed cultures treated with dicationic DTC_{60}^{2+} are exposed to visible light. In particular, the dicationic fullerene exhibits a photosensitizing activity causing a ~3.5 log decrease of cell survival, when the cultures are irradiated for 30 min. These results represent a value greater than 99.97% of cellular inactivation.

The photodynamic activity sensitized by DTC_{60}^{2+} can be directly compared with those of other different family of cationic sensitizers under similar irradiation conditions by using the same *E. coli* strain. Thus, when *E. coli* cultures were treated with 1 μM of a *cis* dicationic porphyrin, 5,10-bis(4-methylphenyl)-15,20-bis(*N*,*N*,*N*-trimethylammoniumphenyl)-porphyrin iodide, 70% of cellular inactivation was found after 5 min of irradiation with visible light, whereas under comparable condition, DTC_{60}^{2+} inactivates 86% of bacterial cells [23]. When the *E. coli* cultures were incubated with 5 μM of *cis* dicationic porphyrin and irradiated for 30 min, the PDI effect produces a ~3.2 log decrease of cell survival. This value is comparable with that obtained using 1 μM of DTC_{60}^{2+} (Fig. 4) [32]. Also, the behavior of dicationic fullerene derivative can be compared with that of a *trans* dicationic porphyrin, 5,15-bis[(4-3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl]-10, 20-bis(4-trifluoromethylphenyl)porphyrin iodide [33]. Irradiation of *E. coli* cultures treated with 1 μM of this *trans* dicationic porphyrin photoinactivates ~87% of the cells even after 20 min of irradiation. Therefore these comparisons indicate that 1 μM of DTC_{60}^{2+} produces a similar photoinactivation of *E. coli* cells than those of dicationic porphyrin after 5 min of irradiation; though DTC_{60}^{2+} causes a higher decrease of cell survival after 30 min of irradiation. On the other hand, porphyrin derivatives bearing three of four cationic charges produce a higher photoinactivation than that found for DTC_{60}^{2+}, probably due to a tighter electrostatic interaction with negatively charged sites at the outer surface of *E. coli* cells [23,32,33]. Also, DTC_{60}^{2+} can be compared with cationic phthalocyanine derivatives. Per example, 1 μM of dicationic fullerene is more effective than 10 μM of a cationic Zn(II) tetramethyltetrapyridinoporphyrazinium salt, which produces ~2 log decrease of cell survival after 30 min of irradiation [26]. The photoinactivation sensitized by 10 μM of a cationic Zn(II) *N*-methylpyridyloxyphthalocyanine causes a ~4.5 log decrease of *E. coli* cell survival, even so it required 10 times higher concentration than that used with DTC_{60}^{2+} [27]. Also, in previous studies cationic fullerenes bearing two quaternary pyrrolidinium groups on sphere were evaluated to eradicate microbes. After incubation, the bis-cationic fullerene was highly active in killing *E. coli* cells producing a 4 log of killing [31].

On the other hand, after one washing step the photoinactivation of *E. coli* cells follows the tendency shown in Fig. 4. Under this condition, the photodynamic effect is mainly associated with porphyrins that are tightly bound to cells. As can be observed, the photoinactivation activity of dicationic fullerene remains similar to those found for unwashed cells. Thus, the enhanced photodynamic activity imputed to the interactions of positive charges on the *E. coli* cellular surface is stable enough to survive the washing.

Photosensitized growth delay of *E. coli* cultures by fullerenes was carried out in medium (10% w/v TS broth). This experiment was performed to ensure that PDI of cells is still possible when the cultures were not under starvation conditions or the potential damaging effects of phosphate buffer washing [18,27,33,49]. The cultures of *E. coli* in lag phase were treated with 2 μM of sensitizer and the flasks were irradiated with visible light at 37 °C. As can be observed in Fig. 5, growth was arrested when *E. coli* cultures were treated with DTC_{60}^{2+} and illuminated. After irradiation in the presence of dicationic fullerene, the cells no longer appeared to be growing as measured by turbidity at 660 nm. Furthermore, a minor effect in the growth delay was found for cells treated with MAC_{60}. On the other hand, *E. coli* cells exposed to sensitizers in the dark or not treated with sensitizer and illuminated showed no growth delay

