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Photodynamic inactivation mechanism of *Streptococcus mitis* sensitized by zinc(II) 2,9,16,23-tetrakis[2-(*N*,*N*,*N*-trimethylamino) ethoxy]phthalocyanine

Mariana B. Spesia, Edgardo N. Durantini *

Departamento de Química, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X5804BYA Río Cuarto, Córdoba, Argentina

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ABSTRACT

Photoinactivation of *Streptococcus mitis* induced by zinc(II) 2,9,16,23-tetrakis[2-(*N*,*N*,*N*-trimethylamino)ethoxy]phthalocyanine ($ZnEPc^{4+}$) was studied under different experimental condition in order to obtain information about the photodynamic processes and the cellular damage. A 3 log decrease in *S. mitis* survival was found in cell suspensions ($\sim 2 \times 10^8$ cells/mL) incubated with 2 μM $ZnEPc^{4+}$ and irradiated for 30 min with visible light (54 J/cm^2). Also, *S. mitis* cells growth was not detected in broth treated with 5 μM $ZnEPc^{4+}$ under continuous irradiation. Studies of photodynamic action mechanism showed that the cells were protected in the presence of azide ion, while the addition of mannitol did not produce a significant effect on the survival. Moreover, the photocytotoxicity was increased in D_2O indicating the interference of singlet molecular oxygen. On the other hand, it was found that $ZnEPc^{4+}$ interacts strongly with calf thymus DNA in solution but photocleavage of DNA was only detected after long irradiation periods. After *S. mitis* photoinactivation, modifications of genomic DNA were not observed by electrophoresis. In contrast, the transmission electron microscopy showed structural changes in the *S. mitis* cells, exhibiting mesosome-like structures. After 2 h irradiation, the cytoplasm showed segregation patterns and PDI appeared to have effects on the cell wall, including variability in wall thickness. Also, the presence of bubbles was detected on the cell surface by scanning electron microscopy. However, the photodamage to the cell envelope was insufficient to cause the release of intracellular biopolymers. Therefore, modifications in the cytoplasmic biomolecules and alteration in the cell barriers could be mainly involved in *S. mitis* photoinactivation. It can be concluded that photosensitization by $ZnEPc^{4+}$ mainly involved a type II photoprocess, while alteration in the cytoplasmatic components and modifications in the cell envelope were the major cause for the photoinactivation of *S. mitis*.

1. Introduction

The human oral cavity is constantly exposed to a wide variety of microorganisms that interact among themselves and with hard and soft tissue surfaces. Several studies have demonstrated that streptococci are the predominant colonizers of early enamel biofilms and, in particular, *Streptococcus mitis* is the species mainly identified [1]. *S. mitis* is a viridans streptococcus and a normal commensal of the human oropharynx. This bacterium is isolated as the predominant species in saliva and in soft tissue surfaces [2,3]. However, *S. mitis* can escape from this niche and cause a variety of infectious complications including infective endocarditis, bacteraemia and septicaemia [4]. Various colonization factors allow *S. mitis* to compete for space and nutrients in the face of its more pathogenic oropharyngeal microbial neighbours. However, it is likely that in vulnerable immune-compromised patients *S. mitis* can use the same colonization and immune modulation factors as virulence factors to promote its opportunistic pathogenesis.

Consequently, the search for new effective antibiotic treatments is awfully necessary. Photodynamic inactivation (PDI) of microorganisms has been proposed as an alternative to controlling bacterial infections [5,6]. This approach combines a photosensitizer, visible light and oxygen to yield 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 [7].

Previous studies have shown that cationic phthalocyanines are effective photosensitizers against bacteria [8–11]. It was established that a tetracationic phthalocyanine, zinc(II) 2,9,16,23-tetrakis[2-(*N*,*N*,*N*-trimethylamino)ethoxy)]phthalocyanine ($ZnEPc^{4+}$, Scheme 1) is an effective photosensitizer for the eradication of

* Corresponding author. Tel.: +54 358 4676157; fax.: +54 358 4676233.
E-mail address: edurantini@exa.unrc.edu.ar (E.N. Durantini).

