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Approaches to unravel pathways of reactive oxygen species in the photoinactivation of bacteria induced by a dicationic fulleropyrrolidinium derivative

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ABSTRACT

The photodynamic mechanism sensitized by *N*,*N*-dimethyl-2-[4-(3-*N*,*N*,*N*-trimethylammoniopropoxy)phenyl]fulleropyrrolidinium (DPC_{60}^{2+}) was investigated in *Staphylococcus aureus* cells. Different experimental conditions were used to detect reactive oxygen species (ROS) in *S. aureus* cell suspensions. First, a photoinactivation of 4 log decrease of *S. aureus* viability was chosen using 0.5 μM DPC_{60}^{2+} and 15 min irradiation. An anoxic atmosphere indicated that oxygen was required for an effective photoinactivation. Also, photoprotection was found in the presence of sodium azide, whereas the photocytotoxicity induced by DPC_{60}^{2+} increased in D_2O. The addition of diazabicyclo[2.2.2]octane or D-mannitol produced a reduction in the *S. aureus* photokilling. Moreover, singlet molecular oxygen, $O_2(^1\Delta_g)$, was detected by the reaction with 9,10-dimethylanthracene into the *S. aureus* cells. A decrease in the photoinactivation of *S. aureus* was observed in the presence of β–nicotinamide adenine dinucleotide reduced form, which was dependent on the NADH concentration. Therefore, under aerobic condition the photocytotoxicity activity induced by DPC_{60}^{2+} was mediated by mainly a contribution of type II process. Moreover, photoinactivation of *S. aureus* was possible with DPC_{60}^{2+} in the presence of azide anions under anoxic condition. However, these conditions were not effective to photoinactivate *Escherichia coli*. On the other hand, the addition of potassium iodide produced an increase in the photokilling of bacteria, depending on the KI concentration and irradiation times. The formation of reactive iodine species may be contributing to inactivate *S. aureus* cells photoinduced by DPC_{60}^{2+}.

1. Introduction

The use of antibiotics to eradicate microorganisms means one of the most revolutionary progresses made in scientific medicine. However, the changing patterns of infectious diseases and the emergence of antibiotic-resistant microbes have posed new challenge to researchers [1]. This is essentially due to inappropriate prescriptions of antibiotics, the application in prophylaxis and the systemic use that can also affect the normal flora and the failure of patients to complete the treatments. Well known resistance carriers with high clinical impact include the Gram-positive organism *Staphylococcus aureus*. This is an opportunistic pathogen responsible for a diverse spectrum of human and livestock diseases; and it is also associated with asymptomatic colonization of the skin and mucosal surfaces of normal humans. Most *S. aureus* infections occur in previously colonized persons who also act as reservoirs for continued dissemination [2]. Historically, *S. aureus* has been a serious human pathogen, and recent few decades it has become a more serious threatening agent due to acquisition of antibiotic resistance [3]. Thus, infection with *S. aureus* has become endemic in hospitals around the world. This Gram-positive bacterium is an important human pathogen and a common etiological factor of health care associated as well as community acquired infections. Due to its growing antibiotic resistance it is crucial to find alternative antimicrobial treatment to which bacteria will not easily develop resistance. Therefore, it is imperative to provide perspectives for the future prophylaxis or new treatments for *S. aureus* infections [1]. Also, some Gram-negative bacteria, particularly *Escherichia coli*, have developed resistance to most or all available antibiotics [4]. Thus, the photodynamic inactivation (PDI) of microbes has been suggested to controlling microbial diseases [5]. PDI involves the addition of a photosensitizer that which preferentially binds to the microorganisms. The irradiation with

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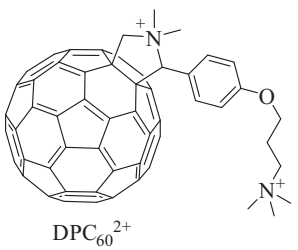
visible light in the presence of oxygen yields highly reactive oxygen species (ROS), which rapidly react with a variety of substrates producing damages in the biomolecules. These changes generate a loss of biological functionality leading to cell inactivation [6,7].

Fullerenes can act as photosensitizers due to their visible light absorption and a long lifetime of triplet excited state. Irradiation of solubilized fullerenes in the presence of oxygen leads to the production of ROS. Therefore, fullerenes have been proposed as phototherapeutic agents for photodynamic therapy of neoplastic diseases [8]. The lack of solubility in biological environments represents an inconvenient in the development of this field. However, the progress of covalent chemistry of fullerene C_{60} can be used to attach this sphere structure with several functional groups, which allows increment the biological activity. Also, interesting results have been found using amphiphilic cationic fullerenes as photosensitizers to photoinactivate microorganisms [9]. Thus, functionalized fullerene derivatives with cationic charges were obtained as broad-spectrum antimicrobial photosensitizers against a panel of human pathogens.

It was previously established that *N*,*N*-dimethyl-2-[4-(3-*N*,*N*,*N*-trimethylammoniopropoxy)phenyl]fulleropyrrolidinium (DPC_{60}^{2+}, Scheme 1) is an active photosensitizer for the eradication of *S. aureus* [10]. The photoreaction mechanisms by which light and fullerene derivatives cause biological damage have been the subject of many studies [11,12]. Reaction of ROS with various biological targets, including unsaturated lipids, proteins, and nucleic acids, resulting in cell damage or functional alterations is thought to be responsible for cell inactivation. Therefore, in the present study we are interested in to obtain mechanistic insight about type I/type II processes involved in the photoinactivation of *S. aureus* cells mediated by DPC_{60}^{2+}. In this way, PDI of *S. aureus* was investigated under different conditions to find information about the photoprocesses that produces the cell death. Photoinactivation experiments were performed varying the atmosphere and the composition of cell suspensions and using scavengers of ROS. 9,10-Dimethylantracene (DMA) was used as molecular probe to sense the formation of $O_2(^1\Delta_g)$ induced by DPC_{60}^{2+} into the cells. Also, *S. aureus* photokilling was evaluated in the presence of different concentrations of β-nicotinamide adenine dinucleotide reduced form (NADH). These studies were used to increase the knowledge of the balance between type I and type II mechanisms mediated by dicationic fullerene derivatives in Gram-positive and Gram-negative bacteria, which may depend on the microenvironment where the photosensitizer is bound to the target microbial cells. Moreover, the effect produced by sodium azide (NaN_3) on the photoinactivation of *S. aureus* and *E. coli* was compared under aerobic condition and a free-oxygen atmosphere. In the present study, the effect of the inert inorganic salt potassium iodide (KI) was also investigated in combination with PDI sensitized by DPC_{60}^{2+}. Thus, bacterial photokilling was determined against *S. aureus* and *E. coli* using different concentrations of KI to stablish conditions that increase the microbial photoinactivation.



Scheme 1. Chemical structure of fullerene derivative DPC_{60}^{2+}.

2. Materials and methods

2.1. General

UV–visible absorption spectra were performed in a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Steady-state fluorescence emission spectra were recorded using a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc, Edison, NJ, USA). Fluence rates were obtained with a Radiometer Laser Mate-Q (Coherent, Santa Clara, CA, USA). Cell suspensions were irradiated with a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector containing a 150 W lamp. A wavelength range between 350 and 800 nm was selected by optical filters. Experiments were performed at room temperature with a fluence rate of 90 mW/cm^2. Photooxidation of DMA in cell suspensions was studied using a Cole-Parmer illuminator 41720-series (Cole-Parmer, Vernon Hills, IL, USA) with a 150 W halogen lamp. A wavelength range between 455 and 800 nm (30 mW/cm^2) was selected using an optical filter (GG455 cutoff filter). All chemicals were purchased from Aldrich (Milwaukee, WI) and used without further purification.