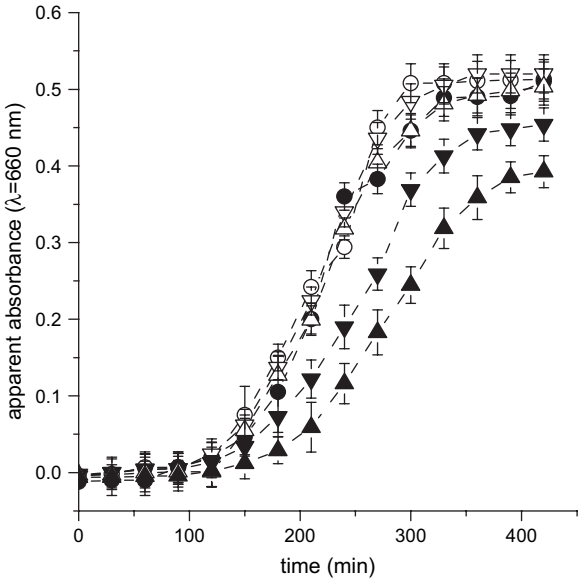


Fig. 5. Photosensitized growth delay curves of *E. coli* cells incubated with 2 μM of MAC_{60} (▼) and DTC_{60}^{2+} (▲) and exposed to different irradiation times with visible light in 10% w/v TS broth at 37 °C. Control culture untreated irradiated (●), untreated in dark (○), treated with 2 μM of MAC_{60} (▽) and DTC_{60}^{2+} (△) in dark. Values represent mean ± standard deviation of three separate experiments.

compared with controls. Therefore, the data illustrate that the observed growth delay is due to the photoinactivation effect of the sensitizers on the cells [32]. Similar behavior was previously observed for *E. coli* cultures in the presence of 10 μM of dicationic porphyrin derivatives under analogous experimental conditions [32,33].

4. Conclusions

In summary, a novel dicationic fullerene derivative has been conveniently synthesized in two-step procedure, which involves a 1,3-dipolar cycloaddition of azomethine ylides to C_{60} with 38% yield and an exhaustive methylation with methyl iodide yielded 95% of DTC_{60}^{2+}. In the structure of DTC_{60}^{2+}, the distribution of cationic groups on the hydrophobic carbon sphere of C_{60} produces a considerable increase in the amphiphilic character of the sensitizer, as indicated by the increase in the dipolar moment. This effect could help fullerene derivatives to pass through or accumulate in biomembranes, enhancing the effective photosensitization [16,50].

Fullerene DTC_{60}^{2+} form aggregates in several solvents of different polarities, however, monomerization increases in a microheterogenic medium, such as benzene/BHDC/water reverse micelles, probably due to its amphiphilic structure. An enhancement in the monomeric form of DTC_{60}^{2+} in the micellar system produces an increase in the $O_2(^1\Delta_g)$ production in comparison with DMF/water medium. Thus, its photodynamic efficiency in biological systems is not directly predictable on the basis of photophysical investigations in solution and DTC_{60}^{2+} can be an efficient sensitizer mainly dependent on the microenvironment where it is localized.

Studies on PDI *in vitro* on *E. coli* cells provide information on the photodynamic activity of this new cationic fullerene DTC_{60}^{2+} in comparison with a non-charged MAC_{60} and a monocationic DAC_{60}^{+} fullerene derivates. Photosensitized inactivation of *E. coli* cellular suspensions by DTC_{60}^{2+} exhibits a ~3.5 log decrease of cell survival after 30 min of irradiation, which represents about 99.97% of cellular inactivation. On the other hand, under the same conditions non-charged MAC_{60} produces a negligible effect on *E. coli* cells, whereas DAC_{60}^{+}, which has an average dipole moment value, produces a ~1.5 log (~96.8%) decrease of cell survival. The photodynamic activity of DTC_{60}^{2+} was confirmed by growth delay of *E. coli* cultures. As previously discussed, DTC_{60}^{2+} appears to be so efficient as some cationic porphyrins and phthalocyanines. Therefore, these results indicate that DTC_{60}^{2+} is an interesting agent with potential applications in photodynamic inactivation of bacteria.

