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Mechanistic studies on the photodynamic effect induced by a dicationic fullerene C_{60} derivative on *Escherichia coli* and *Candida albicans* cells

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Summary The photodynamic mechanism of action induced by *N*,*N*-dimethyl-2-(4′-*N*,*N*,*N*-trimethylaminophenyl)fulleropyrrolidinium iodide (DTC_{60}^{2+}) was investigated on *Candida albicans* and *Escherichia coli* cells. First, photogeneration of superoxide anion radical by DTC_{60}^{2+} in the presence of NADH was detected using nitro blue tetrazolium method in reverse micelles. In *C. albicans* suspensions, 10 μM DTC_{60}^{2+} was an effective photosensitizer, producing a ~5 log decrease of cell survival when the cultures were irradiated for 30 min with visible light. Also, *C. albicans* cells growth was not detected in the presence of 10 μM DTC_{60}^{2+} and irradiation. Photodynamic mechanism investigations were compared in both *C. albicans* and *E. coli* cells. Studies under anoxic conditions indicated that oxygen was required for the photodynamic inactivation of these microorganisms. The photocytotoxicity induced by DTC_{60}^{2+} was similar in D_2O than in water cell suspensions. Furthermore, photoinactivation of microbial cells was negligible in the presence of azide ion, while the addition of mannitol produced a photoprotective effect on the cellular survival. These results indicate that DTC_{60}^{2+} has potential as agent to the photodynamic inactivation of microbial cells. Also, the photocytotoxicity activity induced by this cationic fullerene derivative can involve the intermediacy of both superoxide anion radical and singlet molecular oxygen.

Introduction

The field of antimicrobial chemotherapy is in constant modification due to the wide variety of pathogens encountered and their rapid evolutionary changes [1]. Thus, the great successes in the war against microorganisms are probably coming to the end. For this reason, it is imperative the development of new drugs and therapies. An alternative method includes a non-oncologic application of photodynamic therapy (PDT), named photodynamic inactivation (PDI) of microorganisms [2]. Although the photodynamic action was discovered over 100 years ago as a means for killing microorganisms, it was not until recent years that

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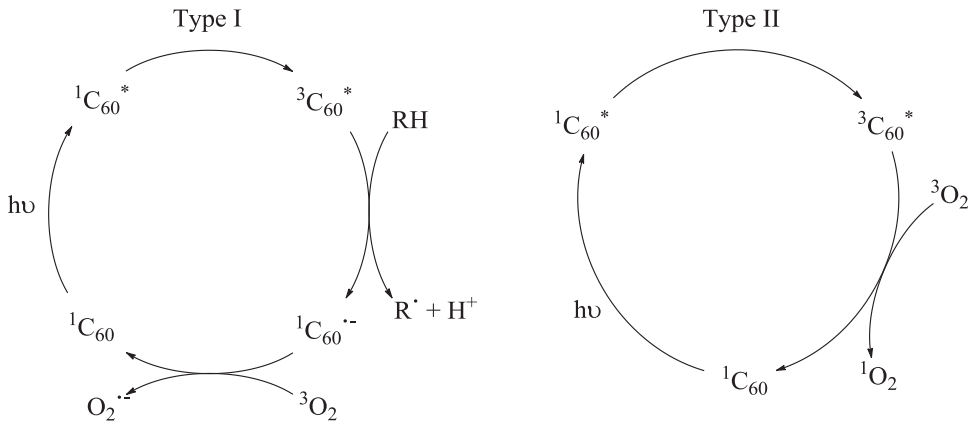


Scheme 1 Schematic representation of type I (charge transfer) and type II (energy transfer) photochemical mechanisms.

interest in the antimicrobial effects of PDT has been proposed as a therapy for a large variety of localized infections [3]. Essentially, 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 generates a cascade of biochemical events that produce cell damages leading to the inactivation of the microorganisms [4].

Potential biological activities of fullerenes have been investigated with the aim of using these small nanoparticles in the field of medicine [5,6]. According with its photochemical properties, these nanostructures have been used as efficient agents to mediate PDI of various classes of microbial cells [7]. The combination of visible light absorption and a long lifetime of triplet excited state allow fullerenes to act as photosensitizers. Irradiation of solubilized fullerenes in the presence of oxygen leads to the generation of reactive oxygen species (ROS) [8,9]. Therefore, they are interesting candidates for use in photosensitization processes, especially in situations where absorption in the red part of the spectra is not completely required.

