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Mechanistic insight of the photodynamic inactivation of *Escherichia coli* by a tetracationic zinc(II) phthalocyanine derivative

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Photosensitizer

Summary Photodynamic inactivation (PDI) of *Escherichia coli* has been studied in cultures treated with zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine ($ZnPPc^{+4}$) to obtain insight about the mechanism of damage. This phthalocyanine is rapidly bound to cells, reaching a value of ~0.8 nmol/10^6 cells when the cultures were incubated with 2 μM sensitizer. After 30 min of irradiation, a 4 log decrease of *E. coli* survival was observed. The photocytotoxic action was investigated in plasmid and genomic DNA by electrophoretic analysis. Absorption spectroscopic studies showed that this cationic phthalocyanine interacts strongly with DNA ($K_{DNA} = 4.7 \times 10^6\ M^{-1}$). Photocleavage of calf thymus DNA sensitized by $ZnPPc^{+}4$ was not found even after long irradiation periods. Similar results were also observed in genomic DNA extracted from *E. coli* cells after PDI treatment. Modifications of plasmid DNA isolated from bacteria were only observed after long irradiation periods. However, under these conditions transmission electron microscopy of the PDI bacteria revealed an aggregation of cytoplasmic macromolecules and irregularities in cell barriers. Also, scanning electron microscopy showed a shrunken appearance in cells after PDI. Even so, release of intracellular biopolymers was not detected by absorption. On the other hand, outer and inner membranes permeabilization assays showed an increase in the permeability. Consequently, alterations in the cell membrane functionality induced by $ZnPPc^{+4}$ appear to be the major cause of *E. coli* inactivation upon PDI.

Introduction

The antimicrobial chemotherapy field is in constant changes, because of the great variety of found pathogenic species and their rapid evolutionary changes [1]. The great successes in the war against microorganism are probable coming to the end. The medical advance gained with the technology of β-lactamic agents probably will not be equal in the near future. For this reason, it is imperative the development of new drugs and therapies [2]. The new antibiotics methods include bacteriophages, bacterial phenotype modification, genomic library to find unknown target sites for new drugs and a non-oncologic applications of photodynamic therapy, named photodynamic inactivation (PDI) of microorganisms [3]. Essentially, PDI is based on the admin-

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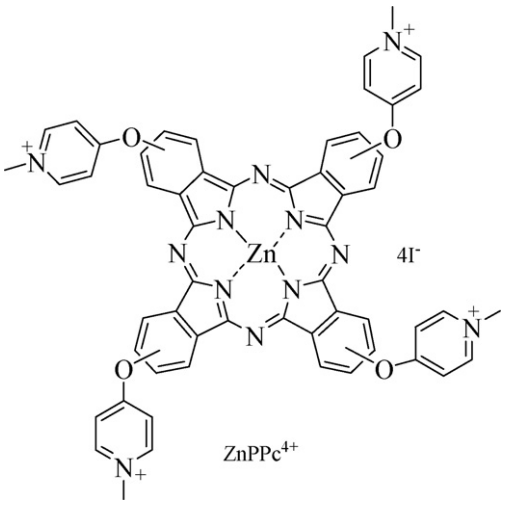
istration of a photosensitizer, which is preferentially accumulated in the microbial cells. The subsequent irradiation with visible light, in the presence of oxygen, specifically generates a cascade of biochemical events that produce cell damages that inactivate the microorganisms [4].

Studies of PDI have shown that Gram-positive bacteria are efficiently photoinactivated by a variety of sensitizers, whereas Gram-negative bacteria are usually resistant to the action of negatively charged or neutral agents [2]. The resistance of Gram-negative bacteria to the action of photoactivated sensitizers has been ascribed to the presence of a highly organized outer membrane, which hinders the interaction of the photosensitizer with the cytoplasmic membrane and intercepts the photogenerated reactive species [5—7]. Previous investigation showed that phthalocyanine derivatives can photosensitize the inactivation of various microbial pathogens [8—10]. In particular, cationic phthalocyanines are effective sensitizers inducing direct inactivation of Gram-negative bacteria even in absence of an additional permeabilization agent [10,11]. Although, photoinactivation of microorganisms sensitized by phthalocyanines in liquid suspensions has been studied in detail, the complete mechanism of action of these photosensitizers in bacteria inactivation is still unknown [2,4]. In cells, several biostructures can be target of the photodynamic activity induced by sensitizers. For instance, the photoinduced reactions can oxidize biomolecules, such as proteins, nucleic acids and lipids. Actually exist two principal ways that have been proposed in the PDI treatment of bacteria. The photodynamic activity can produce changes in cytoplasmatic membrane and/or in DNA structure [12]. The cytoplasmatic membrane deterioration can involve the loss of cellular content, membrane transport functionality and enzymatic inactivation [13]. The outer membranes of Gram-negative bacteria constitute a semi-permeable barrier, which are constituted externally by a high net negative charge of LPS molecules [14]. Its net provides a polyanionic external surface that is partly neutralized by divalent cations Mg^{2+} and Ca^{2+}. Consequently, the outer membrane is relatively impermeable to neutral or anionic drugs, however, polycations sensitizers can disturb the outer membrane of Gram-negative bacteria [15,16]. In previous investigations, photodamage to the cytoplasmic membrane of *Escherichia coli* was observed concomitantly to bacterial death [7,17,18]. Alternatively, it is known that cationic phthalocyanines form complex with nucleic acids [19,20]. When DNA-bound macrocycles are photoexcited, cleavage of DNA can be initiated by photochemical reactions. In this way, the guanine residues have shown to be easier oxidized than other DNA bases [21—24]. Nevertheless, some damages can be corrected by several DNA repairing systems [25]. Previous investigations have concluded that though DNA damage occurs, it cannot be the primary cause of bacterial cell photoinactivation [2]. Actually, there are only a small number of studies on the affected sub-cellular sites and the nature of macromolecular damage caused by PDI treatment of bacteria with phthalocyanines. The main site of photodynamic action depends on the sensitizer structure, which influences the interaction with microbes and its cellular localization.