2.2. Photosensitizer

N,*N*-Dimethyl-2-[4-(3-*N*,*N*,*N*-trimethylammoniopropoxy)phenyl]fulleropyrrolidinium iodide (DPC_{60}^{2+}) was synthesized as previously described [10]. 5,10,15,20-Tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin p-tosylate ($TMAP^{4+}$) was purchased from Aldrich. Stock solutions (0.5 mM) of photosensitizers were prepared by dissolution in 1 mL of *N*,*N*-dimethylformamide (DMF).

2.3. Bacterial strain and preparation of cultures

The Gram-positive strain *S. aureus* ATCC 25923 and Gram-negative *E. coli* EC7 were used in this study [10,12]. This bacterium was aerobically grown on a rotator shaker (100 rpm) at 37 °C in tryptic soy (TS, Britania, Buenos Aires, Argentina) broth overnight. An aliquot (60 μL) of this culture was aseptically transferred to 4 mL of fresh medium (TS broth) and incubated at 37 °C to mid logarithmic phase of growth (absorbance ~0.6 at 660 nm). After that, cells were harvested by centrifugation (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 1/10 or 1/1000 in PBS, corresponding to ~10^8 and ~10^6 colony forming units (CFU)/mL, respectively. After each experiment, cell 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 TS agar plates after ~24 h incubation at 37 °C.

2.4. Photosensitized inactivation of bacteria

Cell suspensions of *S. aureus* (~10^8 CFU/mL) in PBS were obtained as described above. Bacterial cells (2 mL) were treated with 0.5 μM of DPC_{60}^{2+} in Pyrex culture tubes (13 × 100 mm) in the dark for 10 min at 37 °C. DPC_{60}^{2+} was added from a stock solution in DMF (0.5 mM). Then, the cell suspension (0.2 mL) was placed in each well of 96-well microtiter plates (Deltalab, Barcelona, Spain). The cultures were irradiated with visible light for 15 min. Experiments under anoxic conditions were carried out with argon to remove the oxygen. Thus, cell suspension (0.2 mL) was transferred to a thin tube (5 × 20 mm) to allow bubbling with argon. The culture tube was resealed with a rubber septum in the dark. The septum was pierced with a hollow needle connected to argon line and samples were bubbled with this gas for 10 min before irradiation.

An argon atmosphere was keeping in the sample during the irradiation. Studies in deuterated saline water (D_2O) were performed using cell suspensions (2 mL) in PBS, which were centrifuged (3000 rpm for 15 min) and re-suspended in D_2O (2 mL). Then the cell suspensions in D_2O were incubated with 0.5 μM DPC_{60}^{2+} for 10 min in dark at 37 °C. Stock solutions (2 M) of sodium azide and D-mannitol were prepared in water. Cells were treated with 50 mM sodium azide or D-mannitol in dark for 10 min at 37 °C previous to the incubation with 0.5 μM DPC_{60}^{2+} as described above. Diazabicyclo[2.2.2]octane (DABCO) was added from a stock solution (0.2 M) in water. *S. aureus* cells were incubated with 10 mM DABCO for 10 min in dark at 37 °C previous to the addition of 0.5 μM DPC_{60}^{2+}. Stock solution (0.2 M) of NADH was prepared in water. Bacterial cells were also incubated with different concentration of NADH (10, 25 and 50 mM) for 10 min in dark at 37 °C and then were treated with 0.5 μM of DPC_{60}^{2+} as previously described. Experiments in the presence of KI were carried out using a stock solution (1 M) in water. *S. aureus* cells were incubated with different concentrations of IK (10, 25 and 50 mM) for 10 min in dark at 37 °C. After that, 0.5 μM DPC_{60}^{2+} was added as described above. Cell suspensions were immediately irradiated for 15 min with visible light. The number of cells was determined as described above. Similar procedure was used with *E. coli* but incubating with 10 μM of DPC_{60}^{2+}.

2.5. Steady state photolysis

Cell suspensions of *S. aureus* (2 mL, ~10^6 CFU/mL) were treated with DMA (10 μM) for 10 min in dark at 37 °C. Cells were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in 2 mL PBS. Then, different protocols were followed: a) cells were incubated with 0.5 μM DPC_{60}^{2+} for 10 min in dark at 37 °C, b) cells were incubated with 0.5 μM DPC_{60}^{2+} for 10 min in dark and then treated with 50 mM sodium azide for 10 min in dark at 37 °C; and c) cells were treated with 0.5 μM $TMAP^{4+}$, which was used as a reference, for 10 min in dark at 37 °C. *S. aureus* suspensions were irradiated in a quartz cell of 1 cm path length. Photooxidation of DMA was studied by following the decrease of the fluorescence intensity at λ = 428 nm, exciting the samples at λ_{exc} = 375 nm. Control experiments showed that under these conditions the fluorescence intensity correlates linearly with substrate concentrations. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of semi-logarithmic plots of ln (I_0/I) *vs.* time [13].

2.6. Controls and statistical analysis

Control experiments were carried out in the presence and absence of DPC_{60}^{2+} in the dark and in the absence of DPC_{60}^{2+} with cells irradiated. The amount of DMF (<1% v/v) used in each experiment was not toxic to *S. aureus* cells. Three values were obtained per each condition and each experiment was repeated separately three times. Differences between means were tested for significance by one-way ANOVA. Results were considered statistically significant with a confidence level of 95% ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

3. Results

3.1. Effect of media on the photoinactivation of S. aureus

Bacterial cells were treated with 0.5 μM DPC_{60}^{2+} and irradiated for 15 min with visible light. PDI mediated by DPC_{60}^{2+} showed a photosensitizing activity causing a 4 log decrease of cell survival (Fig. 1, line 4). Furthermore, no toxicity was detected for *S. aureus* cells irradiated for 15 min within DPC_{60}^{2+} (Fig. 1, line 2). Also, 0.5 μM DPC_{60}^{2+} was not toxic in dark (Fig. 1, line 3). Therefore, this concentration and an irradiation time of 15 min were chosen to not produce a complete eradication of *S. aureus*, which allow observing a photoprotective effect or an increase in the photokilling under different experimental conditions used to inactivate of *S. aureus* cells.

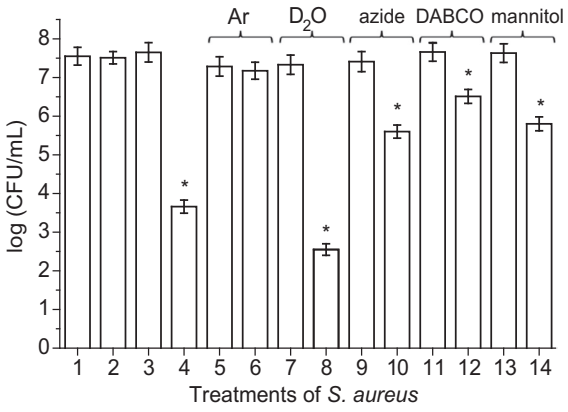


Fig. 1. Survival of *S. aureus* cell suspensions (~10^8 CFU/mL) incubated with 0.5 μM DPC_{60}^{2+} for 10 min in dark at 37 °C and irradiated with visible light for 15 min; 1) cells in dark; 2) irradiated cells; 3) cells treated with DPC_{60}^{2+} in dark; 4) irradiated cells treated with DPC_{60}^{2+}; 5) irradiated cells under Ar; 6) irradiated cells treated with DPC_{60}^{2+} under Ar; 7) irradiated cells in D_2O; 8) irradiated cells treated with DPC_{60}^{2+} in D_2O; 9) irradiated cells treated with 50 mM azide; 10) irradiated cells treated with DPC_{60}^{2+} and 50 mM azide; 11) irradiated cells treated with 10 mM DABCO, 12) irradiated cells treated with DPC_{60}^{2+} and 10 mM DABCO, 13) irradiated cells treated with 50 mM D-mannitol; 12) irradiated cells treated with DPC_{60}^{2+} and 50 mM D-mannitol (*$p < 0.05$, compared with control).