Under aerobic conditions, the triplet excited state of fullerene ($^3C_{60}$*) can interact with ground state molecular oxygen to form ROS (Scheme 1). This process can occur by energy transfer from the $^3C_{60}$* to produce singlet molecular oxygen, $O_2(^1\Delta_g)$ (or simply $^1O_2$) or by electron transfer to form superoxide anion radical ($O_2{}^{\bullet-}$) [10]. Fullerenes are extremely efficient $^1O_2$ generators with a quantum yield that is near unity [11,12]. On the other hand, fullerenes can be easily reduced to C_{60} radical anion ($C_{60}{}^{\bullet-}$) by electron transfer [8]. Thus, the $^3C_{60}$* or $C_{60}{}^{\bullet-}$ can transfer an electron to molecular oxygen forming $O_2{}^{\bullet-}$. In contrast to $^1O_2$ generation, the electron transfer type of reaction preferentially occurs in polar solvents, particularly in the presence of reducing agents such as NADH [13]. These pathways, yielding $^1O_2$ and $O_2{}^{\bullet-}$, are analogous to the two main photochemical reaction types known as type II and type I mechanisms, respectively.

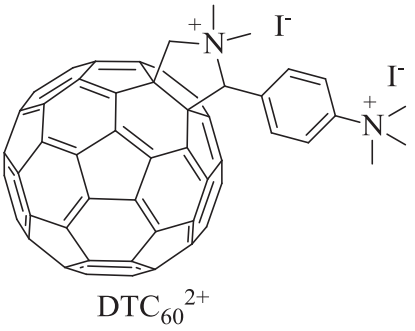
In the last years, several fullerene derivatives were evaluated as potential photosensitizers to inactivate microorganisms [14]. Tegos et al. have reported on the relative ability of two series of functionalized fullerenes to mediate photoinactivation of Gram-positive, Gram-negative bacteria, and fungi [15]. Also, there is a high level of selectivity for microbial cells over mammalian cells [16]. In previous studies, we have investigated the photodynamic activity of fullerene derivatives with different number of cationic charges as agents to eradicate *Escherichia coli* [17]. The amphiphilic structure of *N*,*N*-dimethyl-2-(4′-*N*,*N*,*N*-trimethylaminophenyl)fulleropyrrolidinium iodide ($DTC_{60}{}^{2+}$, Scheme 2) bearing two cationic groups showed to be an active photosensitizer.

In the present study, we examined the photogeneration of $O_2{}^{\bullet-}$ induced by $DTC_{60}{}^{2+}$ in a biomimetic medium containing NADH and its photodynamic action to eliminate *Candida albicans* cells. The photoinactivation of yeast was compared with that of *E. coli* under different experimental conditions. These studies provide insights into the specific mechanism of the photoreaction process which cause the cellular death of microorganisms after PDI treatment with $DTC_{60}{}^{2+}$.

Materials and methods

General

Absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan) using 1 cm path length quartz cuvettes at room temperature. Irradiation experiments were performed using a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) 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



Scheme 2 Structure of $DTC_{60}{}^{2+}$ fullerene derivative.

by optical filters. The light fluence rate at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Chemicals from Aldrich (Milwaukee, WI, USA) were used without additional purification. Benzyl-*n*-hexadecyldimethylamonium chloride (BHDC) from Sigma (St. Louis, MO, USA) was recrystallized twice from ethyl acetate and dried under vacuum over P_2O_5. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

Photosensitizers

N,*N*-Dimethyl-2-(4′-*N*,*N*,*N*-trimethylaminophenyl)fulleropyrrolidinium iodide (DTC_{60}^{2+}) was synthesized as previously described [17]. Stock solution of 0.5 mM DTC_{60}^{2+} was prepared by dissolution in 1 mL of *N*,*N*-dimethylformamide (DMF). The concentration was checked by spectroscopy, taking into account the value of molar extinction coefficient, $\varepsilon = 2.1 \times 10^3\ M^{-1}\ cm^{-1}$ at 429 nm in DMF [17].

Detection of superoxide radical anion by the nitro blue tetrazolium method

The nitro blue tetrazolium (NBT) method was used to detect $O_2^{\bullet -}$ formation in BHDC reverse micelles [13,18]. The micellar system was formed from a stock solution of 0.1 M BHDC, 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 ($W_0 = [H_2O]/[BHDC]$. The NBT method was carried out using 0.2 mM NBT, 0.5 mM NADH and 10 μM DTC_{60}^{2+} in benzene/BHDC (0.1 M)/water ($W_0 = 10$). Control experiments were performed in absence of NBT, NADH or DTC_{60}^{2+}. Samples were irradiated in 1 cm path length quartz cells (2 mL) under aerobic condition as described above. The progress of the reaction was monitored by following the increase of the absorbance at $\lambda = 560$ nm.