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References

[1] S. Bosi, T. Da Ros, G. Spalluto, M. Prato, Fullerene derivatives: an attractive tool for biological applications, Eur. J. Med. Chem. 38 (2003) 913–923.
[2] M.E. El-Khouly, O. Ito, P.M. Smith, F. D'Souza, Intermolecular and supramolecular photoinduced electron transfer processes of fullerene–porphyrin/phthalocyanine systems, J. Photochem. Photobiol. C: Photochem. Rev. 5 (2004) 79–104.
[3] M. Prato, M. Maggini, Fulleropyrrolidines: a family of full-fledged fullerene derivatives, Acc. Chem. Res. 31 (1998) 519–526.
[4] H. Imahori, Y. Sakata, Fullerenes as novel acceptors in photosynthetic electron transfer, Eur. J. Org. Chem. (1999) 2445–2457.
[5] Y. Tabata, Y. Ikada, Biological functions of fullerene, Pure Appl. Chem. 71 (1999) 2047–2053.
[6] N. Tagmatarchis, H. Shinohara, Fullerenes in medicinal chemistry and their biological applications, Mini Rev. Med. Chem. 1 (2001) 339–348.
[7] Y. Tabata, Y. Murakami, Y. Ikada, Photodynamic effect of polyethylene glycol-modified fullerene on tumor, Jpn. J. Cancer Res. 88 (1997) 1108–1116.
[8] F. Diederich, L. Isaacs, D. Philp, Syntheses, structures, and properties of methanofullerenes, Chem. Soc. Rev. (1994) 243–255.
[9] P.W. Taylor, P.D. Stapleton, J.P. Luzio, New ways to treat bacterial infections, Drug Discov. Today 7 (2002) 1086–1091.
[10] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT), J. Antimicrob. Chemother. 42 (1998) 13–28.
[11] M.R. Hamblin, T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem. Photobiol. Sci. 3 (2004) 436–450.
[12] M. Ochsner, Photophysical and photobiological processes in photodynamic therapy of tumours, J. Photochem. Photobiol., B: Biol. 39 (1997) 1–18.
[13] M.C. DeRosa, R.J. Crutchley, Photosensitized singlet oxygen and its applications, Coord. Chem. Rev. 233–234 (2002) 351–371.
[14] Y. Yamakoshi, S. Sueyoshi, K. Fukuhara, N. Miyata, $OH^{\bullet}$ and $O_2^{\bullet-}$ generation in aqueous C_{60} and C_{70} solution by photoirradiation: an EPR study, J. Am. Chem. Soc. 120 (1998) 12363–12364.
[15] G. Jori, S.B. Brown, Photosensitized inactivation of microorganisms, Photochem. Photobiol. Sci. 5 (2004) 403–405.
[16] E.N. Durantini, Photodynamic inactivation of bacteria, Curr. Bioact. Comp. 2 (2006) 127–142.
[17] M. Merchat, G. Spikes, G. Bertoloni, G. Jori, Studies on the mechanism of bacteria photosensitization by *meso*-substituted cationic porphyrins, J. Photochem. Photobiol., B: Biol. 35 (1996) 149–157.
[18] A. Minnock, D.I. Vernon, J. Schofield, J. Griffiths, J.H. Parish, S.B. Brown, Mechanism of uptake of a cationic water-soluble pyridinium zinc phthalocyanine across the outer membrane of *Escherichia coli*, Antimicrob. Agents Chemother. 44 (2000) 522–527.
[19] M. Salmon-Divon, Y. Nitzan, Z. Malik, Mechanistic aspect of *Escherichia coli* photodynamic inactivation by cationic tetra-*meso*(*N*-methylpyridyl)-porphine, Photochem. Photobiol. Sci. 3 (2004) 423–429.
[20] Y. Nitzan, H. Ashkenazi, Photoinactivation of *Acinetobacter baumannii* and *Escherichia coli* B by cationic hydrophilic porphyrin at various light wavelengths, Curr. Microbiol. 42 (2001) 408–414.
[21] M. Merchat, G. Bertoloni, P. Giacomini, A. Villanueva, G. Jori, *meso*-Substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria, J. Photochem. Photobiol., B: Biol. 32 (1996) 153–157.
[22] E. Reddi, M. Ceccon, G. Valduga, G. Jori, J.C. Bommer, F. Elisei, L. Latterini, U. Mazzucato, Photophysical properties and antibacterial activity of *meso*-substituted cationic porphyrin, Photochem. Photobiol. 75 (2002) 462–470.
[23] M.B. Spesia, D. Lazzeri, L. Pascual, M. Rovera, E.N. Durantini, Photoinactivation of *Escherichia coli* using porphyrin derivatives with different