Photoinactivation of *S. aureus* cell by DPC_{60}^{2+} was investigated under an argon atmosphere. This anoxic condition was used to establishing the implication of oxygen on the PDI of *S. aureus*. Under an argon atmosphere, cell viability was not changed after irradiation without DPC_{60}^{2+} (Fig. 1, line 5) or in the presence of DPC_{60}^{2+} kept in the dark (result not shown). Moreover, cell viability was not affected for cells incubated with DPC_{60}^{2+} after 15 min irradiation (Fig. 1, line 6). Therefore, aerobic condition was necessary to produce photosensitized inactivation of *S. aureus*.

Photoinactivation of *S. aureus* mediated by DPC_{60}^{2+} was performed in cells suspended in D_2O to evaluate the $O_2(^1\Delta_g)$-mediated damage of microbial cells. A medium with D_2O was used to increase the lifetime of $O_2(^1\Delta_g)$ in the biological system [14]. No toxicity was detected in the presence of D_2O under irradiation without DPC_{60}^{2+} (Fig. 1, line 7). Also, DPC_{60}^{2+} was not toxic for cells in dark (result not shown). However, a high cell photoinactivation of 5 log decrease was observed for *S. aureus* cells incubated with DPC_{60}^{2+} in D_2O (Fig. 1, line 8), which was 1 log more effective than that found in PBS cell suspension after 15 min irradiation.

3.2. Effect of scavengers of ROS on the photoinactivation of S. aureus

The effect of suppressors of ROS, sodium azide, DABCO and D-mannitol, were investigated to obtain insight about the photodynamic mechanism involved in the photosensitized inactivation of *S. aureus* cells mediated by DPC_{60}^{2+}. Azide anion can be used as a quencher of intracellular $O_2(^1\Delta_g)$ [15]. However, it also can deactivate photosensitizers in their triplet excited state preventing both type I and type II photoprocesses. Bacterial cells were treated with 50 mM sodium azide and 0.5 μM DPC_{60}^{2+}. No toxicity was detected using this concentration of azide ions under irradiation without DPC_{60}^{2+} for 15 min with visible light (Fig. 1, line 9) or in the dark containing the fullerene (result not shown). A photoinactivation of 2 log decrease was found in cell viability (Fig. 1, line 10). Thus, a

reduction in the cell inactivation photosensitized by DPC_{60}^{2+} was obtained in the medium containing the azide ions. Therefore, the addition of sodium azide quenched the photocytotoxic species, producing a protective effect on microbial cells.

To evaluate the possible involvement of $O_2(^1\Delta_g)$ on the photosensitized inactivation of *S. aureus* by DPC_{60}^{2+}, experiments were performed treating the cell suspensions with 10 mM DABCO. A charge transfer-induced mechanism was suggested for the quenching of $O_2(^1\Delta_g)$ by DABCO [16]. This compound was used to inhibit $O_2(^1\Delta_g)$-mediated oxidations and to suppress $O_2(^1\Delta_g)$-sensitized fluorescence. No toxicity was found with this DABCO concentration under irradiation without DPC_{60}^{2+} (Fig. 1, line 11) or containing the fullerene in the dark (result not shown). A protective effect on PDI of *S. aureus* mediated by DPC_{60}^{2+} was observed in the presence of DABCO, producing a photoinactivation of *S. aureus* of 1 log after 15 min irradiation (Fig. 1, line 12).

Likewise, the photoinactivation of *S. aureus* mediated by 0.5 μM DPC_{60}^{2+} was examined after incubation with 50 mM D-mannitol. This compound can be used as a scavenger of the superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical (type I reaction) [17]. The presence of 50 mM D-mannitol was not toxic to irradiated cells without DPC_{60}^{2+} (Fig. 1, line 13). Also, it was not toxic for *S. aureus* cells treated with D-mannitol and DPC_{60}^{2+} in dark (result not shown). However, cell inactivation mediated by DPC_{60}^{2+} exhibited a photoprotective effect of about 2 log in suspensions containing D-mannitol after irradiation (Fig. 1, line 14).

3.3. Photooxidation of DMA in S. aureus cells

Photooxidation of DMA induced by DPC_{60}^{2+} was investigated in *S. aureus* cell suspensions under aerobic conditions. DMA quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction and it can be used to evaluate the ability of the photosensitizers to produce $O_2(^1\Delta_g)$ [18]. In all cases, cells were first treated with 10 μM DMA followed by a washing step to remove unbound molecules to cells. This substrate can itself act as photosensitizer producing $O_2(^1\Delta_g)$ [19]. However, DMA do not absorb in the wavelength range used to irradiate (455–800 nm). Thus, it was found that PDI of *S. aureus* was not affected by the addition of 10 μM DMA. Furthermore, short irradiation times were used in kinetic studies with a total time of about 3 min. Photooxidation of DMA was detected by the decay in its fluorescence intensity after different irradiation periods. Results for DMA decomposition are shown in Fig. 2. The values of the observed rate constant (k_{obs}) were calculated from first-order kinetic plots of the DMA emission intensities with time. Photooxidation of DMA was not detected in absence of DPC_{60}^{2+}, while a value of $k_{obs} = (6.76 \pm 0.08) \times 10^{-4}\ s^{-1}$ was calculated for DMA decomposition photosensitized by DPC_{60}^{2+}. Moreover, photooxidation of DMA induced by DPC_{60}^{2+} in *S. aureus* cells was evaluated in suspensions containing 50 mM sodium azide. In the presence of azide ions, a high photoprotective effect was found for DMA and not significant decomposition of this substrate was observed in *S. aureus* cells. The reaction rate mediated by DPC_{60}^{2+} was compared with that produced by $TMAP^{4+}$. This cationic porphyrin was used as a photosensitizer of reference due to it is an active compound established to eradicate microorganisms [20]. In the presence of $TMAP^{4+}$, a value of $k_{obs} = (27.3 \pm 0.2) \times 10^{-4}\ s^{-1}$ was obtained for DMA photooxidation. Thus, the photodecomposition of DMA mediated by $TMAP^{4+}$ considerably increase (~4 times) with respect to the cells treated with DPC_{60}^{2+}.

3.4. Effect of NADH on photoinactivation of S. aureus

Photoinactivation of *S. aureus* was investigated in the presence of different concentration of NADH. It is well-known that NADH can act as both an electron donor for $O_2^{\cdot-}$ formation and a substrate for $O_2(^1\Delta_g)$ [21]. Cells were treated with 10–50 mM NADH and then with 0.5 μM DPC_{60}^{2+}. Fig. 3 shows the survival of *S. aureus* after 15 min irradiation. The highest concentration of NADH used was not toxic in the dark (Fig. 3, line 2). The presence of NADH produced a photoprotective effect, which was dependent on the concentration of NADH (Fig. 3, line 4-6). The cytotoxic activity mediated by DPC_{60}^{2+} was reduced and the cell survival was only about 1 log decreased in the presence of 50 mM NADH (Fig. 3, line 6).