Microorganisms and growth conditions

The strains of *C. albicans* (PC31) and *E. coli* (EC7) were previously characterized and identified [19,20]. Cultivation and microorganisms handling were performed in sterile condition in order to avoid contamination with other strains. Microbial cells were grown aerobically overnight at 37 °C in 4 mL Sabouraud and tryptic soy (Britania, Buenos Aires, Argentina) broths for cultures of yeast and bacterium, respectively. Cell suspensions corresponding to ~10^6 colony forming units (CFU)/mL in 10 mM phosphate-buffered saline (PBS, pH 7.0) solution were prepared as earlier described [19,20]. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 mm × 100 mm) were used. Cellular suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable microbial cells were monitored and the number of CFU was determined on Sabouraud agar plates (~48 h incubation at 37 °C) for *C. albicans* or TS agar plates (~24 h incubation at 37 °C) for *E. coli*.

Photosensitized inactivation of *C. albicans* in PBS suspensions

Cellular suspensions of *C. albicans* (2 mL, ~10^6 CFU/mL) in PBS were incubated with 10 μM DTC_{60}^{2+} for 30 min in the dark at 37 °C. After that, the cultures were exposed to visible light as described above. Different irradiation times (0, 5, 15 and 30 min) were used to obtain a variation in the light fluence between 0 and 162 J/cm^2.

Growth curves of *C. albicans* cultures

A portion (1 mL) of overnight culture in Sabouraud broth was transfer to 20 mL of fresh Sabouraud broth medium and the suspension was homogenized. Then, 10 μM DTC_{60}^{2+} was added to aliquots of 2 mL culture and the flasks were immediately irradiated with visible light at 37 °C. In this experiment, the irradiation was performed without incubation because this protocol was used to evaluate the ability of the photosensitizer to prevent the growth of the cell culture. Control containing 10 μM DTC_{60}^{2+} was kept in dark. The culture grown was measured by turbidity at 660 nm using a Tuner SP-830 spectrophotometer (Dubuque, IA, USA).

Photoinactivation of microbial suspensions cells under different conditions

Cellular suspensions of microorganisms (2 mL, ~10^6 CFU/mL) in PBS were incubated with 10 μM DTC_{60}^{2+} (*C. albicans*) or 1 μM DTC_{60}^{2+} (*E. coli*) for 30 min in the dark at 37 °C. Different concentrations of DTC_{60}^{2+} were used because the photocytotoxic effect was dissimilar for cultures of fungi and bacteria. A higher concentration of fullerene was necessary to obtain an efficient inactivation of *C. albicans* cells. After that, the cultures were exposed to visible light for 30 min. Studies in the absence of oxygen were performed by displacing the oxygen with argon in the cultures flasks for 15 min before irradiation and maintaining argon atmosphere during the illumination. Sodium azide or mannitol were added to yeast suspensions from 2.5 M stock solutions in water and the cells were incubated for 30 min at 37 °C in dark previous to the treatment with the photosensitizer. For photoinactivation assays in D_2O, cells were centrifuged (3000 rpm for 15 min) and re-suspended in 2 mL D_2O, then the cell suspensions were incubated with the corresponding amount of DTC_{60}^{2+} as described above.

Controls and statistical analysis

In all cases, control experiments were conducted by irradiating the cell suspensions in the absence of DTC_{60}^{2+} and keeping DTC_{60}^{2+} treated cells in the dark. DMF was not toxic at the concentrations used. Each experiment was repeated separately three times. Variation between groups was evaluated using Student's *t*-test, with a confidence level of 95% ($p < 0.05$) considered statistically significant.