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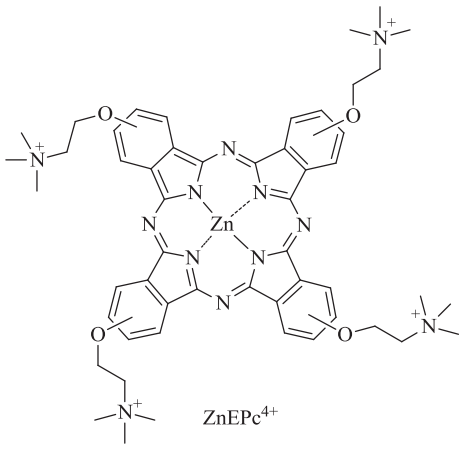

Scheme 1. Molecular structure of $ZnEPc^{4+}$.

Escherichia coli and *S. mitis* [12]. In particular, PDI of *S. mitis* by $ZnEPc^{4+}$ was even possible in presence of blood derivatives with a low hemolysis of erythrocytes. Therefore, in this work we are interested in investigating mechanistic aspects of the photodynamic action of $ZnEPc^{4+}$ to inactivate *S. mitis* cell suspensions. Thus, photoinactivation of *S. mitis* under different conditions was used to obtain insights about the mechanism of the photoreaction that produces the cellular death. Moreover, the purpose was to evaluate the possible photodynamic damages in the cells at the DNA level and in the cell components. These studies provide a better understanding of the mechanism of the cellular death of *S. mitis* after the photoinactivation sensitized by $ZnEPc^{4+}$.

2. Materials and methods

2.1. General

UV–visible absorption spectra were performed on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Quartz cuvettes of 1 cm path length was used at room temperature. Irradiation experiments were carried out with a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. Irradiation of the cultures was performed as previously described [13]. Experiments were carried out at room temperature and there was no heating during irradiation. A wavelength range between 350 and 800 nm was selected by optical filters. The light 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 Shelton Scientific (Peosta, IA, USA) IBI MP-1015 Horizontal Gel Electrophoresis Unit and the electric field was generated by a power supply PS251-2 (Sigma–Aldrich Techware). Images of the stained gel were acquired using UV transillumination with an Alpha Innotech Corporation (San Leandro, CA, USA) Multi Image Light Cabinet. Chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Calf thymus double-stranded DNA was 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.

2.2. Photosensitizer

Zinc(II) 2,9,16,23-tetrakis[2-(*N*,*N*,*N*-trimethylamino)ethoxy)]phthalocyanine iodide ($ZnEPc^{4+}$) were synthesized as previously described [14]. Stock solution (0.45 mM) of $ZnEPc^{4+}$ was obtained by dissolution in 1 mL of *N*,*N*-dimethylformamide (DMF) and checking the final concentration by spectroscopy. This solvent was used because $ZnEPc^{4+}$ exhibited in water a pronounced monomer/dimer equilibrium whereas in DMF only the monomer exists [14].

2.3. Bacterial strain and cultures preparation

The Gram-positive bacterium, *S. mitis* (MS171) was isolated from maxillary first molar abscess and identified as previously described [12]. Bacteria were grown aerobically at 37 °C in tryptic soy (TS) broth overnight. Aliquots (~70 μL) of this culture was aseptically transferred to 4 mL of fresh TS broth and incubated at 37 °C to middle of the logarithmic phase (absorbance ~0.7 at 660 nm). Under this condition, cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 3 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0) solution, corresponding to $\sim 2 \times 10^8$ colony forming units (CFU)/mL. Then the cells were diluted 1/400 in PBS to obtain $\sim 5 \times 10^5$ CFU/mL. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and $ZnEPc^{4+}$ was added from a stock solution (0.45 mM) in DMF and kept in dark for 30 min at 37 °C. After each experiment, cellular suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable *S. mitis* were monitored and the number of CFU was determined on Columbia blood agar after 18–24 h incubation at 37 °C.