3.5. Effect of sodium azide on photoinactivation of bacteria

The effect of azide ions on the photokilling of *S. aureus* was compared in aerobic and anoxic conditions. Thus, cell suspensions were incubated with 50 mM sodium azide and treated 0.5 μM DPC_{60}^{2+}. Samples were irradiated for 15 min both in air and in absence of oxygen. The results are summarized in Fig. 4A. No toxicity was found for the cultures treated with DPC_{60}^{2+} (Fig. 4A, line 2), with sodium azide (Fig. 4A, line 3), with DPC_{60}^{2+} and argon (Fig. 4A, line 4) or with DPC_{60}^{2+}, azide and argon (Fig. 4A, line 5) in dark respect

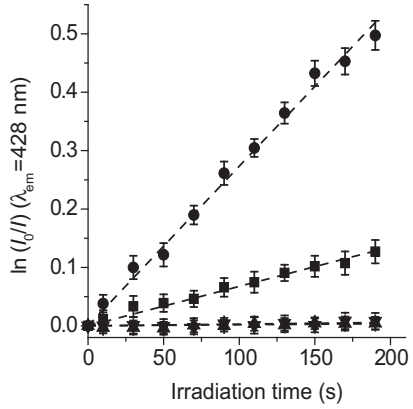


Fig. 2. First-order plots for the photooxidation of DMA in *S. aureus* cells (~10^6 CFU/mL) incubated with: (■) 10 μM DMA for 10 min followed by a washing step and treated with 0.5 μM DPC_{60}^{2+} for 10 min, (●) 10 μM DMA for 10 min followed by a washing step and treated with 0.5 μM $TMAP^{4+}$ for 10 min, (▲) 10 μM DMA for 10 min followed by a washing step, (▼) 10 μM DMA for 10 min followed by a washing step and treated with 50 mM sodium azide and 0.5 μM DPC_{60}^{2+} for 10 min.

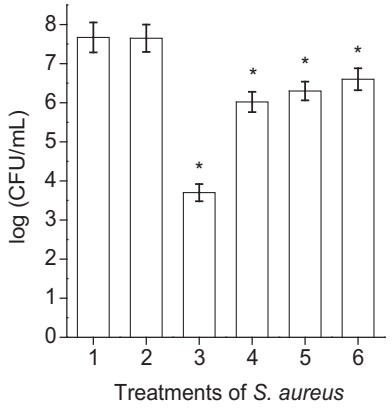


Fig. 3. Survival of *S. aureus* cell suspensions (~10^8 CFU/mL) incubated with 0.5 μM DPC_{60}^{2+} for 10 min in dark at 37 °C and irradiated with visible light for 15 min; 1) cells in dark; 2) cells containing 50 mM NADH in dark; 3) irradiated cells treated with DPC_{60}^{2+}; 4) irradiated cells treated with DPC_{60}^{2+} containing 10 mM NADH; 5) irradiated cells treated with DPC_{60}^{2+} containing 25 mM NADH; 6) irradiated cells treated with DPC_{60}^{2+} containing 50 mM NADH irradiated cells treated with DPC_{60}^{2+} ($^*p < 0.05$, compared with control).

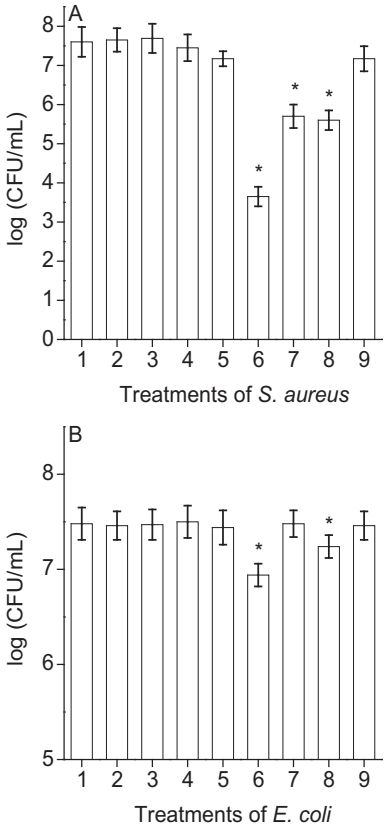


Fig. 4. Survival of (A) *S. aureus* and (B) *E. coli* cell suspensions (~10^8 CFU/mL) incubated with 0.5 μM DPC_{60}^{2+} and 10 μM DPC_{60}^{2+}, respectively, for 10 min in dark at 37 °C and irradiated with visible light for 15 min; 1) cells in dark; 2) cells treated with DPC_{60}^{2+} in dark; 3) cells treated with 50 mM sodium azide in dark; 4) cell treated with DPC_{60}^{2+} under argon atmosphere in dark; 5) cells treated with DPC_{60}^{2+} and 50 mM sodium azide under argon in dark; 6) irradiated cells treated with DPC_{60}^{2+}; 7) irradiated cells treated with DPC_{60}^{2+} and 50 mM sodium azide under argon atmosphere; 8) irradiated cells treated with DPC_{60}^{2+} and 50 mM azide; 9) irradiated cells treated with DPC_{60}^{2+} under argon ($^*p < 0.05$, compared with control).

to no treated cells (Fig. 4A, line 1). PDI of *S. aureus* incubated with DPC_{60}^{2+} produced a 4 log decrease of cell viability after 15 min irradiation (Fig. 4A, line 6). As it was shown above, a photoprotective effect of 2 log was found in presence of azide ions under an aerobic condition (Fig. 4A, line 8). Also, when PDI was carried out in atmosphere of argon, photoinactivation of bacteria was practically negligible as expected for an oxygen-dependent photodynamic process (Fig. 4A, line 9). However, about 2 log decrease in *S. aureus* survival was observed when PDI was performed in the presence of 10 mM sodium azide in absence of oxygen (Fig. 4A, line 7). Photoinactivation of *E. coli* incubated with 10 μM DPC_{60}^{2+} produced 1 log decrease in cell survival after 15 min irradiation (Fig. 4B, line 6). Control experiments showed not toxicity in dark (Fig. 4B, line 1 to 5). *E. coli* cells were not photoinactivated under an argon atmosphere (Fig. 4B, line 9). The addition of sodium azide produced a slight protection on cell viability (Fig. 4B, line 8). Moreover, negligible difference in the photoinactivation of *E. coli* was found between experiments under argon atmosphere in presence or absence of azide ions (Fig. 4B, lines 7 and 9).

3.6. Effect of KI on photoinactivation of bacteria

Bacterial killing mediated by DPC_{60}^{2+} was evaluated in the presence of different concentrations of KI. In water, the reaction of iodine anion and $O_2(^1\Delta_g)$ results in the production of tri-iodide (I_3^-). In this process hydrogen peroxide (H_2O_2) is formed, which can react further with iodide anions [22]. Therefore, cells were incubated with KI in the range of 10–50 mM and after that with 0.5 μM DPC_{60}^{2+}. A concentration of 50 mM KI was not toxic to *S. aureus* in dark (Fig. 5A, line 2). Nor in the presence of DPC_{60}^{2+} (Fig. 5A, line 3). After 15 min irradiation, a potentiation in photoinactivation of *S. aureus* was observed in presence of iodide anions (Fig. 5A, lines 4–7). The photokilling was increase with the KI concentration. A complete eradication of *S. aureus* cells was found when PDI was carried out in the presence of 50 mM KI (Fig. 5A, line 7). In the case of *E. coli*, the addition of 50 mM KI alone or combined with 10 μM DPC_{60}^{2+} were not toxic in dark (Fig. 5B, line 2–3). Photoinactivation of *E. coli* was slightly affected by the presence of KI (Fig. 5B, line 4–7) after 15 min irradiation. However, cell survival decreases 1 log respect to the controls after 30 min irradiation (6.5 log cell survival).