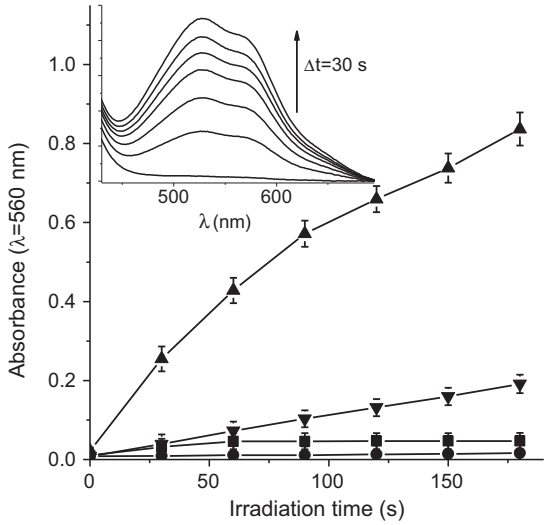


Figure 1 Time course of $O_2^{\bullet -}$ generation detected by the NBT method as an increase in the absorption at 560 nm. Samples contain 10 μM DTC_{60}^{2+}, 0.2 mM NTB and 0.5 mM NADH (▲); without DTC_{60}^{2+} (▼); without NADH (●) and without NBT (■) in benzene/BHDC (0.1 M)/W_0 = 10 micelles irradiated with visible light for different times. Values represent mean ± standard deviation of three separate experiments. Insert: absorption spectra changes of NTB after different irradiation times (Δt = 30 s).

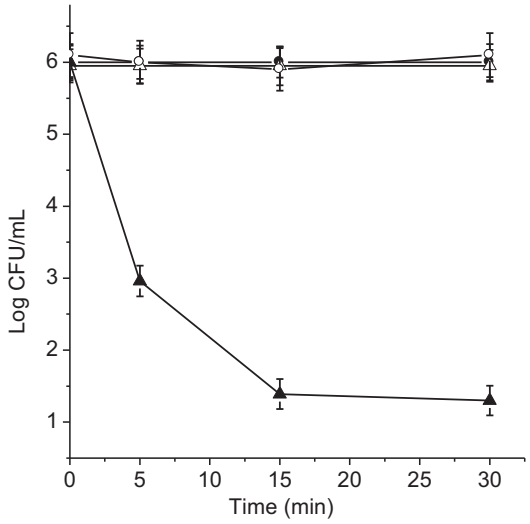


Figure 2 Survival curves of *C. albicans* cells (~10^6 CFU/mL) incubated with 10 μM DTC_{60}^{2+} for 30 min at 37 °C in dark and exposed to visible light for different irradiation times (▲) and keeping in dark (△). Control culture untreated with DTC_{60}^{2+} and irradiated (●) and untreated with DTC_{60}^{2+} in dark (◯). Values represent mean ± standard deviation of three separate experiments.

Results

Detection of $O_2^{\bullet -}$

Generation of $O_2^{\bullet -}$ was detected by NBT reduction to formazan following the absorption at 560 nm in benzene/BHDC (0.1 M)/W_0 = 10 micelles (Fig. 1, inset). The increase of formazan absorption was monitored as a function of time after irradiation of samples with visible light. As shown in Fig. 1, reduction of NBT by $O_2^{\bullet -}$ was not detected in the photoirradiated samples without NADH or NBT. The effect of light irradiation on the decomposition of NBT considerably increases in the micellar system containing DTC_{60}^{2+} and NADH with respect to solution without the fullerene derivative. These results indicate an important contribution of the photodynamic activity induced by DTC_{60}^{2+} in the formazan production.

Photosensitized inactivation of *C. albicans*

Photoinactivation of *C. albicans* was evaluated in PBS cellular suspensions treated with 10 μM DTC_{60}^{2+} for 30 min in dark at 37 °C and irradiated for different times with visible light (Fig. 2). Control experiments showed that the viability of *C. albicans* was unaffected by illumination alone or by dark incubation with 10 μM DTC_{60}^{2+}. Therefore, the cell inactivation obtained after irradiation of the cultures treated with the DTC_{60}^{2+} was due to the photosensitization effect of the fullerene derivative. As seen in Fig. 2, the *C. albicans* cells were rapidly photoinactivated when the cultures treated with 10 μM DTC_{60}^{2+} were exposed to visible light. Thus, the dicationic fullerene exhibited a photosensitizing activity causing ~3 log decrease of cell survival after 5 min irradiation. An increase in light exposure of the cultures was accompanied by an enhancement in the PDI efficiency, producing ~5 log decrease after 15 min irradiation.

Photosensitized growth delay of *C. albicans* cultures

The photocytotoxic activity on growth of *C. albicans* cultures sensitized by DTC_{60}^{2+} was achieved in Sabouraud medium. Thus, 10 μM DTC_{60}^{2+} was added to fresh cultures of *C. albicans* reaching the log phase and the flasks were continuously irradiated with visible light for 11 h at 37 °C. The effect induced by DTC_{60}^{2+} on growth of cells is shown in Fig. 3. As can be observed, cells of *C. albicans* treated with DTC_{60}^{2+} in the dark or not treated with the fullerene derivative and illuminated showed similar behavior as the controls. On the contrary, growth was delayed when *C. albicans* cultures were treated with DTC_{60}^{2+} and illuminated. After irradiation in the presence of 10 μM DTC_{60}^{2+}, the cells did not appear to be growing as measured by turbidity at 660 nm. Also, the cell viability evaluations post treatments by spread plate technique did not show colonies formation, indicating a complete eradication of *C. albicans* cells.