2.4. Photoinactivation of S. mitis in PBS suspension

Suspensions of *S. mitis* (2 mL, $\sim 2 \times 10^8$ CFU/mL) in PBS were dark incubated with 2 μM $ZnEPc^{4+}$ for 30 min at 37 °C and then the culture tubes were exposed to visible light for 30 and 120 min. After that, cellular suspensions were serially diluted with PBS and the number of colonies formed was counted as described above.

2.5. Photosensitized growth delay experiment

A portion (60 μL) of *S. mitis* cells grown overnight was transferred to 20 mL of fresh TS broth medium. The suspension was homogenized and aliquots of 2 mL were placed in culture tubes with 5 μM $ZnEPc^{4+}$ and incubated at 37 °C with continuous irradiation along 10 h. The cell culture growth was monitored by turbidity at 550 nm every 60 min using a Turner SP-830 spectrophotometer (Dubuque, IA, USA).

2.6. Effect of additives and media on S. mitis photoinactivation in PBS suspension

S. mitis cell suspensions (2 mL, $\sim 5 \times 10^5$ CFU/mL) in PBS were treated with 1 μM $ZnEPc^{4+}$ for 30 min in dark at 37 °C. Photodynamic activity of $ZnEPc^{4+}$ was studied in presence of sodium azide and mannitol. These compounds were added from a stock solution (2.5 M) in water to obtain a concentration of 50 mM in the culture tubes. Studies under anoxic conditions were performed displacing the oxygen with argon in the culture tubes for 30 min before irradiation and maintaining an argon atmosphere during the experiment. To obtain *S. mitis* cell suspensions in D_2O, the cell cultures were centrifuged (3000 rpm for 15 min) and resuspended in 2 mL of PBS solution in D_2O. After that, the cultures were exposed to visible light for 5 min and the samples were treated as described above.

2.7. Phthalocyanine binding to calf thymus DNA

Stock solution of calf thymus double-stranded DNA (1.3×10^{-5} M base pairs) was prepared by dissolution in PBS. The DNA concentration was determined by spectroscopy [15]. The apparent binding constant (K_{DNA}) for phthalocyanine-DNA complex was calculated assuming 1:1 stoichiometry by Eq. (1), where (ΔA) represents the absorbance changes at the Q-band maximum and $(\Delta A)_\infty$ symbolizes the extrapolated absorbance change at [DNA] → ∞[16]. From a plot of $1/\Delta A$ *vs.* 1/[DNA] was calculated the value of K_{DNA} considering the ratio of the intercept to the slope.

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_\infty} + \frac{1}{\Delta A_\infty K_{DNA}[DNA]} \quad (1)$$

2.8. Steady state photolysis of calf thymus DNA

Culture tubes containing 1 mL solutions of 1.3×10^{-5} M calf thymus DNA and 20 μM $ZnEPc^{4+}$ in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) were irradiated with visible light (30 mW/cm^2) along 4 h at room temperature. Every hour, samples of 30 μL were taken at different times after irradiation. After that, each aliquot was mixed with 3 μL of Loading Buffer 6× (bromophenol blue 0.25% w/v, sucrose 40% w/v, acetic acid 1.15%, Tris 40 mM, EDTA 1 mM). Then, DNA was analyzed by electrophoresis using 1% agarose gel in TBE buffer (90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA, pH = 8) at 2.9 V/cm for 2 h. Ethidium bromide (1 mg/mL) was added into the agarose gel. The Lamda DNA/*Hind* III (Promega, Madison, Wi, USA) was used as a molecular weight marker (MK) (0.5 μg/μL) with DNA fragments between 125 and 23,130 base pairs.

2.9. Purification of S. mitis genomic DNA

Cell suspensions of *S. mitis* (∼2×10^8 CFU/mL) in PBS were treated with 2 μM $ZnEPc^{4+}$ as previously indicated. Culture tubes were irradiated for 30, 60 and 120 min with visible light. Post irradiation, the genomic DNA was extraction from the *S. mitis* cells using a Wizard Genomic DNA Purification Kit, (Promega). Genomic DNA samples were analyzed by electrophoresis as described above.