4. Discussion

Fullerene derivatives are accepted as nanoparticles with several potential biomedical applications [8,9]. In the present study, DPC_{60}^{2+} contains a hydrophobic carbon sphere with two intrinsic cationic charges that provide an amphiphilic character. Furthermore, in this structure the cationic group at the end of aliphatic chain is isolated from the fullerene by a propoxy bridge. Thus, this charge has minimal effect on the electronic density of the fullerene. This can help to keep the photophysical properties of the fullerene. Moreover, this chain allows a higher mobility of the charge facilitating the interaction with the membrane of the bacteria. Photodynamic

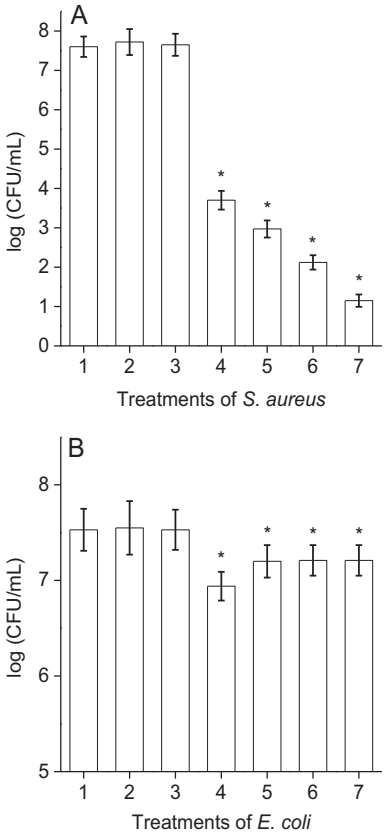
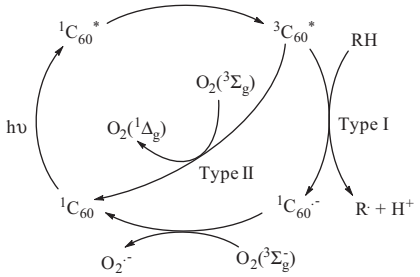


Fig. 5. Survival of (A) *S. aureus* and (B) *E. coli* cell suspensions (~10^8 CFU/mL) incubated with 0.5 μM DPC_{60}^{2+} and 10 μM DPC_{60}^{2+}, respectively, for 10 min in dark at 37 °C and irradiated with visible light for 15 min; 1) cells in dark; 2) cells containing 50 mM KI in dark; 3) cells treated with DPC_{60}^{2+} containing 50 mM KI in dark; 4) irradiated cells treated with DPC_{60}^{2+}; 5) irradiated cells treated with DPC_{60}^{2+} containing 10 mM KI; 6) irradiated cells treated with DPC_{60}^{2+} containing 25 mM KI; 7) irradiated cells treated with DPC_{60}^{2+} containing 50 mM KI ($^*p < 0.05$, compared with control).

activity of DPC_{60}^{2+} was studied in the presence of different substrates to sense ROS [10]. The $O_2(^1\Delta_g)$ production quantum yield (Φ_Δ) of DPC_{60}^{2+} was 0.44 in DMF. The photodynamic activity of these photosensitizers remained high in a simple biomimetic system formed by AOT reverse micelles. Also, the formation of $O_2^{\cdot-}$ induced by DPC_{60}^{2+} was detected in the presence of NADH. Decomposition of L-tryptophan mediated by DPC_{60}^{2+} in DMF indicated a possible contribution of type I pathway, together with type II photoprocess. However, there are limitations to predict photodynamic activity of DPC_{60}^{2+} in microbial cells on the basic of photooxidation processes determined in solutions. Under aerobic conditions, the triplet excited state of fullerene ($^3C_{60}^*$) can interact with $O_2(^3\sum_g^-)$ to form ROS (Scheme 2). This process can occur by energy transfer from the $^3C_{60}^*$ to produce $O_2(^1\Delta_g)$ or by electron transfer to form $O_2^{\cdot-}$ [23]. Fullerene derivatives are efficient $O_2(^1\Delta_g)$ generators in solution with a quantum yield that is near unity [24]. Moreover, the $^3C_{60}^*$ or $C_{60}^{\cdot-}$ can transfer an electron to molecular oxygen forming $O_2^{\cdot-}$. In contrast to $O_2(^1\Delta_g)$ generation, the electron transfer type of reaction preferentially occurs in polar solvents, particularly in the presence of reducing agents, such as NADH [34,25]. These two pathways, yielding $O_2(^1\Delta_g)$ and $O_2^{\cdot-}$, are analogous to the two main photochemical reaction types known as type II and type I photochemical mechanisms, respectively.

The photodynamic action mechanism mediated by DPC_{60}^{2+} was studied analyzing different effects on the photoinactivation of *S. aureus*. After 15 min irradiation, a photoinactivation of 99.99% (4 log decrease) was found for cells incubated with 0.5 μM DPC_{60}^{2+}. However, the photokilling of *S. aureus* cells sensitized by DPC_{60}^{2+} was negligible under an anoxic atmosphere. Oxygen molecules are decisive for the formation of $O_2(^1\Delta_g)$ through the type II photosensitization pathway. Moreover, oxygen can be involved in the type I mechanism [5]. In a type I process the triplet excited state of the photosensitizer can directly interact with substrates to produce radical ions by a hydrogen atom or electron transfer reaction. These radicals instantaneously react with oxygen molecules to generate a mixture of highly reactive oxygen intermediates. Also, $O_2^{\cdot-}$ can be generated by the reaction of molecular oxygen with the photosensitizer radical anion.

Photoinactivation of *S. aureus* induced by DPC_{60}^{2+} was determined in D_2O to evaluate the $O_2(^1\Delta_g)$-mediated inactivation of microbial cells. An increase in the $O_2(^1\Delta_g)$ lifetime is expected when D_2O was used instead of water [14]. The lifetime of $O_2(^1\Delta_g)$ is 67 μs in D_2O but only 3.5 μs in H_2O [19]. Thus, in previous investigations the intracellular water was replaced with D_2O to facilitate the detection of $O_2(^1\Delta_g)$ [26]. The present results showed that the photocytotoxic effect sensitized by DPC_{60}^{2+} was higher in D_2O than in aqueous medium, revealing a contribution of type II photosensitization in the PDI of *S. aureus*. Moreover, experiments were performed in the presence of sodium azide to determinate the participation of $O_2(^1\Delta_g)$. Quenching experiments showed the benefit of its use to characterize the effects of intracellular



Scheme 2. Schematic representation of type I (charge transfer) and type II (energy transfer) photochemical mechanisms mediated by fullerenes.