Studies of the photodynamic action mechanism in microbial cells

In order to obtain insight about the photodynamic mechanism, the photosensitized inactivation of *C. albicans* and *E. coli* cells was examined under different experimental conditions, such as an argon atmosphere, cellular suspensions in D_2O and addition of azide ion or mannitol.

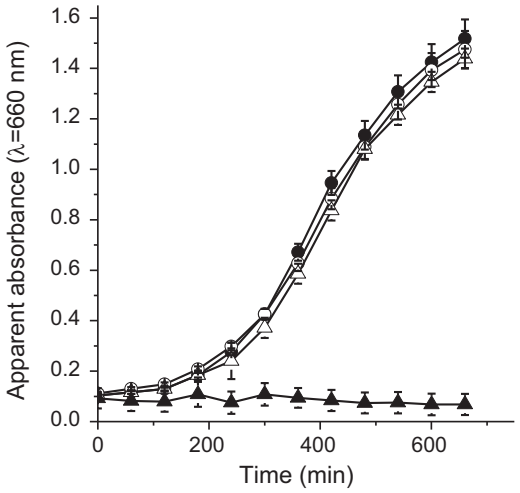


Figure 3 Growth curves of *C. albicans* cells incubated with 10 μM DTC_{60}^{2+} and exposed to different irradiation times with visible light in Sabouraud broth at 37 °C (▲). Control cultures: cells treated 10 μM of DTC_{60}^{2+} in dark (△), cells untreated irradiated (●) and in dark (○). Values represent mean ± standard deviation of three separate experiments.

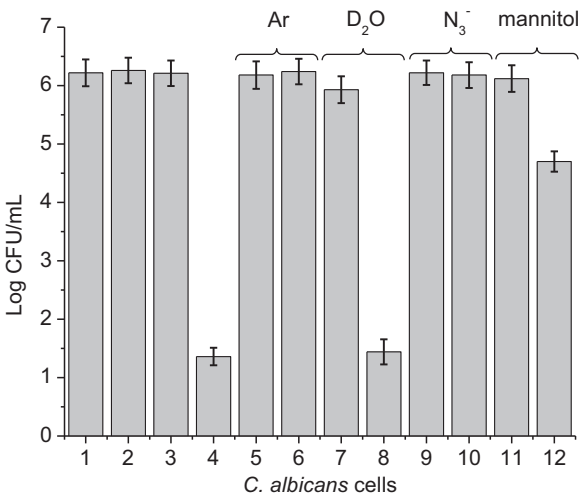


Figure 4 Survival of *C. albicans* cells (~10^6 CFU/mL) incubated with 10 μM DTC_{60}^{2+} in dark for 30 min at 37 °C and exposed to visible light for 30 min: (1) control culture in dark; (2) control culture irradiated; (3) control culture treated with DTC_{60}^{2+} and kept in dark; (4) culture treated with DTC_{60}^{2+} and irradiated; (5) control culture under argon and irradiated; (6) culture treated with DTC_{60}^{2+} under argon and irradiated; (7) control culture in D_2O and irradiated; (8) culture treated with DTC_{60}^{2+} in D_2O and irradiated; (9) control culture containing 100 mM azide and irradiated; (10) culture treated with DTC_{60}^{2+} containing 100 mM azide and irradiated; (11) control culture containing 100 mM mannitol and irradiated; (12) culture treated with DTC_{60}^{2+} containing 100 mM mannitol and irradiated. Values represent mean ± standard deviation of three separate experiments.

PDI under anoxic atmosphere

The irradiation of *C. albicans* and *E. coli* cellular suspensions treated with DTC_{60}^{2+} was performed under argon atmosphere. These experiments were used to evaluate the influence of oxygen atmosphere on the cell viability. No toxicity was observed for cell suspensions irradiated under an argon atmosphere (Figs. 4 and 5, line 5) or cells treated with DTC_{60}^{2+} under an argon atmosphere in the dark (result not shown). As can be observed, the loss of viability was highly oxygen dependent for *C. albicans* (Fig. 4, line 6) and *E. coli* (Fig. 5, line 6) cultures. Almost, cell inactivation did not take place using DTC_{60}^{2+} as photosensitizer under a low oxygen concentration.