2.10. Transmission electron microscopy (TEM)

Cell suspensions of *S. mitis* (∼2×10^8 CFU/mL) in PBS were treated with 2 μM $ZnEPc^{4+}$ for 30 min in dark. After that, culture tubes were irradiated for 30 and 120 min. Cell suspensions were centrifuged (3000 rpm for 10 min) and the cells were fixed in 4% w/v formaldehyde and 2% v/v glutaraldehyde mixture in cacodylate buffer 0.1 M for 2 h at room temperature [17]. Then, the bacterial cells were washed three times with cacodylate buffer and post-fixed in 1% osmium tetroxide in the same buffer solution for 1–2 h at room temperature. *S. mitis* cells were dehydrated with gradients of acetone and embedded in Araldite epoxy resin and polymerized at 60 °C for 24 h. Sections of 80–100 nm thick were cut with a diamond knife on a JEOL JUM-7 ultramicrotome (Nikon, Tokyo, Japan). Samples were stained with uranyl acetate in alcoholic solution (2 min) and lead citrate (2 min). Sections were examined and digitally photographed with a transmission electronic microscopy Leo 906E equipped with a MegaView III camera.

2.11. Scanning electron microscopy (SEM)

Cell suspensions of *S. mitis* (∼2×10^8 CFU/mL) were treated as described in TEM experiments. Samples were centrifuged (3000 rpm for 10 min) and supernatant were separated. The bacterial pellets were fixed in a 4% formaldehyde and 2% glutaraldehyde mix in buffer cacodilate 0.1 M by a minimum period about 2 h at room temperature [17]. After that, all material was dehydrated in water solutions of alcohol of different graduation (30–100%) along 15 min in each one. Samples were dropped on round glass cover slip and dried by critical-point method (EMITECH, Model K850, East Grinstead, West Sussex, UK). Then they were sputter coating with gold (Balzers Instruments SCD 030, Liechtenstein) to provide electrical conductivity to the non-conductive samples and observed under the scanning electronic microscopy Philips SEM 505 (Philips, Eindhoven, NL, USA), equipped with a program for digital imaging (Soft Imaging System ADDA II).

2.12. Measurement of DNA and protein leakage

S. mitis cells (∼2×10^8 CFU/mL) in PBS (2 mL) were incubated with 2 μM $ZnEPc^{4+}$. Culture tubes were irradiated with visible light for 30 and 120 min. Each irradiated tube was centrifuged at 5000 rpm for 10 min. The pellet was detached and the release of materials in the supernatant was examined by UV–visible spectroscopy [17].

2.13. Controls and statistical analysis

In all cases, control experiments were carried out in presence of $ZnEPc^{4+}$ in the dark and in the absence of $ZnEPc^{4+}$ with cellular suspensions irradiated and in the dark. Each experiment was repeated separately three times. Data were depicted as the mean ± standard deviation of each group. Variation between each experiments was calculated using the Kruskal–Wallis test, with a confidence level of 95% ($p < 0.05$) considered statistically significant.

3. Results

3.1. Photosensitized inactivation of S. mitis in PBS suspension

Photoinactivation of *S. mitis* was evaluated in PBS cell suspensions using ∼2×10^8 CFU/mL. This cell density was chosen to obtain a significant number of cells in the pellet for further studies of cell damage. Thus, cells were treated with 2 μM $ZnEPc^{4+}$ for 30 min in dark at 37 °C and irradiated with visible light for 30 and 120 min. As can be seen in Fig. 1, a reduction of 3 log on cell

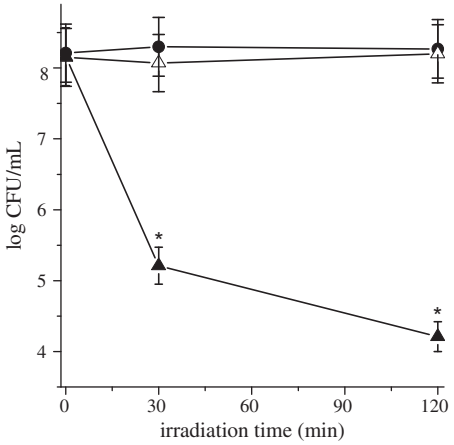

Fig. 1. Survival curves of *S. mitis* (∼2×10^8 CFU/mL) incubated with 2 μM $ZnEPc^{4+}$ (▲) for 30 min at 37 °C in dark and exposed to visible light (30 mW/cm^2) for different irradiation times. Control cultures untreated with $ZnEPc^{4+}$and irradiated (●) and treated with 2 μM $ZnEPc^{4+}$and keep in dark (△). Values represent mean ± standard deviation of three separate experiments. *$p < 0.05$, compared with untreated cells.