In previous works, we have investigated the photodynamic activity of cationic phthalocyanine derivatives in different biomimetic media and *in vitro* as sensitizers to eradicate *E. coli* cells [26,27]. In the present paper, the photodynamic action of zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine iodide ($ZnPPc^{4+}$) phthalocyanine (Scheme 1) was examined *in vitro* to inactivate *E. coli* cells in cellular suspensions. The aim was to obtain more insight into the specific mechanism of cellular photodamage, which cause the bacterial cell death after PDI treatment with this photosensitizer.



Scheme 1 Molecular structure of $ZnPPc^{4+}$ phthalocyanine.

Materials and methods

General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Spectra were recorded using 1 cm path length quartz cuvettes at $25.0 \pm 0.5\,^{\circ}C$. Cultures absorption was determinate at 660 nm in a Barnstead Turner SP-830 (Dubuque, IA) spectrophotometer. The light source used was a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) 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. The fluence rate at the treatment site was 30 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Electrophoresis was performed on a IBI MP-1015 Horizontal Gel Electrophoresis Unit Shelton Scientific (Peosta, IA, USA) and the electric field was generated by a power supply PS251-2 (Sigma—Aldrich Techware).

All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Calf thymus double-stranded DNA, *o*-nitrophenyl-β-D-galactoside (ONPG) and 1-*N*-phenylnaphthylamine (NPN) were purchased from Sigma (St. Louis, MO, USA). Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

Photosensitizer

Zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine iodide ($ZnPPc^{4+}$) was synthesized as previously described [27]. A sensitizer stock solution (~0.5 mM) was prepared by dissolution in 1 mL of *N*,*N*-dimethylformamide (DMF). The concentration was confirmed by spectroscopy, taking into account the value of molar coefficient ($\varepsilon^{678} = 1.1 \times 10^5\ M^{-1}\ cm^{-1}$ $ZnPPc^{4+}$ in DMF) [27].

Phthalocyanine binding to DNA

The concentration of calf thymus double-stranded DNA stock solution (2.7 mM), calculated in base pairs, was determined spectrophotometrically using molar absorptivity $\varepsilon_{260} = 1.31 \times 10^4\ M^{-1}\ cm^{-1}$ [28]. Absorbance titrations were conduced by adding concentrated stock solution of DNA directly to a cuvette containing phthalocyanine solution (2 mL, ~3 μM) in methanol. The apparent binding constants (K_{DNA}) for phthalocyanine-DNA complex were calculated from the absorbance changes at the *Q*-band maximum (ΔA), assuming a 1:1 stoichiometry and that the DNA concentration is always significantly larger than the porphyrin concentration, using Eq. (1), where $(\Delta A)_\infty$ represents the extrapolated absorbance change at [DNA] → ∞ [29]:

$$\frac{1}{\Delta A} = \frac{1}{(\Delta A)_\infty} + \frac{1}{(\Delta A)_\infty K[\mathrm{DNA}]} \quad (1)$$

A plot of $1/\Delta A$ *vs.* 1/[DNA] was used to calculate the value of K_{DNA} from the ratio of the intercept to the slope.

Bacterial strain and preparation of cultures

The *E. coli* strain (EC7), recovered from clinical urogenital material, was used as previously described [27]. The bacteria were grown aerobically at 37 °C in 30% (w/v) tryptic soy (TS) broth overnight. Aliquots (~40 μL) of this culture was aseptically transferred to 4 mL of fresh medium (30%, w/v, TS broth) and incubated at 37 °C to middle of the 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 equal amount of 10 mM phosphate-buffered saline (PBS, pH 7.0), corresponding to ~10^9 colony forming units (CFU)/mL. Depending on the experiment, cellular suspensions of ~10^9 CFU/mL were used or the cells were diluted 1/1000 in PBS to obtain ~10^6 CFU/mL. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 mm × 100 mm) were used and the sensitizer was added from a stock solution of phthalocyanine (~0.5 mM) in DMF. Viable bacteria were monitored and their number calculated by counting the number of CFU after appropriated dilution on TS agar plates and 18—24 h incubation at 37 °C.

Sensitizer binding to bacterial cells

Suspensions of *E. coli* (2 mL, ~10^6 CFU/mL) in PBS were incubated in dark at 37 °C with 2 μM of $ZnPPc^{4+}$ for 5, 10 and 30 min. The cultures were centrifuged (3000 rpm for 15 min) and the cell pellets were re-suspended in 2% aqueous SDS (2 mL), incubated overnight at 4 °C and sonicated for 30 min. The concentration of sensitizer in the supernatant was measured by spectrofluorimetry (λ_{exc} = 670 nm and λ_{em} = 684 nm) in solution of 2% SDS in PBS. The fluorescence values obtained from each sample were referred to the total number of bacteria contained in the suspension [27].

Photosensitized inactivation of bacteria cells

Cell suspensions of *E. coli* (2 mL) in PBS were incubated with 2 μM phthalocyanine for 30 min in the dark at 37 °C. The cultures were exposed for different time intervals to visible light [27]. Control experiments were carried out without illumination in the absence and in the presence of sensitizer. Each experiment was repeated separately three times.

DNA purification and electrophoresis

Bacterial cultures of *E. coli* cells (10^9 CFU/mL) were grown as described above. Plasmid DNA was extracted following the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). Genomic DNA was purified using a Wizard Genomic DNA Purification Kit (Promega). In PDI experiments, the cellular suspensions were treated as described above and plasmid or genomic DNA was extracted from the cells immediately post-irradiation.

DNA samples were gently mixed with 3 μL of Loading Buffer 6× (0.25%, w/v, bromophenol blue, 40%, w/v, sucrose, 1.15% acetic acid, 40 mM Tris, 1 mM EDTA). DNA was analyzed by electrophoresis 1% agarose gel in TBE buffer (90 mM Tris—HCl, 90 mM boric acid and 2 mM EDTA, pH 8) at 2.9 V/cm for 2 h. Ethidium bromide (1 mg/mL) was incorporated into de agarose gel. The Lamda DNA/*Hind* III (Promega) was used as molecular weight marker (MK) (0.5 μg/μL) with DNA fragments between 125 and 23,130 bp.