PDI of cell suspensions in D_2O

The photoinactivation was carried out in D_2O to evaluate the $^1O_2$-mediated damage of microbial cells induced by DTC_{60}^{2+}. This medium was used to increase the lifetime of $^1O_2$ [21]. Thus, cell viabilities treated with DTC_{60}^{2+} were not affected in D_2O for 30 min in dark (result not shown) nor in D_2O under irradiation without photosensitizer (Figs. 4 and 5, line 7). Irradiation of *C. albicans* (Fig. 4, line 8) and *E. coli* (Fig. 5, line 8) cells incubated with DTC_{60}^{2+} in D_2O produced a similar cell photoinactivation than those found in PBS cell suspensions.

Effect of azide ion on PDI

The cell suspensions of *C. albicans* and *E. coli* were treated with DTC_{60}^{2+} in the presence of 100 mM and 50 mM azide ion, respectively. The azide ion is a quencher of $^1O_2$ but it also can deactivate compounds in their triplet excited state [22]. These concentrations of sodium azide were not toxic in the dark containing DTC_{60}^{2+} (result not shown) or under irradiation without DTC_{60}^{2+} (Figs. 4 and 5, line 9). The resulting photoinactivation was greatly affected by the azide ion, the addition of which produced a high reduction in the cell inactivation photosensitized by DTC_{60}^{2+}. Differences with the controls were not found for *C. albicans* (Fig. 4, line 10) and *E. coli* (Fig. 5, line 10) after 30 min irradiation. Thus, the presence of azide ion quenched the photocytotoxic species, producing a protective effect on microbial cells.

Effect of mannitol on PDI

The photoinactivation of microorganisms was investigated after treatment with mannitol and DTC_{60}^{2+}. This compound acts as scavenger of the superoxide anion radical and hydroxyl radical [23]. The addition of 100 mM mannitol for *C. albicans* and 50 mM mannitol for *E. coli* were not cytotoxic for cells treated with DTC_{60}^{2+} in dark (result not shown). Also, no toxicity was found for cells irradiated in the presence of mannitol (Figs. 4 and 5, line 11). After 30 min illumination, cell inactivation incubated with DTC_{60}^{2+} showed a photoprotective effect in the presence of mannitol, causing a ~1.5 log decrease in *C. albicans* cell survival (Fig. 4, line 12). Also, a reduction of ~1 log in the photoinactivation of *E. coli* was found in cultures containing mannitol (Fig. 5, line 12).

Discussion

In last years, fullerene derivatives attracted an increased interest because these nanostructures prove characteristics for biomedical applications [24]. In the present case, the compound DTC_{60}^{2+} bears a hydrophobic carbon sphere

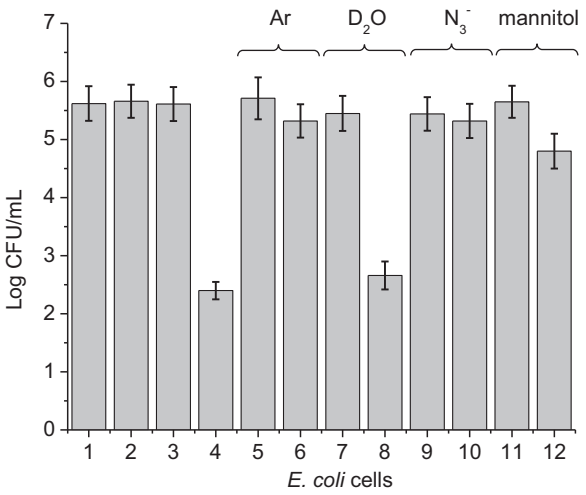


Figure 5 Survival of *E. coli* cells (~10^6 CFU/mL) incubated with 1 μM DTC_{60}^{2+} in dark for 30 min at 37 °C and exposed to visible light for 30 min: (1) control culture in dark; (2) control culture irradiated; (3) control culture treated with DTC_{60}^{2+} and kept in dark; (4) culture treated with DTC_{60}^{2+} and irradiated; (5) control culture under argon and irradiated; (6) culture treated with DTC_{60}^{2+} under argon and irradiated; (7) control culture in D_2O and irradiated; (8) culture treated with DTC_{60}^{2+} in D_2O and irradiated; (9) control culture containing 50 mM azide and irradiated; (10) culture treated with DTC_{60}^{2+} containing 50 mM azide and irradiated; (11) control culture containing 50 mM mannitol and irradiated; (12) culture treated with DTC_{60}^{2+} containing 50 mM mannitol and irradiated. Values represent mean ± standard deviation of three separate experiments.