inhomogeneity on the reactivity of $O_2(^1\Delta_g)$ [27]. In a cellular environment, the lifetime of $O_2(^1\Delta_g)$ is limited by quenching and reactions with cell constituents. Therefore, the cell damage mediated by $O_2(^1\Delta_g)$ occurs close to its site of generation [14]. The $O_2(^1\Delta_g)$ lifetime and the rate constant for $O_2(^1\Delta_g)$ quenching by added azide ion depend on the location of the photosensitizer in different domains of a living cell. Thus, azide ion should be located in the subcellular compartments close to the photosensitizer because $O_2(^1\Delta_g)$ does not diffuse a great distance from its site of production. The presence of sodium azide produced a photoprotection of 2 log in *S. aureus* cells survival. This suggests that azide ions cause significant lowering of the photodynamic effects of the DPC_{60}^{2+} by quenching $O_2(^1\Delta_g)$. Previous studies demonstrated that intracellular quenching by azide ions of $O_2(^1\Delta_g)$ sensitized by the tetracationic 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin (TMPyP) occurs at the diffusion-controlled limit [27]. Also, photoinactivation of *S. aureus* by DPC_{60}^{2+} was investigated in the presence of DABCO, which was used as a $O_2(^1\Delta_g)$ quencher [16]. Bacterial cells were protected from the photodynamic activity by the addition of DABCO and photoinactivation was only 1 log decrease in cell survival. Photoprotection using DABCO was previously observed in the photoinactivation of *S. aureus* by deuteroporphyrin [28]. The formation of $O_2(^1\Delta_g)$ photoinduced by DPC_{60}^{2+} was determined by the decomposition of DMA inside the *S. aureus* cells. Considering that DMA quenches $O_2(^1\Delta_g)$ by means of chemical reaction to form the corresponding endoperoxide, it is used as a method to determinate the ability of DPC_{60}^{2+} to produce $O_2(^1\Delta_g)$ in a cellular media [18]. In *S. aureus* cells, photooxidation rates of DMA significantly increased in the presence of DPC_{60}^{2+}, indicating the generation of $O_2(^1\Delta_g)$. The effectiveness of DPC_{60}^{2+} to produce $O_2(^1\Delta_g)$ into *S. aureus* cells was compared with that produced by $TMAP^{4+}$. A higher photodecomposition rate of DMA was observed using $TMAP^{4+}$ as photosensitizer. This tetracationic porphyrin represents an active photosensitizer to eradicate bacteria [20]. Also, $TMAP^{4+}$ showed high quantum yields of $O_2(^1\Delta_g)$ with a value of 0.77 in water [29]. This is an important photosensitizer property because the reactions induced by $O_2(^1\Delta_g)$ can be the mainly cause of cell damage photosensitized by porphyrins under aerobic conditions [30]. Therefore, the slower photooxidation rate of DMA mediated by DPC_{60}^{2+} could be indicative of a lower $O_2(^1\Delta_g)$ production than $TMAP^{4+}$ in *S. aureus* cells. However, a different intracellular localization of the photosensitizers could also be affecting the decomposition rate of DMA due to the short lifetime of $O_2(^1\Delta_g)$ inside the cells [14]. Anthracene derivatives were also previously used to detect the formation of $O_2(^1\Delta_g)$ by 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine in *Candida albicans* cells [13]. Moreover, sodium azide was used to establish the presence of $O_2(^1\Delta_g)$, preventing type II photoprocess [31]. The deactivation of $O_2(^1\Delta_g)$ by physical quenching decreases the formation of endoperoxide inside the cells and the photooxidation of DMA by DPC_{60}^{2+} was negligible with the addition of azide ions in the cell suspensions. On the other hand, D-mannitol was used as a free-radical scavenger to investigate the contribution of type I mechanism [17,32]. Photocytotoxicity of *S. aureus* cells mediated by DPC_{60}^{2+} was affected when D-mannitol was added to cells, producing a photoprotection of 2 log. In earlier studies, the addition of D-mannitol produced a photoprotective effect on the cellular survival, when *N*,*N*-dimethyl-2-(4-*N*,*N*,*N*-trimethylaminophenyl)fulleropyrrolidinium (DTC_{60}^{2+}) was used as photosensitizers to inactivate *E. coli* [12]. The photoprotective effect caused by D-mannitol was indicative of a contribution of type I pathway.

When the *S. aureus* cells were treated with NADH, photoinactivation activity mediated by DPC_{60}^{2+} was decreased. NADH is the most important redox coenzyme acting as the source of electrons in the living system. In the presence of NADH, $O_2(^1\Delta_g)$ can be quenched by both the physical and electron-transfer processes in

competition with the decay of $O_2(^1\Delta_g)$ to the ground state [33,34]. The physical quenching process gives the ground-state reactant pair, NADH and $O_2(^3\sum_g^-)$, whereas the electron transfer produces $NADH^{\cdot+}$ and $O_2^{\cdot-}$. In solution, the generation of $O_2^{\cdot-}$ by DPC_{60}^{2+} was enhanced by addition of NADH [10]. Previous experiments both in the cell-free system and in primary cultured rat hepatocytes, strongly suggest that NADH was the primary and enzymatically restorable target of $O_2(^1\Delta_g)$ within mitochondria of viable cells [21]. It was suggested that mitochondrial NADH was likely to act as a directly operating antioxidant and thus provides protection when $O_2(^1\Delta_g)$ was generated within this organelle. Therefore, the findings of photoprotection by NADH are indicative that DPC_{60}^{2+} operates primarily via a type II reaction.

Therefore, the photodamage produced to the *S. aureus* cells mediated DPC_{60}^{2+} appears to be mainly facilitated by the intermediacy of $O_2(^1\Delta_g)$. Although in a minor contribution, the participation of other ROS could not be neglected. It was previously demonstrated that the killing of *E. coli* cells by DTC_{60}^{2+} and visible light irradiation can be mediated by a contribution of type I photoprocess [12]. This finding is in agreement with previous report that type II are better at killing Gram-positive bacteria than type I, while the reverse is true for Gram-negative [9,35]. It was proposed that $O_2(^1\Delta_g)$ 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 hydroxyl radicals to cause real damage.

Under aerobic condition, a photoprotective effect was found for PDI of *S. aureus* induced by DPC_{60}^{2+} in the presence of azide ions. Moreover, photoinactivation under an anoxic atmosphere was not as effective as in presence de oxygen. Despite this, in the absence of oxygen 2 log decrease of bacterial photokilling was obtained when azide anions were added. The aerobic respiration of same bacterial strains is susceptible to sodium azide. *S. aureus* is an anaerobic facultative bacterium, therefore it can grow under aerobic conditions if oxygen is present, but is capable of switching to fermentation or anaerobic respiration if oxygen is absent. Addition of sodium azide and as well as oxygen removal was previously used to investigate the photochemical mechanisms of microbial killing mediated by decacationic fullerene derivatives [36]. It was proposed that the photoactivated fullerene directly can accept an electron transferred from azide anion to produce azidyl radicals and fullerenyl radical anions. The azidyl radical was presumed to mediate bacterial killing especially in the absence of oxygen. Therefore, the fact that azide anions allow the photoactivated fullerenes to kill the bacteria in the absence of oxygen does suggest that azidyl radicals can be involved in the cytotoxic activity. Azidyl radicals may be formed from sodium azide and hydroxyl radicals. The differences in sodium azide inhibition may reflect differences in the extent of photosensitizer binding to microorganisms or differences in penetration of azide anions into cell walls of bacteria [35]. Thus, sodium azide can protected against *S. aureus* killing mediated by a polyethylenimine chlorin(e6) conjugate (PEI-ce6) but not against killing with a cationic fullerene. Also, the addition of sodium azide to *S. aureus* and *E. coli* incubated with methylene blue gave significantly increased bacterial killing [37]. This observation suggests a possible mechanism to carry out oxygen-independent PDT. The effect of azide ions was compared using six different homologous phenothiazinium dyes [38]. Killing of *E. coli* was potentiated with all six dyes after a wash, while *S. aureus* killing was only potentiated by methylene blue and toluidine blue O with a wash and with no wash. More lipophilic dyes, such as dimethylmethylene blue, were more likely to show potentiation. It was concluded that the type I photochemical mechanism, potentiation with azide ions, likely depends on the microenvironment.