number of cationic charges, FEMS Immunol. Med. Microbiol. 44 (2005) 289–295.
[24] A. Minnock, D.I. Vernon, J. Schofield, J. Griffiths, J.H. Parish, S.B. Brown, Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanines to photoinactivate both Gram-negative and Gram-positive bacteria, J. Photochem. Photobiol., B: Biol. 32 (1996) 159–164.
[25] A. Segalla, C.D. Borsarelli, S.E. Braslavsky, J.D. Spikes, G. Roncucci, D. Dei, G. Chiti, G. Jori, E. Reddi, Photophysical, photochemical and antibacterial photosensitizing properties of a novel octacationic Zn(II)-phthalocyanine, Photochem. Photobiol. Sci. 1 (2002) 641–648.
[26] E.A. Dupouy, D. Lazzeri, E.N. Durantini, Photodynamic activity of cationic and non-charged Zn(II) tetrapyridinoporphyrazine derivatives: biological consequences in human erythrocytes and *Escherichia coli*, Photochem. Photobiol. Sci. 3 (2004) 992–998.
[27] I. Scalise, E.N. Durantini, Synthesis, properties, and photodynamic inactivation of *Escherichia coli* using a cationic and noncharged Zn(II) pyridyloxyphthalocyanine derivatives, Bioorg. Med. Chem. 13 (2005) 3037–3045.
[28] K.G. Thomas, V. Biju, M.V. George, D.M. Guldi, P.V. Kamat, Excited-state interactions in pyrrolidinofullerenes, J. Phys. Chem. A. 102 (1998) 5341–5348.
[29] F. Käsermann, C. Kempf, Buckminsterfullerene and photodynamic inactivation of viruses, Rev. Med. Virol. 8 (1998) 143–151.
[30] S. Marchesan, T. Da Ros, G. Spalluto, J. Balzarini, M. Prato, Anti-HIV properties of cationic fullerene derivatives, Bioorg. Med. Chem. Lett. 15 (2005) 3615–3618.
[31] G.P. Tegos, T.N. Demidova, D. Arcila-Lopez, H. Lee, T. Wharton, H. Gali, M.R. Hamblin, Cationic fullerenes are effective and selective antimicrobial photosensitizers, Chem. Biol. 12 (2005) 1127–1135.
[32] D. Lazzeri, M. Rovera, L. Pascual, E.N. Durantini, Photodynamic studies and photoinactivation of *Escherichia coli* using *meso*-substituted cationic derivatives with asymmetric charge distribution, Photochem. Photobiol. 80 (2004) 286–293.
[33] D.A. Caminos, M.B. Spesia, E.N. Durantini, Photodynamic inactivation of *Escherichia coli* by novel *meso*-substituted porphyrins by 4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy) phenyl and 4-(trifluoromethyl)phenyl groups, Photochem. Photobiol. Sci. 5 (2006) 56–65.
[34] M.E. Milanesio, M.G. Alvarez, V. Rivarola, J.J. Silber, E.N. Durantini, Porphyrin–fullerene C_{60} dyads with high ability to form photoinduced charge-separated state as novel sensitizers for photodynamic therapy, Photochem. Photobiol. 81 (2005) 891–897.
[35] M.G. Alvarez, C. Prucca, M.E. Milanesio, E.N. Durantini, V. Rivarola, Photodynamic activity of a new sensitizer derived from porphyrin-C_{60} dyad and its biological consequences in a human carcinoma cell line, Int. J. Biochem. Cell Biol. 38 (2006) 2092–2101.