substituted by two cationic groups forming one amphiphilic monoadduct. The presence of a dicationic moiety in the periphery of fullerene derivative considerably enhances the dipole moment with respect to the non-charged structures. This effect helps the fullerene derivatives to pass through or accumulate in biomembranes, enhancing the effective photosensitization. Besides, the generation of ROS by fullerenes through energy transfer and electron transfer was studied by a combination of methods [13]. Thus, the photodynamic activity of DTC_{60}^{2+} was studied in different media. The $^1O_2$ production quantum yield (Φ_Δ) of this fullerene was 0.07 in DMF/water (10%, v/v). This low $^1O_2$ production of DTC_{60}^{2+} was probably due to an incomplete monomerization of the cationic fullerene in the DMF/water medium. However, an increase in the $^1O_2$ production of DTC_{60}^{2+} was found in a biomimetic microenvironment formed by BHDC micelles. This enhancement in the microheterogeneous medium was facilitated by a better solubilization as monomer of DTC_{60}^{2+} [17]. Also, it was demonstrate that under aerobic conditions the decomposition of NBT occurred predominantly through a type I photoreaction process, indicating the formation of $O_2^{\bullet-}$ [13]. Thus, when DTC_{60}^{2+} was irradiated in benzene/BHDC/water reverse micelles containing NADH, the production of $O_2^{\bullet-}$ was observed by the appearance of formazan (Fig. 1). Therefore, whereas $^1O_2$ can be generated effectively by photoexcited $^3C_{60}$* in nonpolar solvents such as benzene and benzonitrile, it was found that $O_2^{\bullet-}$ and hydroxyl radical (HO•) were produced instead of $^1O_2$ in polar solvents such as water, especially in the presence of a physiological concentration of a reductant, including NADH. It was also showed that visible light irradiation of solubilized fullerenes into water, with polyvinylpyrrolidone as a detergent, resulted in the formation of $^1O_2$ and $O_2^{\bullet-}$, when experiments were performed in the presence of NADH [25]. However, the biological microenvironment of the photosensitizer can induce important modifications in the photophysics of the fullerene derivatives established in solution. In consequence, there are limitations to predict photodynamic efficiencies of DTC_{60}^{2+} in biological systems on the basic of photophysical investigations in solutions. Therefore, it is interesting to obtain information about ROS generation that predominantly occurs in the microbial experiments. First, appropriate conditions were determined for an effective photoinactivation of microorganisms in PBS suspensions. In previous investigations, the photodynamic activity of DTC_{60}^{2+} was evaluated in *E. coli* cells [17]. The results showed that cell suspensions of this Gram-negative bacterium were rapidly photoinactivated when the cultures treated with 1 μM DTC_{60}^{2+} were exposed to visible light. In particular, the dicationic fullerene exhibited a photosensitizing activity causing a ~3.5 log decrease of cell survival, when the cultures were irradiated for 30 min. Moreover, DTC_{60}^{2+} showed to be an active photosensitizer to eradicate *C. albicans* cells (Fig. 2). After 30 min incubation with 10 μM DTC_{60}^{2+} in dark, the tendency observed was a fast reduction in the yeast viability for the irradiated cultures. Thus, an exposure to visible light of 15 min produced a photoinactivation greater than 99.999%. Furthermore, the photodynamic activity of this dicationic fullerene was established by growth delay of *C. albicans* cultures (Fig. 3). Under these conditions, the growth was suppressed when *C. albicans* cells were treated with 10 μM DTC_{60}^{2+} and continuously irradiated with visible light. Also, it was previously showed that growth of *E. coli* cultures was delayer when treated with 2 μM DTC_{60}^{2+} under continuous irradiation [17]. These experiments confirm that PDI of cells is still possible when the cultures were not under starvation conditions.

The photoreaction mechanisms by which light and fullerene derivatives cause biological damage have been the subject of many studies [9]. In this work, to obtain information about the main photoprocesses involved in the inactivation of *C. albicans* and *E. coli* cells by DTC_{60}^{2+}, the PDI was investigated under different experimental conditions (Figs. 4 and 5). The results showed that the photocytotoxic effect induced by DTC_{60}^{2+} was negligible under anoxic conditions. Although, the presence of oxygen is essential for the generation of $^1O_2$ through the type II photosensitization mechanism that involves a triplet energy transfer reaction. Also, oxygen plays a major role in the type I mechanism by adding to biochemical radicals. In a type I process, the light-excited photosensitizers directly interact with substrate to yield radical ions in a hydrogen atom or electron transfer reaction. The majority of these radicals instantaneously reacts with oxygen and generates a complicated mixture of highly reactive oxygen intermediates, which can oxidize a wide variety of biomolecules [22]. Oxygen is also necessary for the formation of $O_2^{\bullet-}$ that can occur as the result of the reaction of molecular oxygen with the radical anion of the photosensitizer. Therefore, these experiments are not decisive in establishing the predominant photoreaction process involved in the cytotoxicity.