The photographs were analyzed using ImageJ (National Institute of Health, USA) software to quantify the amount of DNA remaining after different treatments.

Steady state photolysis of DNA

Solutions of DNA and 20 μM sensitizer in 15 μL of TE buffer (10 mM Tris—HCl, 1 mM EDTA, pH 7.6) was irradiated in polypropylene 1.5 mL Eppendorf tubes with visible light (30 mW/cm^2) at room temperature. The tubes were placed vertically with the cap open in the path of the visible beam and irradiated for different times. After irradiation, the samples were analyzed by electrophoresis.

Transmission electron microscopy (TEM)

Cultures of *E. coli* (~10^9 CFU/mL) were incubated with 2 μM sensitizer by 30 min in dark. The cultures were irradiated as described above by different times. Samples were centrifuged and the cells were fixed in 4% formaldehyde and 2% glutaraldehyde mixture in cacodylate buffer 0.1 M for 2 h at room temperature. After that, the bacteria were washed three times with cacodylate buffer and postfixed in 1% osmium tetraoxide in the same buffer solution for 1—2 h

at room temperature. The cells were dehydrated with gradients of acetone and embedded in Araldite epoxy resin at 60 °C for 24 h. Thin-sectioned samples (80—100 nm width) were prepared using a Jeol Jum-7 ultratome. The samples were stained with uranyl acetate in alcoholic solution (2 min) and lead citrate (2 min). Finally, the samples were viewed and digitally photographed with a transmission electronic microscopy Leo 906E with a MegaView III camera.

Scanning electron microscopy (SEM)

Cultures of *E. coli* ($\sim 10^6$ CFU/mL) were incubated with 2 μM sensitizer by 30 min in dark and irradiated as described above for 30 min. Samples of 500 μL were mixed with 500 μL of glutaraldehyde (2%) and formaldehyde (4%) in cacodylate buffer 0.1 M for 2 h at room temperature. Then, they were dehydrated gradually by successive passage through ethanol from 10 to 100%. Samples were placed on isoamylate and dried in a critical-point drying apparatus in liquid CO_2 (Baltec CP-30). Cells were gold coated (Jeol FineCoat Sputter JFC-1100) and examined using a Jeol model JSM 6360 LV (Tokyo, Japan) scanning electron microscope. SEM images were performed in Facultad de Ciencias Naturales y Museo, UNLP, Argentina.

Integrity of *E. coli* cell membrane

Bacterial cell membrane integrity was examined by determination of the release of material absorbing at 260 nm [30,31]. *E. coli* cellular suspension (10^9 CFU/mL) in PBS (2 mL) were obtained and treated as described above with 2 μM sensitizer. After that, the tubes were irradiated with visible light for different times. Each irradiated tube was centrifuged at 5000 rpm by 10 min, the pellet was separated and the release of materials in the supernatant was monitored by UV—vis spectroscopy.

Outer membrane (OM) permeabilization assays

OM permeabilization activity of the phthalocyanine was determined by the NPN assay previously described [32,33]. *E. coli* cultures (10^9 CFU/mL) re-suspended in PBS (2 mL) were treated with 2 μM $ZnPPc^{4+}$ and keep in dark by 30 min and then irradiated by 30 min. After that, the culture was transfer to a quartz cuvette and 40 μL of 1 mM NPN was added. Fluorescence spectra were recorder at different time. Excitation and emission wavelengths were set at 350 and 429 nm, respectively. Increase in fluorescence due the partitioning of NPN into the OM was recorder as a function of time until no further increase in intensity. Control tests without irradiation were performed to verify that the enhance fluorescence was due to NPN uptake by bacteria [34].

Inner membrane (IM) permeabilization assay

IM permeabilization was determined by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate [35]. Logarithmic-phase bacteria grown in tryptic soy broth containing 2% lactose were harvested by centrifugation and re-suspended in PBS. Bacteria suspensions (2 mL) were incubated with 2 μM of $ZnPPc^{4+}$ in dark for 30 min and then exposes for 30 min to visible light. Then, the cultures were drop in a quartz cuvette and 100 μL ONPG (30 mM) added. The production of *o*-nitrophenol over time was determined by monitoring the absorbance increase at 420 nm using a spectrophotometer.

Results

Interaction of $ZnPPc^{4+}$ with calf thymus DNA

Affinity of $ZnPPc^{4+}$ to calf thymus DNA was evaluated by absorption spectroscopic analysis in methanol. This solvent was chosen to solubilize the phthalocyanine as monomer. Titration of $ZnPPc^{4+}$ with DNA produces the spectral changes in the *Q*-band showed in Fig. 1. The extensive hypochromicity (~80%) of *Q*-band observed indicates that this phthalocyanine binds to DNA. It is assumed that the electronic structure of the phthalocyanine is not greatly affected by interaction with DNA, because the positively charged groups are separated by an ether bridge from the chromophore. Apparent binding constant (K_{DNA}) of tetracationic phthalocyanine was calculated giving values of $\sim 4.7 \times 10^6\ M^{-1}$.