viability was obtained for cells treated with the photosensitizer after 30 min irradiation. The photocytotoxic effect produced a ~4 log decrease in cell survival when the cultures were irradiated for 120 min. In contrast, *S. mitis* cells viability were not affected in control assays, where cultures were incubated with the same concentration of $ZnEPc^{4+}$ in dark or cultures were exposed to visible light without phthalocyanine.

3.2. Photosensitized growth delay of S. mitis cultures

The photoinactivation capacity induced by $ZnEPc^{4+}$ was evaluated on the growth of *S. mitis* cultures in TS broth. Thus, 5 μM $ZnEPc^{4+}$ was added to fresh cultures and the tubes were irradiated with visible light at 37 °C. The effect produced by $ZnEPc^{4+}$ on growth of *S. mitis* is shown in Fig. 2. As can be observed, cultures without phthalocyanine, both irradiated and in dark, showed that the middle of the exponential phase was reached in ~5 h. When the cultures were incubated with 5 μM $ZnEPc^{4+}$ in dark, the curve shape showed a slight decline, indicating a low toxicity of this phthalocyanine in dark at this concentration. In spite of this delay in the curve, the growth stationary phase reached similar values than untreated controls after 9 h incubation. Instead, *S. mitis* growth was suppressed when cultures containing 5 μM $ZnEPc^{4+}$ were continually irradiated and the cells no longer appeared to be growing as measured by turbidity at 550 nm. This result shows that photoinactivation by $ZnEPc^{4+}$ is an effective inhibitor of the *S. mitis* growth in this culture media.

3.3. Studies of the photodynamic action mechanism

Photoinactivation of *S. mitis* cells was studied under different experimental conditions, such as addition of azide ion or mannitol, an argon atmosphere and cell suspensions in D_2O, to obtain insight about the photodynamic mechanism. Thus, *S. mitis* cells (~5×10^5 CFU/mL) were treated with 1 μM $ZnEPc^{4+}$ for 30 min in dark at 37 °C and then the culture tubes were irradiated for 5 min with visible light. This PDI treatment produced a ~2.5 log reduction in the viability (Fig. 3, line 4). Therefore, these conditions were chosen because they allowed observing either possible protective effects or an increase in the inactivation of cells.

First, the cell suspensions of *S. mitis* were treated with $ZnEPc^{4+}$ containing azide ion. This molecule is a quencher of intracellular singlet molecular oxygen, $O_2(^1\Delta_g)$ [18]. The presence of 50 mM sodium azide in *S. mitis* cells was not toxic in the dark (Fig. 3, line 5). However, the addition of azide ion produced a high reduction in the cell inactivation photosensitized by $ZnEPc^{4+}$ and no change with respect to the control was found after 5 min irradiation (Fig. 3, line 6). Therefore, the presence of azide ion quenched the photocytotoxic species, producing a protective effect on *S. mitis* cells.

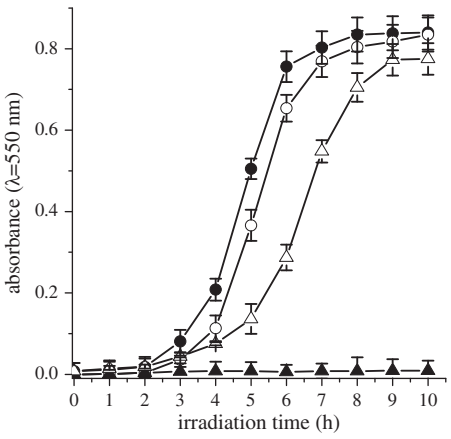

Fig. 2. Growth curves of *S. mitis* cells treated with 5 μM $ZnEPc^{4+}$ (▲) and irradiated with visible light (30 mW/cm²) for different times in TS broth at 37 °C. Control cultures treated with 5 μM $ZnEPc^{4+}$ (△) in dark, untreated cells irradiated (●) and in dark (○). Values represent mean ± standard deviation of three separate experiments.