To evaluate the $^1O_2$-mediated photoinactivation of microorganisms, the PDI was performed in D_2O. Under this condition, the photocytotoxic activity of DTC_{60}^{2+} was similar to that found in PBS cell suspensions. These results reveal a low contribution of type II photosensitization in the inactivation of these microorganisms. Also, in order to assess the involvement of $^1O_2$, experiments were carried out in the presence of sodium azide, a known quencher of $^1O_2$ [26]. The presence of sodium azide produced almost complete photoprotection of *C. albicans* and *E. coli* cells. This suggests that azide ions cause significant lowering of the photodynamic effects of the DTC_{60}^{2+} by quenching $^1O_2$. The azide ion prevents both type I and type II photoprocesses [22]. Therefore, to investigate the predominant mechanisms of action mannitol was used as a type I scavenger [23]. The results indicated that phototoxicity efficacy was significantly affected when mannitol was added to *C. albicans* or *E. coli* cells sensitized by DTC_{60}^{2+}. Thus, the photoprotective effect produced by mannitol was indicative of some contribution of type I reactions in the inactivation process.

In a complex environment of intracellular space, which contains reducing agents, such as NADH, the electron transfer from the $C_{60}^{\bullet-}$ to molecular oxygen (type I photochemical mechanism) or directly from the reducing agent to $^1O_2$ could also yield $O_2^{\bullet-}$ and, subsequently, hydrogen peroxide (H_2O_2) and $HO^{\bullet}$ [27]. It is therefore an interesting strategy to employ ROS-generating/quenching capacity of C_{60} in designing biologically active agents with diverse functions. Type I mechanisms can be similarly effective or even more effective than type II. This is because hydroxyl radicals are the most reactive and potentially the most cytotoxic of all ROS. It is assumed that hydroxyl radicals are formed from H_2O_2 by Fenton chemistry reactions catalyzed by Fe^{2+} or Cu^{+} ions, and that the H_2O_2 is produced by dismutation of $O_2^{\bullet-}$ either by enzyme catalysis or naturally [13]. Another possible mechanism of cytotoxicity is the diffusion-controlled reaction between superoxide and nitric oxide to form peroxynitrite, a highly toxic species [28]. The photochemical mechanism studies with cationic fullerenes confirmed that depending on the precise conditions of the experiment, illuminated fullerenes can produce both $O_2^{\bullet-}$ and $^1O_2$ [9].

Conclusions

Whereas in previous studies $^1O_2$ was generated by photoexcited DTC_{60}^{2+} in DMF, we found that $O_2^{\bullet-}$ was produced in benzene/BHDC/water reverse micelles, especially in the presence of a biological reducing agent, NADH. Studies of PDI *in vitro* on *C. albicans* cells provide information on the photodynamic activity as antifungal of this cationic fullerene DTC_{60}^{2+}. Photosensitized inactivation of *C. albicans* cellular suspensions by DTC_{60}^{2+} exhibits a ~5 log decrease of cell survival after 30 min of irradiation, which represents about 99.999% of cellular inactivation. On the other hand, the present study provides knowledge about the photodynamic mechanism that takes place in the PDI of *C. albicans* and *E. coli* cells sensitized by DTC_{60}^{2+}. To elucidate the oxidative processes that occur during the killing of microbial cells, first, the effect of the media was analyzed on cell photoinactivation. It was observed that an oxygen atmosphere is necessary for an efficient photoinactivation. Moreover, photooxidative cell killing was similar in D_2O and PBS, although the lifetime of $^1O_2$ is longer in D_2O. Photoprotection was found using sodium azide as type II scavengers. Also, phototoxicity efficacy was affected when mannitol was used as a type I scavenger. Therefore, the present *in vitro* experiments have shown that DTC_{60}^{2+} is a highly effective photosensitizer to eradicate *C. albicans*. The killing of microbial cells by DTC_{60}^{2+} and visible light irradiation can be mediated by a contribution of both type I and type II processes.

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