Photocleavage of calf thymus DNA

Photoinduced damage of calf thymus DNA sensitized by this phthalocyanine was studied in TE buffer solution. Samples of DNA and photosensitizer were irradiated with

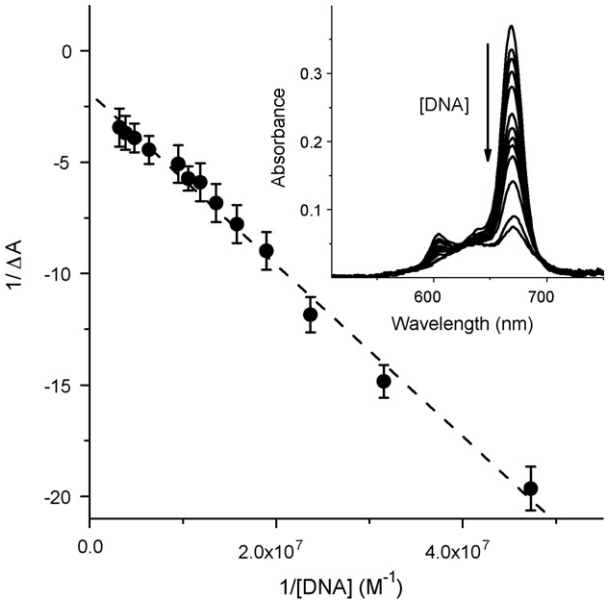


Figure 1 Variation of $1/(\Delta A)$ *vs.* 1/[DNA] for spectral titration of $ZnPPc^{4+}$ phthalocyanine with calf thymus DNA in PBS, λ_{max} = 669 nm. Dashed line: linear fit by Eq. (1). Inset: absorption spectra of $ZnPPc^{4+}$ in methanol at different DNA concentrations (0.01; 0.02; 0.03; 0.04; 0.05; 0.06; 0.07; 0.08; 0.09; 0.11; 0.16; 0.21; 0.26; 0.32 μM). Values represent mean ± standard deviation of three separate experiments.

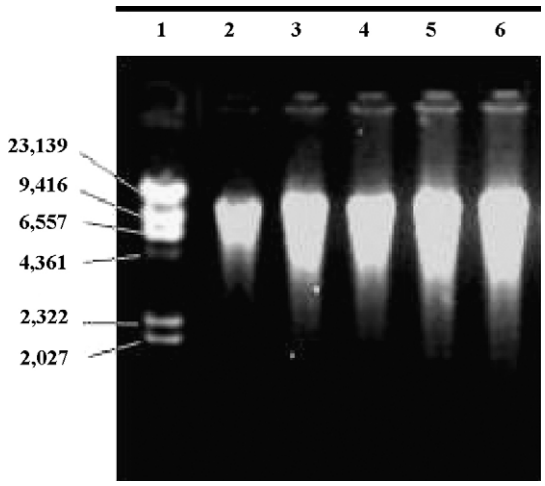


Figure 2 Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated by 1, 2, 3 and 4 h (lines 3—6, respectively) with visible light (30 mW/cm^2) in the presence of ZnPPc^{4+} (20 μM). Line 1: Lamda-*Hind* III DNA weight maker and line 2: control with sensitizer in dark.

visible light under aerobic condition for different times. The DNA integrity was analyzed by agarose gel electrophoresis (Fig. 2). DNA photodamage is observed by the appearance of a remarkable smearing after electrophoresis. As can be observed, a slightly modified pattern for DNA with respect to control (Fig. 2, line 2) was found for samples sensitized by ZnPPc^{4+}. Therefore, the photocleavage effect on DNA was not very significant even after longer irradiation periods (Fig. 2).

Binding of ZnPPc^{4+} to *E. coli* and photosensitized inactivation

The *E. coli* cells were incubated with 2 μM ZnPPc^{4+} for different times (5, 10, 30 min) in dark at 37 °C. The amount of sensitizer bound to cells was not significantly changed in these periods, reaching a value of ∼0.8 nmol/10^6 cells. The photoinactivation effect of ZnPPc^{4+} was evaluated to inactivate *E. coli* cellular suspensions in PBS. The results for *E. coli* cultures treated with 2 μM sensitizer for 30 min in dark and irradiated are shown in Fig. 3. As can be seen, cationic phthalocyanine produces a ∼4 log decrease in the cellular survival after a light fluence of 54 J/cm^2.

Photosensitized cleavage of *E. coli* plasmid DNA

One of the most important antibiotic resistant mechanisms involves the transfer of plasmids from one bacterium to another, for this reason is interesting to evaluate the photodynamic effect on plasmid DNA. A convenient way to assess the ability of a cationic phthalocyanine to induce photodegradation of plasmid DNA is to measure the conversion of supercoiled plasmid DNA (form I) to relaxed circular DNA (form II) [36]. In this way, the photodynamic activity of ZnPPc^{4+} was first studied in plasmid DNA purified from *E. coli* cells. Solutions of plasmid DNA and 2 μM phthalocyanine in TE buffer were irradiated with visible light for different times and the samples were examined by agarose electrophoretic analysis. As can be observed in Fig. 4, ZnPPc^{4+} produced the conversion of supercoiled DNA (form I) to nicked circular DNA (form II) in the presence of light. On the other hand, no cleavage occurred without irradiation (Fig. 4, line 1), indicating that photodamage of DNA was caused by sensitization activity raised from irradiation of the phthalocyanine. After a light fluence of 108 J/cm^2 (60 min), plasmid DNA is observed like circular form II (∼40%) in the presence of ZnPPc^{4+}.

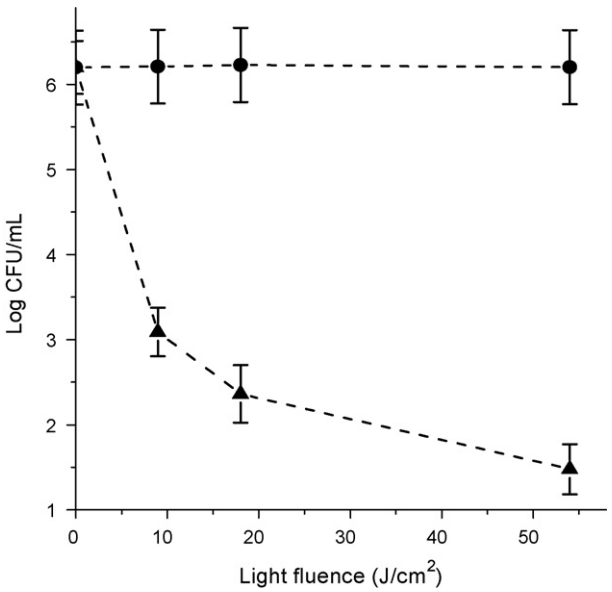


Figure 3 Survival curves of *Escherichia coli* cells incubated with 2 μM ZnPPc^{4+} (▲) for 30 min at 37 °C in dark and exposed to visible light (30 mW/cm^2) for different irradiation times. Control culture untreated with ZnPPc^{4+} (●) and irradiated. Values represent mean ± standard deviation of three separate experiments.