PDI of bacteria sensitized by DPC_{60}^{2+} was investigated in cell suspensions containing KI. This inert salt produced a dose-dependent (10–50 mM KI) increase of light-mediated bacterial killing of *S. aureus*. Thus, in the presence of 50 mM iodide ions the photoinactivation was over 7 log decrease in cell survival after 15 min irradiation, which means 2.5 log more effective than in absence of KI. Under these PDI conditions, potentiation of photokilling was not found in *E. coli*. However, an increase in the photoinactivation of this Gram-negative bacterium was observed after 30 min irradiation. Previously, the efficacy of PDI mediated by methylene blue was investigated in bacteria by addition of KI [39]. A consistent increase of red light-mediated bacterial killing was found by adding KI to *S. aureus*. Moreover, it was found that PDI of *Acinetobacter baumannii*, *S. aureus* and *C. albicans* with cationic fullerenes could be potentiated by iodide both *in vitro* and *in vivo* [40]. The mechanism of action was probably due to formation of reactive iodine species that are produced quickly with a short lifetime. In this system, iodine atoms ($I^{\cdot}$) and iodine radical anions ($I_2^{\cdot-}$) can be generated by interaction of the photosensitizer excited state or hydroxyl radicals with iodide ions that would increase the damage to bacterial cell wall constituents [41]. Moreover, the interaction of the ROS and KI during light exposure, biocidal molecular iodine (I_2) or tri-iodide (I_3^-) can be formed improving bacterial inactivation. Therefore, this complementary pathway of photodamage can be used to improve the photodynamic activity produced by DPC_{60}^{2+} in *S. aureus* cells.

5. Conclusions

This study provides insight into the photodynamic action pathways that occurs in the PDI of *S. aureus* cells mediated by DPC_{60}^{2+}. In particular, the main mechanism was influenced by the location of the photosensitizer in the different domains of the target microbial cells. To elucidate the oxidative processes that occur during the killing of cells, first, the effect of the media was analyzed on cell photoinactivation. It was found that an oxygen atmosphere was required for an efficient photoinactivation. Moreover, photoinactivation of *S. aureus* was increased in D_2O due to a longer lifetime of $O_2(^1\Delta_g)$. When ROS scavengers were added, photoprotection was observed with azide ions as $O_2(^1\Delta_g)$ quencher in aerobic medium. Moreover, photoprotection was found using DABCO as $O_2(^1\Delta_g)$ trapping. The formation of $O_2(^1\Delta_g)$ was detected within the *S. aureus* cells by photo decomposition of DMA that serve as a sensitive probe. Also, photoinactivation activity induced by DPC_{60}^{2+} was decreased when NADH was added to cell suspensions. Therefore, these finding indicate that the killing of *S. aureus* cells by DPC_{60}^{2+} and visible light irradiation seem to be mediated mainly by $O_2(^1\Delta_g)$. However, some contribution of type I pathway cannot be completely ruled out due to the protective effect of D-mannitol. On the other hand, the addition of sodium azide to cell suspensions can be used to photoinactivate *S. aureus* in an anoxic atmosphere. Moreover, the photokilling produced by DTC_{60}^{2+} can be potentiated by the addition of KI to obtain a better rate of inactivation of microorganisms. Thus, the balance between type I and type II mechanisms may be influenced by the microenvironment of the cationic fullerene. This in turn may be influenced by the strength of binding by the target microbial cells. Gram-negative bacteria have a more pronounced negative charge in the cell envelope than those of Gram-positive bacteria. Therefore, the cationic charged fullerenes may be supposed to bind more strongly to Gram-negatives than they do to Gram-positives. In the present case, the results are in agreement with previous report that type II photoprocess is mainly involved in the killing of Gram-positive bacteria.