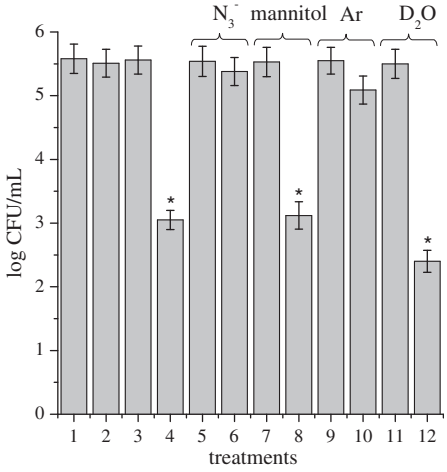

Fig. 3. Survival of *S. mitis* cells (~5×10^5 CFU/mL) incubated with 1 μM $ZnEPc^{4+}$ in dark for 30 min at 37 °C. (1) Control culture kept in dark, (2) control culture irradiated for 5 min, (3) control culture treated with $ZnEPc^{4+}$ and kept in dark; (4) culture treated with $ZnEPc^{4+}$ and irradiated for 5 min; (5) control culture with 50 mM azide in dark; (6) culture treated with $ZnEPc^{4+}$ in presence of 50 mM azide and irradiated for 5 min; (7) control culture with 50 mM mannitol in dark; (8) culture treated with $ZnEPc^{4+}$ in presence of 50 mM mannitol and irradiated for 5 min; (9) control culture under argon atmosphere in dark; (10) culture treated with $ZnEPc^{4+}$ under argon and irradiated for 5 min; (11) control culture in D_2O and kept in dark; (12) culture treated with $ZnEPc^{4+}$ in D_2O and irradiated for 5 min. Values represent mean ± standard deviation of three separate experiments. $^*p < 0.05$, compared with untreated cells.

On the other hand, mannitol acts as scavenger of the superoxide anion radical and hydroxyl radical [19]. The addition of 50 mM mannitol was not cytotoxic for *S. mitis* cells in dark (Fig. 3, line 7). After 5 min illumination, cell inactivation was very similar in presence or absence of mannitol (Fig. 3, lines 8 and 4), indicating a negligible effect on the *S. mitis* photoinactivation.

Also, *S. mitis* cell suspensions treated with $ZnEPc^{4+}$ were irradiated under anoxic conditions to evaluate the influence of oxygen on the cell viability. No toxicity was observed for cells under an argon atmosphere in the dark (Fig. 3, line 9). As can be seen, almost no photoinactivation was found in *S. mitis* cells treated with $ZnEPc^{4+}$ under a low oxygen concentration after 5 min irradiation (Fig. 3, line 10). Therefore, the loss of viability of *S. mitis* cells was highly oxygen dependent.

Moreover, the photoinactivation was assayed using *S. mitis* cells in saline D_2O. The lifetime of $O_2(^1\Delta_g)$ is improved in D_2O with respect to the aqueous medium, therefore it can be used to estimate the $O_2(^1\Delta_g)$-mediated damage of cells induced by $ZnEPc^{4+}$ [20]. The cell viability was not affected in dark (Fig. 3, line 11). However, irradiation of *S. mitis* cells incubated with the $ZnEPc^{4+}$ in D_2O produced a higher photoinactivation than that found in PBS cell suspensions (Fig. 3, lines 12 and 4).