Similar experiments with plasmid DNA were also performed *in vitro* on *E. coli* cells treated with 2 μM ZnPPc^{4+} and exposed to visible light for different periods. After PDI, cells were lysed and the plasmid DNA isolated. The purified DNA was analyzed by electrophoresis. As showed in Fig. 5, time dependent modifications of the electrophoretic patterns of the plasmid DNA were also observed *in vitro*. Photocleavage of intracellular plasmid DNA is identified at longer irradiations. After a light dose of 162 J/cm^2, the photodamage in supercoiled DNA is observed in the presence of tetracationic phthalocyanine, diminishing to ∼55%. Under these conditions, intracellular plasmid DNA is converted to a less tensioned form II. At longer periods of irradiation, DNA fragmentation is also evidenced by a smearing of the bands. The amount of DNA form I progressively decreases until disappears at longer periods of irradiation.

Photosensitized cleavage of *E. coli* genomic DNA

The photodynamic activity of this sensitizer was analyzed *in vitro* to observe possible genomic DNA photodegradation purified from *E. coli* cells after PDI treatments. In these experiments, genomic DNA was extracted from cells treated with 2 μM sensitizer and exposed to visible light for dif-

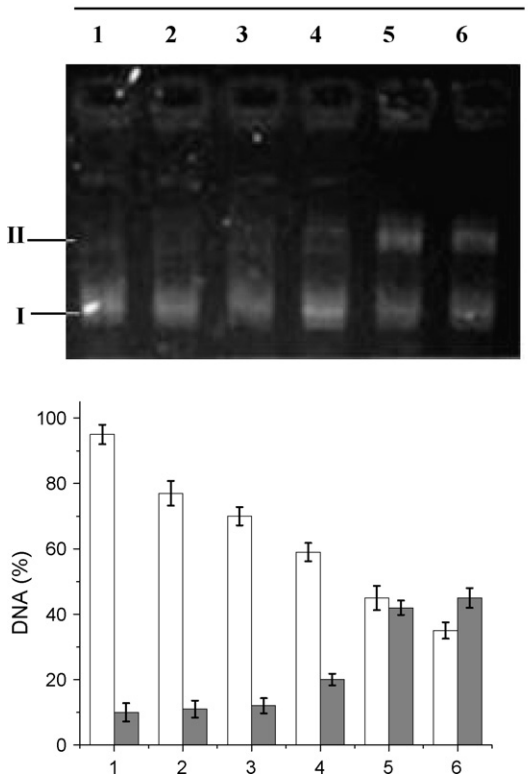


Figure 4 Agarose gel electrophoresis of plasmid DNA samples in TE buffer (pH 7.6) irradiated for 5, 10, 20, 60 and 90 min (lines 2—6, respectively) with visible light (30 mW/cm^2) in presence of 2 μM ZnPPc^{4+}. Line 1: control with sensitizer in dark. Column plots: form I (white bars) and form II (grey bars) plasmid DNA (%) sensitized by ZnPPc^{4+} after different periods of irradiation.

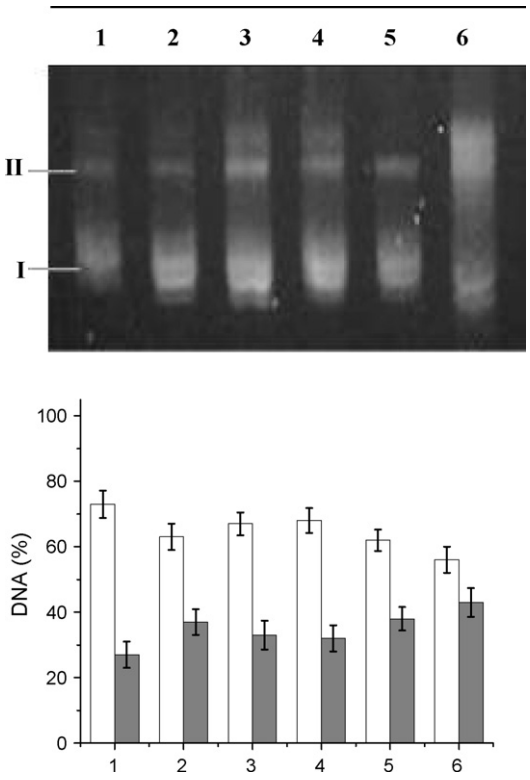


Figure 5 Agarose gel electrophoresis of plasmid DNA samples extracted from *E. coli* cells after PDI treatment. The cultures were irradiated for 5, 10, 20, 60 and 90 min (lines 2—6, respectively) with visible light (30 mW/cm^2) in presence of 2 μM ZnPPc^{4+}. Line 1: control with sensitizer in dark. Line 1: control with sensitizer in dark. Column plots: form I (white bars) and form II (grey bars) plasmid DNA (%) sensitized by ZnPPc^{4+} after different periods of irradiation.

ferent times. The extracts were separated by agarose gel electrophoresis as showed in Fig. 6. However, an unmodified pattern of DNA was found for cells treated with ZnPPc^{4+} with respect to the control assay (Fig. 6, line 2). The photodynamic action of this tetracationic phthalocyanine did not cause significant cleavage of genomic DNA even after an extended light fluence of 432 J/cm^2. Therefore, it can be considered either the genomic DNA was not involved in the photodamage process or the overall DNA degradation of a few lesions was not sufficient to induce DNA strand breaks.

Photodynamic effect on cellular ultrastructure of *E. coli*

Ultrastructural changes in the *E. coli* cells were first analyzed by transmission electron microscopy (TEM). The *E. coli* cells were treated with 2 μM ZnPPc^{4+} for 30 min in dark and the cultures irradiated with visible light for different times. Representative results after a light fluence of 54 J/cm^2 are shown in Fig. 7. As can be observed, alterations in the ultrastructure of the treated *E. coli* cells were formed during the photodynamic process, which were not detected in the untreated cells (Fig. 7A). Photodynamic treatment sensitized by ZnPPc^{4+} produced cells that present the appearance of low-density areas (Fig. 7B and C).