[36] M.E. Milanesio, E.N. Durantini, Synthesis and spectroscopic properties of a covalently linked porphyrin–fullerene C_{60} dyad, Synth. Commun. 36 (2006) 2135–2144.
[37] J.N. Demas, G.A. Crosby, The measurement of photoluminescence quantum yields, J. Phys. Chem. 75 (1971) 991–1024.
[38] M.E. Milanesio, M.G. Alvarez, E.I. Yslas, C.D. Borsarelli, J.J. Silber, V. Rivarola, E.N. Durantini, Photodynamic studies of metallo 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin: photochemical characterization and biological consequences in a human carcinoma cell line, Photochem. Photobiol. 74 (2001) 14–21.
[39] R. Hung, J. Grabowski, A precise determination of the triplet energy of C_{60} by photoacoustic calorimetry, J. Phys. Chem. 95 (1991) 6073–6075.
[40] T. Hamano, K. Okuda, T. Mashino, M. Hirobe, K. Arakane, A. Ryu, S. Mashiko, T. Nagano, J. Chem. Soc., Chem. Commun. (1997) 21–22.
[41] R.W. Redmond, J.N. Gamlin, A compilation of singlet yields from biologically relevant molecules, Photochem. Photobiol. 70 (1999) 391–475.
[42] M.B. Spesia, M. Rovera, L. Pascual, E.N. Durantini, A new antibacterial approach using photodynamic inactivation, Chem. Educator 10 (2005) 126–129.
[43] J.-F. Eckert, J.-F. Nicoud, J.-F. Nierengarten, S.-G. Liu, L. Echegoyen, F. Barigelletti, N. Armaroli, L. Ouali, V. Krasnikov, G. Hadziioannou, Fullerene–oligophenylenevinylene hybrids: synthesis, electronic properties, and incorporation in photovoltaic devices, J. Am. Chem. Soc. 122 (2000) 7467–7479.
[44] P.B. Merker, D.R. Kearns, Comment regarding the rate constant for the reaction between 1,3-diphenylisobenzofuran and singlet oxygen, J. Am. Chem. Soc. 97 (1975) 462–463.
[45] P. Zimcik, M. Miletin, Z. Musil, K. Kopecky, L. Kubza, D. Brault, Cationic azaphthalocyanines bearing aliphatic tertiary amino substituents. Synthesis, singlet oxygen production and spectroscopic studies, J. Photochem. Photobiol., A: Chem. 183 (2006) 59–69.
[46] I. Scalise, E.N. Durantini, Photodynamic effect of metallo 5-(4-carboxyphenyl)-10,15,20-tris(4-methylphenyl) porphyrins in biomimetic media, J. Photochem. Photobiol., A: Chem. 162 (2004) 105–113.
[47] J.J. Silber, A. Biasutti, E. Abuin, E. Lissi, Interactions of small molecules with reverse micelles, Adv. Colloid Interface Sci. 82 (1999) 189–252.
[48] T. Mashino, N. Usui, K. Okuda, T. Hirota, M. Mochizuki, Respiratory chain inhibition by fullerene derivatives: hydrogen peroxide production caused by fullerene derivatives and a respiratory chain system, Bioorg. Med. Chem. 11 (2003) 1433–1438.
[49] Y. Nitzan, A. Balzam-Subakevitz, H. Ashkenazi, Eradication of Acinebacter baumannii by photosensitized agents *in vitro*, J. Photochem. Photobiol., B: Biol. 42 (1998) 211–218.
[50] R.W. Boyle, D. Dolphin, Structure and biodistribution relationships of photodynamic sensitizers, Photochem. Photobiol. 64 (1996) 469–485.