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References

[1] A. Nigama, D. Gupta, A. Sharma, Treatment of infectious disease: beyond antibiotics, Microbiol. Res. 169 (2014) 643–651.
[2] A. Sarkar, A. Raji, G. Garaween, O. Soge, J. Rey-Ladino, W. Al-Kattan, A. Shibl, A. Senok, Antimicrobial resistance and virulence markers in methicillin sensitive *Staphylococcus aureus* isolates associated with nasal colonization, Microb. Pathog. 93 (2016) 8–12.
[3] H. Teramoto, S. Salaheen, D. Biswas, Contamination of post-harvest poultry products with multidrug resistant *Staphylococcus aureus* in Maryland-Washington DC metro area, Food Control 65 (2016) 132–135.
[4] U. Theuretzbacher, Global antibacterial resistance: the never-ending story, J. Glob. Antimicrob. Resist. 1 (2013) 63–69.
[5] E. Alves, M.A.F. Faustino, M.G.P.M.S. Neves, A. Cunha, J. Tome, A. Almeida, An insight on bacterial cellular targets of photodynamic inactivation, Future Med. Chem. 6 (2014) 141–164.
[6] E.N. Durantini, Photodynamic inactivation of bacteria, Curr. Bioact. Compd. 2 (2006) 127–142.
[7] T.G. St, T. Denis, L. Dai, C. Izikson, R.R. Astrakas, M.R. Anderson, G.P.Tegos. Hamblin, Antimicrobial photoinactivation as an evolving and emerging discovery strategy against infectious disease, Virulence 2 (2011) 509–520.
[8] S.K. Sharma, L.Y. Chiang, M.R. Hamblin, Photodynamic therapy with fullerenes *in vivo*: reality or a dream?, Nanomedicine 6 (2011) 1813–1825
[9] Y.-Y. Huang, S.K. Sharma, R. Yin, T. Agrawal, L.Y. Chiang, M.R. Hamblin, Functionalized fullerenes in photodynamic therapy, J. Biomed. Nanotechnol. 10 (2014) 1918–1936.
[10] M.L. Agazzi, M.B. Spesia, N.S. Gsponer, M.E. Milanesio, E.N. Durantini, Synthesis, spectroscopic properties and photodynamic activity of a fulleropyrrolidine bearing a basic amino group and its dicationic analog against *Staphylococcus aureus*, J. Photochem. Photobiol. A: Chem. 310 (2015) 171–179.
[11] P. Mroz, A. Pawlak, M. Satti, H. Lee, T. Wharton, H. Gali, T. Sarna, M.R. Hamblin, Functionalized fullerenes mediate photodynamic killing of cancer cells: Type I versus Type II photochemical mechanism, Free Radical Biol. Med. 43 (2007) 711–719.
[12] M.E. Milanesio, M.B. Spesia, M.P. Cormick, E.N. Durantini, Mechanistic studies on the photodynamic effect induced by a dicationic fullerene C_{60} derivative on *Escherichia coli* and *Candida albicans* cells, Photodiag. Photodyn. Ther. 10 (2013) 320–327.
[13] M.A. Di Palma, M.G. Alvarez, E.N. Durantini, Photodynamic action mechanism mediated by zinc(II) 2,9,16,23-tetrakis[4-(N-methylpyridyloxy)] phthalocyanine in *Candida albicans* cells, Photochem. Photobiol. 91 (2015) 1203–1209.
[14] P.R. Ogilby, Singlet oxygen: there is still something new under the sun, and it is better than ever, Photochem. Photobiol. Sci. 9 (2010) 1543–1560.
[15] E.F.F. da Silva, B.W. Pedersen, T. Breitenbach, R. Toftegaard, M.K. Kuimova, L.G. Arnaut, P.R. Ogilby, Irradiation- and sensitizer-dependent changes in the lifetime of intracellular singlet oxygen produced in a photosensitized process, J. Phys. Chem. B 116 (2012) 445–461.
[16] C. Schweitzer, R. Schmidt, Physical mechanisms of generation and deactivation of singlet oxygen, Chem. Rev. 103 (2003) 1685–1757.
[17] T. Maisch, C. Bosl, R.-M. Szeimies, N. Lehn, C. Abels, Photodynamic effects of novel XF porphyrin derivatives on prokaryotic and eukaryotic cells, Antimicrob. Agents Chemother. 49 (2005) 1542–1552.
[18] A. Gomes, E. Fernandes, J.L.F.C. Lima, Fluorescence probes used for detection of reactive oxygen species, J. Biochem. Biophys. Methods 65 (2005) 45–80.
[19] F. Wilkinson, W.P. Helman, A.B. Ross, Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution. An expanded and revised compilation, J. Phys. Chem. Ref. Data 24 (1995) 663–1021.
[20] N.S. Gsponer, M.B. Spesia, E.N. Durantini, Effects of divalent cations, EDTA and chitosan on the uptake and photoinactivation of *Escherichia coli* mediated by cationic and anionicporphyrins, Photodiag. Photodyn. Ther. 12 (2015) 67–75.
[21] F. Petrat, S. Pindiur, M. Kirsch, H. de Groot, NAD(P)H, a primary target of $^1O_2$ in mitochondria of intact cells, J. Biol. Chem. 278 (2003) 3298–3307.
[22] A. Felgenträger, T. Maisch, A. Späth, J.A. Schröderc, W. Bäumlera, Singlet oxygen generation in porphyrin-doped polymeric surface coating enables antimicrobial effects on *Staphylococcus aureus*, Phys. Chem. Chem. Phys. 16 (2014) 20598–20607.
[23] Y. Yamakoshi, S. Sueyoshi, K. Fukuhara, N. Miyata, T. Masumizu, M. Kohno, $^{\cdot}OH$ and $O2^{\cdot-}$ generation in aqueous C60 and C70 solutions by photoirradiation: an EPR study, J. Am. Chem. Soc. 120 (1998) 12363–12364.
[24] T. Hamano, K. Okuda, T. Mashino, M. Hirobe, K. Arakane, A. Ryu, S. Mashiko, T. Nagano, Singlet oxygen production from fullerene derivatives: effect of sequential functionalization of the fullerene core, Chem. Commun. 1 (1997) 21–22.
[25] Y. Yamakoshi, N. Umezawa, A. Ryu, K. Arakane, N. Miyata, Y. Goda, T. Masumizu, T. Nagano, Active oxygen species generated from photoexcited fullerene (C60) as potential medicines: $O2^{\cdot-}$ versus 1O2, J. Am. Chem. Soc. 125 (2003) 12803–12809.
[26] S. Hatz, J.D.C. Lambertb, P.R. Ogilby, Measuring the lifetime of singlet oxygen in a single cell: addressing the issue of cell viability, Photochem. Photobiol. Sci. 6 (2007) 1106–1116.
[27] M.K. Kuimova, G. Yahioglu, P.R. Ogilby, Singlet oxygen in a cell: spatially dependent lifetimes and quenching rate constants, J. Am. Chem. Soc. 131 (2009) 332–340.
[28] Y. Nitzan, B. Shainberg, Z. Malik, The mechanism of photodynamic inactivation of *Staphylococcus aureus* by deuteroporphyrin, Curr. Microbiol. 19 (1989) 265–269.
[29] J.B. Verlhac, A. Gaudemer, I. Kraljic, Water-soluble porphyrins and metalloporphyrins as photosensitizers in aerated aqueous solutions. I. Detection and determination of quantum yield of formation of singlet oxygen, Nouv. J. Chim. 8 (1984) 401–406.
[30] M.P. Cormick, E.D. Quiroga, S.G. Bertolotti, M.G. Alvarez, E.N. Durantini, Mechanistic insight of the photodynamic effect induced by tri- and tetra-cationic porphyrins on *Candida albicans* cells, Photochem. Photobiol. Sci. 10 (2011) 1556–1561.
[31] K. Ergaieg, M. Chevanne, J. Cillard, R. Seux, Involvement of both type I and type II mechanisms in Gram-positive and Gram-negative bacteria photosensitization by a *meso*-substituted cationic porphyrin, Sol. Energy 82 (2008) 1107–1117.
[32] L.V. Chekulayeva, V.A. Chekulayev, I.N. Shevchuk, Active oxygen intermediates in the degradation of hematoporphyrin derivative in tumor cells subjected to photodynamic therapy, J. Photochem. Photobiol. B: Biol. 93 (2008) 94–107.
[33] I. Nakanishi, S. Fukuzumi, T. Konishi, K. Ohkubo, M. Fujitsuka, O. Ito, N. Miyata, DNA cleavage via superoxide anion formed in photoinduced electron transfer from NADH to γ-cyclodextrin-bicapped C_{60} in an oxygen-saturated aqueous solution, J. Phys. Chem. B 106 (2002) 2372–2380.
[34] B. Zhao, Y.-Y. He, P.J. Bilski, C.F. Chignell, Pristine (C_{60}) and hydroxylated [$C_{60}(OH)_{24}$] fullerene phototoxicity towards HaCaT keratinocytes: type I vs type II mechanisms, Chem. Res. Toxicol. 21 (2008) 1056–1063.
[35] L. Huang, Y. Xuan, Y. Koide, T. Zhiyentayev, M. Tanaka, M.R. Hamblin, Type I and Type II mechanisms of antimicrobial photodynamic therapy: an in vitro study on Gram-negative and Gram-positive bacteria, Lasers Surg. Med. 44 (2012) 490–499.
[36] R. Yin, M. Wang, Y.-Y. Huang, G. Landi, D. Vecchio, L.Y. Chiang, M.R. Hamblin, Antimicrobial photodynamic inactivation with decacationic functionalized fullerenes: oxygen-independent photokilling in presence of azide and new mechanistic insights, Free Radical Biol. Med. 79 (2015) 14–27.
[37] L. Huang, T.G. St, Y.Xuan. Denis, Y.-Y. Huang, M. Tanaka, A. Zadlo, T. Sarna, M.R. Hamblin, Paradoxical potentiation of methylene blue-mediated antimicrobial photodynamic inactivation by sodium azide: role of ambient oxygen and azide radicals, Free Radical Biol. Med. 53 (2012) 2062–2071.
[38] K.R. Kasimova, M. Sadasivam, G. Landi, T. Sarna, M.R. Hamblin, Potentiation of photoinactivation of Gram-positive and Gram-negative bacteria mediated by six phenothiazinium dyes by addition of azide ion, Photochem. Photobiol. Sci. 13 (2014) 1541–1548.
[39] D. Vecchio, A. Gupta, L. Huang, G. Landi, P. Avci, A. Rodas, M.R. Hamblin, Bacterial photodynamic inactivation mediated by methylene blue and red light is enhanced by synergistic effect of potassium iodide, Antimicrob. Agents Chemother. 59 (2015) 5203–5212.
[40] Y. Zhang, T. Dai, M. Wang, D. Vecchio, L.Y. Chiang, M.R. Hamblin, Potentiation of antimicrobial photodynamic inactivation mediated by a cationic fullerene by added iodide: *in vitro* and *in vivo* studies, Nanomedicine 10 (2015) 603–614.
[41] J.M. Gardner, M. Abrahamsson, B.H. Farnum, G.J. Meyer, Visible light generation of iodine atoms and I–I bonds: sensitized I^- oxidation and I_3^- photodissociation, J. Am. Chem. Soc. 131 (2009) 16206–16214.