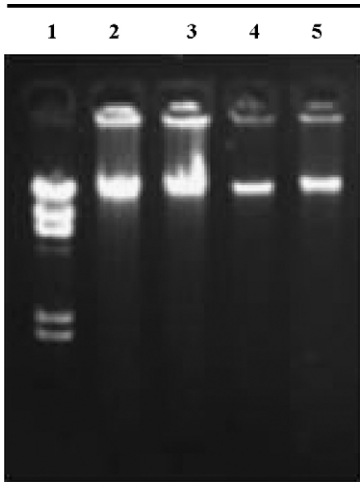


Figure 6 Agarose gel electrophoresis of genomic DNA samples extracted from *E. coli* cells after PDI treatment. The cultures were irradiated for 0.5, 1 and 4 h (lines 3—5, respectively) with visible light (30 mW/cm^2) in the presence of 2 μM ZnPPc^{4+}. Line 1: Lamda-*Hind* III DNA weight maker, line 2: control with sensitizer in dark.

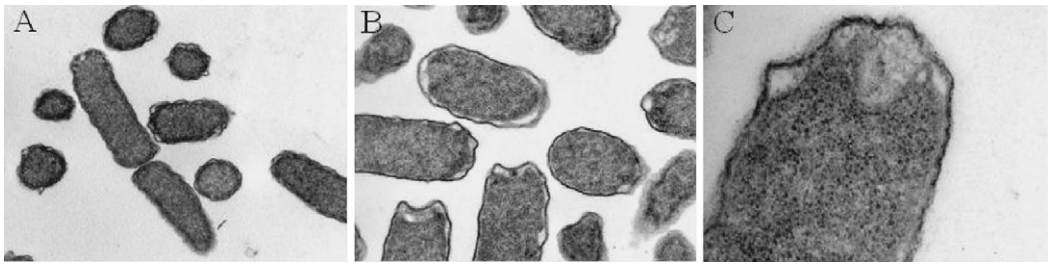


Figure 7 Transmission electron microscopy (TEM) of *E. coli* cells treated with 2 μM $ZnPPc^{4+}$ and irradiated for 30 min (B and C) with visible light (30 mW/cm^2). Control (A) of *E. coli* cells without sensitizer and irradiated for 30 min, magnification: (A) 16,700, (B) 21,560 and (C) 77,500.

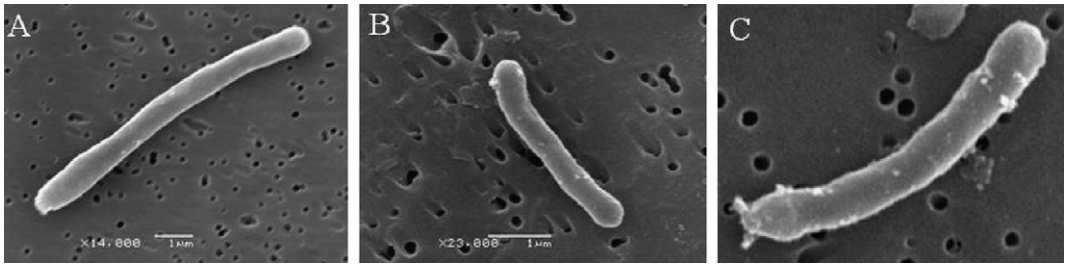


Figure 8 Scanning electron microscopy (SEM) of *E. coli* cells treated with 2 μM $ZnPPc^{4+}$ and irradiated for 30 min (B and C) with visible light (30 mW/cm^2). Control (A) of *E. coli* cells without sensitizer and irradiated for 30 min.

Moreover, the cells were analyzed by scanning electron microscopy (SEM). As can be observed in Fig. 8, changes on cellular surface were found between the untreated cells control (Fig. 8A) and treated cells with 2 μM $ZnPPc^{4+}$ (Fig. 8B) and irradiated with a light dose of 54 J/cm^2. After PDI treatment, *E. coli* cells showed small vesiculations on the cellular surface (Fig. 8B). This effect can be more clearly evidenced in a magnified image showed in Fig. 8C.

Integrity and membrane permeabilization of *E. coli* cells

The cytoplasmic cell membrane is the target for many antimicrobial agents. The damage of bacterial membrane is first accompanied by leached out low molecular weight species and followed by nucleotides, such as DNA, RNA and other materials [30]. The release of these intracellular components with strong UV absorption at 260 nm is an indication of membrane damage [31]. Thus, *E. coli* cellular suspensions were treated with 2 μM $ZnPPc^{4+}$ for 30 min in dark and irradiated with visible light. After different light doses, the cultures were centrifuged and the supernatants analyzed by UV spectroscopy. In all these cases, the absorbance at 260 nm was almost unchanged. Representative results for cultures sensitized by $ZnPPc^{4+}$ are shown in Fig. 9. In contrast, an intense absorption band ($\lambda \sim 260$ nm) appears when the cultures were sonicated by 15 min. Furthermore, this PDI treatment of *E. coli* cells did not induce fast release of $\lambda \sim 260$ nm absorbing materials from bacteria even after 432 J/cm^2 of irradiation, suggesting that although the functionality of the membrane can be damaged, it was not a sufficient amount to produce release of higher molecular weight species.

On the other hand, outer membrane (OM) permeabilization of *E. coli* cells after PDI treatment was evaluated using the hydrophobic NPN fluorescent probe. In this assay, NPN

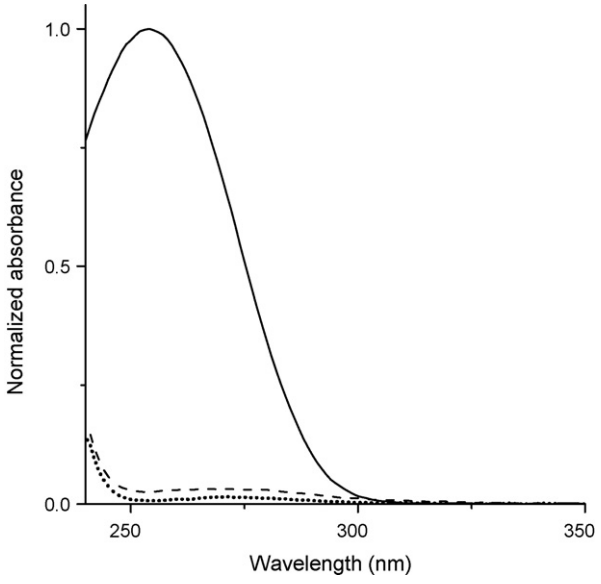


Figure 9 Release of absorbing material from *E. coli* cells treated with 2 μM $ZnPPc^{4+}$ for 30 min in dark and irradiated for 4 h (dashed line) with visible light. Cultures sonicated for 15 min, irradiated control without sensitizer (solid line) and dark control with sensitizer (dotted line).

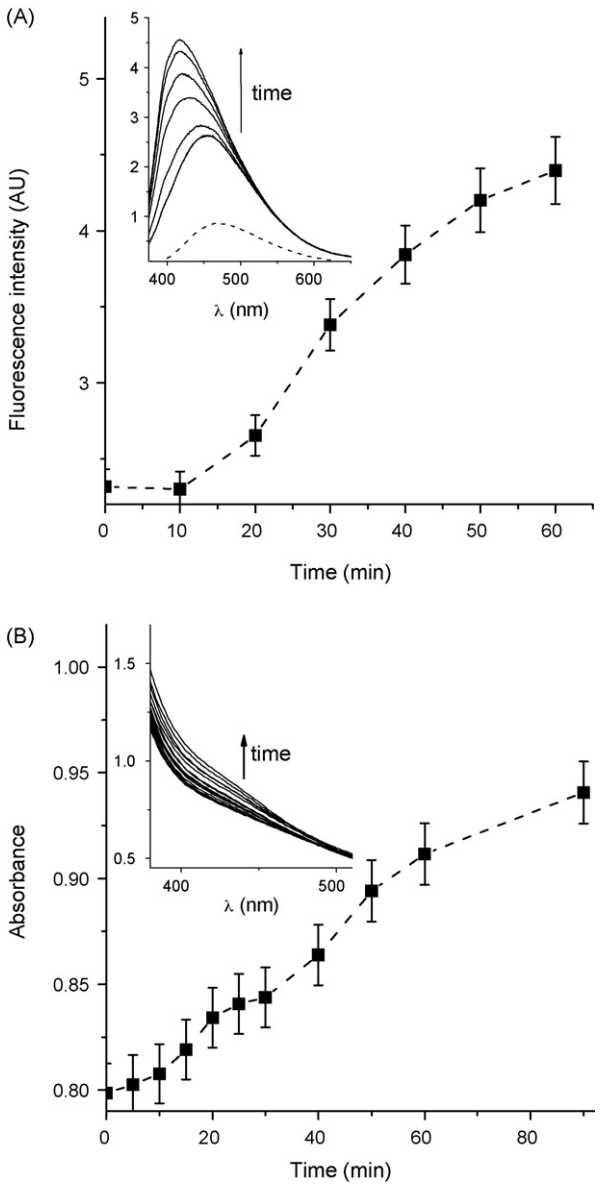


Figure 10 (A) Variation in the fluorescence emission intensity of NPN at different times post-PDI treatment of *E. coli* suspensions. Inset: fluorescence emission spectra of NPN, $\lambda_{exc} = 350$ nm. (B) Changes in the absorbance ($\lambda = 420$ nm) of ONPG. Insert: absorption spectra of *E. coli* cells in the presence of ONPG after PDI. Cultures were incubated with 2 μM $ZnPPc^{4+}$ for 30 min in dark at 37 °C and irradiated with a light fluence of 54 J/cm^2.

is normally excluded from OM but when this membrane is damaged the probe is partitioned into perturbed OM. This effect produces an increase in the fluorescence of NPN [32,33]. The addition of NPN to *E. coli* suspensions caused the increase in fluorescence exhibited in Fig. 10A. In addition, the ability of $ZnPPc^{4+}$ to permeate the *E. coli* IM was investigated as a function of cytoplasmic β-galactosidase release with bacteria grown in medium containing lactose. After PDI treatment, release of cytoplasmic β-galactosidase was detected by a progressive increase in the absorbance at 420 nm (Fig. 10B).

Discussion

In the structure of $ZnPPc^{4+}$, the four cationic centers are attached to the phthalocyanine macrocycle ring by an ether bond (Scheme 1). This spacer provides an appropriated mobility to the charges, which facilitates the interaction with the outer membrane of the *E. coli* cells. Also, this charges distribution has minimal influence on the electronic properties of the phthalocyanine macrocycle. Thus, this phthalocyanine exhibits a high absorption coefficient in the visible region of the spectrum, characterized by the typical *Soret* (∼374 nm) and *Q*-bands (∼678 nm). Also, $ZnPPc^{4+}$ emits two bands (∼687 and 756 nm) with a fluorescence quantum yield of 0.22 in DMF, which allows its detection and quantification in biological media even at very low concentrations [27].

The photodynamic activity of $ZnPPc^{4+}$ indicates a high efficiency in the quantum yield of $O_2(^1\Delta_g)$ production ($\Phi_\Delta = 0.59$) in solution of DMF. Also, it is an efficient photosensitizer to produce photooxidation of biological substrates, such as the amino acid L-tryptophan [27]. The studies in presence of calf thymus DNA showed that $ZnPPc^{4+}$ strongly interacts with the double-stranded DNA in solution (Fig. 1). The value of K_{DNA} for $ZnPPc^{4+}$ is higher than those previously reported for other tetracationic sensitizers, such as porphyrin derivatives [28,37]. Similar tight binding was also found for a cationic copper (II) phthalocyanine, cuprolinic blue, to calf thymus DNA [19]. In contrast, an anionic phthalocyanine does not bind to DNA, reflecting electrostatic contribution to the interaction between cationic sensitizers and the nucleotide chains. Also, thermodynamic and spectroscopic studies showed a higher affinity of the cationic zinc(II) tetrapyridinoporphyrazines towards DNA with respect to the cobalt(II) complex. This effect was attributed to the coordination interaction as the major contributing factor that enables superior interaction of the zinc complex with the DNA duplex [20].

The electrophoretic analysis of calf thymus DNA indicated that photocleavage takes place when the samples were exposed to photoexcited $ZnPPc^{4+}$ in aerobic condition. The photocleavage ability of this tetracationic phthalocyanine was considerable smaller than that found with a tetracationic porphyrin, 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin *p*-tosylate ($TMAP^{4+}$) [28]. Even though $ZnPPc^{4+}$ has a larger K_{DNA} than $TMAP^{4+}$, it is not as effective as this porphyrin to photocleavage DNA. However, an increasing smearing appears after long irradiation periods due to the modification and photocleavage of DNA (Fig. 2).

In vitro experiments showed that $ZnPPc^{4+}$ was rapidly bound to *E. coli* cells when the cultures were treated with 2 μM of sensitizer in dark. Under these conditions, the photodynamic action of $ZnPPc^{4+}$ produced a ∼4 log decrease of *E. coli* cell survival after 30 min of visible light irradiation (54 J/cm^2). This result represents a value greater than 99.99% of cellular inactivation. Also, a high PDI activity was previously reported for a tetracationic pyridinium zinc phthalocyanine (PPC) on *E. coli*, when the cultures are treated with 10 μg/mL sensitizer [8]. The mechanism of uptake of PPC across the outer membrane of *E. coli* appears to occur via the self-promoted uptake pathway [6]. Another proposed photosensitizers to eradicate bacteria involves

an octacationic zinc phthalocyanine, which is peripherally substituted with four bis(*N*,*N*,*N*-trimethyl)amino-2-propoxy groups [10]. This compound exhibited an appreciable PDI of *E. coli*, after irradiation with 675 nm light in the presence of 1 μM of photosensitizer. Also, a tetracationic Zn(II) tetramethyltetrapyridinoporphyrazinium salt was investigated to eradicate microbes. This phthalocyanine produced a ~2.4 log (~99.5%) decrease of *E. coli* cell survival, when the cultures were treated with 10 μM of photosensitizer and irradiated for 30 min [26].

The photodynamic activity produced by $ZnPPc^{4+}$ was analyzed exploring the photodegradation of *E. coli* bacterial nucleic acids and the membrane damage incurred during PDI. In aqueous solution, the disappearance of the supercoiled plasmid band took place during irradiation to produce the closed circular form plasmid DNA band, but the linear form was not detected (Fig. 4). When the photosensitization of plasmid DNA within whole bacterial cells was assayed (Fig. 5), the pattern of supercoiled plasmid DNA degradation was more slowly incremental. Therefore, the photodamage of plasmid DNA appeared at long PDI treatment (>108 J/cm^2), mainly when the *E. coli* cells were inactivated. On the other hand, genomic DNA isolated from *E. coli* after PDI was not cleaved even after a light fluence of 432 J/cm^2 (Fig. 6). In previous studies, it was found that 5,10,15,20-tetra(4-*N*-methylpyridyl)porphine (TMPyP) produced the death of all the *E. coli* cells in the culture, while the chromosomal DNA remained intact [7]. However, it was also reported that TMPyP induces a marked reduction in the chromosomal high-molecular DNA band after PDI at low light dose and 7.3 μM TMPyP [12]. The results of the present study about DNA integrity extracted from *E. coli* indicated that plasmid DNA damage appeared only after a long period of irradiation, whereas genomic DNA remained practically intact.

TEM analysis showed alterations in the ultrastruture of the *E. coli* cells (Fig. 7) treated with $ZnPPc^{4+}$ after PDI treatment. These modifications have been assigned to aggregation of cytoplasmic macromolecules [7]. Also, alterations and irregularities in the cell barriers were observed along the bacterium. Multilamellar membrane structures that are connected directly to the cytoplasmatic membrane appear in the cells after PDI treatment (Fig. 7C). The ultrastructural changes demonstrated by TEM supported the view that photoactivated phthalocyanine may potentially interfere with membrane functions. Oxidative reactions involving $O_2(^1\Delta_g)$ may be responsible for the effects on membrane biosynthesis sites stimulating the accumulation of macromolecules and conducing to alterations of the cell membrane structure. This process can lead to the inability to multiply and to produce viable bacteria [18]. Differences on the cellular surface were also observed via SEM for *E. coli* following PDI with $ZnPPc^{4+}$ (Fig. 8). The presence of bubbles were detected on the cellular surface in Fig. 8B and C. Similar structures were previous observed in Gram-negative bacteria and they were assigned to release of outer membrane vesicles due to stress-induced response [38]. In previous investigation using a tetracationic porphyrin, SEM images showed that *E. coli* sensitized by 11 μM TMPyP produced cell-wall ruptures in a fraction of cells [12]. However, in the present study the results were consistent with the absence of a direct observation of cell-wall rupture and cell lysis after PDI treatment. The release of intracellular biopolymers upon photodynamic action was not detected by absorption at 260 nm (Fig. 9). However, destabilization of the OM of *E. coli* cells was found after PDI treatment. Alteration in OM permeability barrier of *E. coli* was also observed for cells treated with a pyridinium zinc phthalocyanine (PPC) [6]. In previous investigations, it was pointed out that some protein molecules in the outer wall of phototreated *E. coli* cells with an octacationic Zn(II)-phthalocyanine were the target of the initial stages of the photoprocess [10]. Such modifications can induce an increase in the permeability of the wall, which is considered as the structural element responsible for the strong resistance of Gram-negative bacteria to photosensitization.

Therefore, the present results point out that structural changes and alterations in the membrane induced by the photodynamic action of $ZnPPc^{4+}$ play a major role in *E. coli* eradication, while that plasmid DNA damage is mainly involved after long periods of irradiation.

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