3.4. Interaction of $ZnEPc^{4+}$ with calf thymus DNA

The interaction of $ZnEPc^{4+}$ with calf thymus double-stranded DNA was studied by visible absorption spectroscopy in methanol. This solvent was used to solubilize the phthalocyanine as monomer in solution. As can be observed in Fig. 4, titration of $ZnEPc^{4+}$

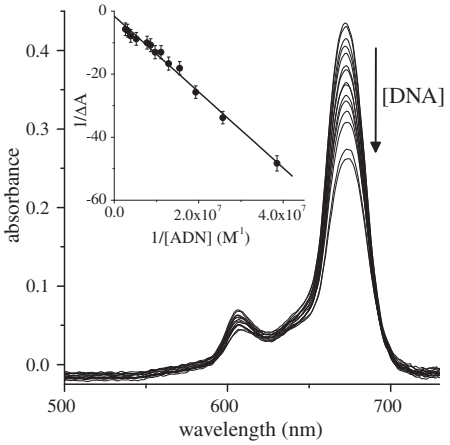

Fig. 4. Absorption spectra of $ZnEPc^{4+}$ in methanol at different calf thymus DNA concentrations (0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.09, 0.10, 0.11, 0.13, 0.19, 0.25, 0.32, 0.38 μM). Inset: Variation of $1/(\Delta A)$ *vs* 1/[DNA] for spectral titration of $ZnEPc^{+4}$ with calf thymus DNA in methanol, λ_{max} = 672 nm. Complete line: linear fit by Eq. (1). Values represent mean ± standard deviation of three separate experiments.

with DNA produced mainly spectral changes in the *Q* band at 672 nm. Spectral changes upon addition of DNA were assumed to the interaction of the $ZnEPc^{4+}$ with the DNA structure. The effect was characterized by a bathochromic shift of the *Q* maximum and by a hypochromicity of 45% (Fig. 4). The large hypochromicity can be assigned to perturbations of phthalocyanine π electrons by the association with DNA. Apparent binding constant (K_{DNA}) of $ZnEPc^{4+}$-DNA was calculated by Eq. (1) (Fig. 4, insert), giving a value of $1.3 \times 10^6\ M^{-1}$. This high value of K_{DNA} was indicative of the formation of stable complexes with calf thymus DNA.

3.5. Photocleavage of calf thymus DNA

Photocleavege of calf thymus DNA induced by $ZnEPc^{4+}$ was evaluated in TE buffer solution. Solutions of DNA and photosensitizer were irradiated with visible light for 1, 2, 3 and 4 h under aerobic condition. Then, agarose gel electrophoresis was used to evaluate the DNA integrity after each irradiation periods (Fig. 5). A slightly modified pattern for DNA was found for samples photosensitized by $ZnEPc^{4+}$ with respect to control (Fig. 5). The photodamage on DNA appears to be not significant after 1 h irradiation (Fig. 5, line 3). Only after 4 h irradiation, the DNA photocleavege was more evident by the appearance of fragments of lower molecular weights (Fig. 5, line 6).

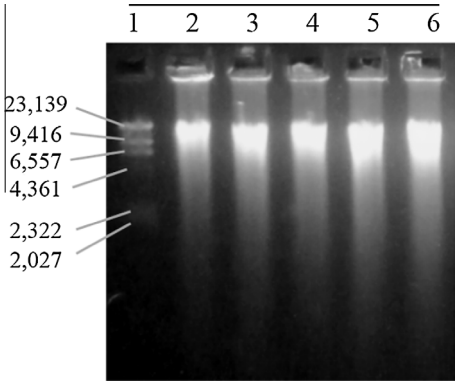

Fig. 5. Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated with visible light (30 mW/cm^2) for 1, 2, 3 and 4 h (lines 3–6, respectively) in the presence of $ZnEPc^{4+}$ (20 μM). Line 1: Lamda-*Hin*d III DNA weight maker and line 2: control with $ZnEPc^{4+}$ (20 μM) in dark.

3.6. PDI effect on S. mitis genomic DNA

S. mitis genomic DNA integrity was compared before and after PDI treatment with $ZnEPc^{4+}$. Thus, cells ($\sim 10^8$ CFU/mL) were treated with 2 μM $ZnEPc^{4+}$ and exposed to visible light for different times. Genomic DNA extracts were analyzed by agarose gel electrophoresis as showed in Fig. 6. The photodynamic action of $ZnEPc^{4+}$ did not produce significant cleavage of genomic DNA, even after 2 h photodynamic treatment (Fig. 6, line 5). Although, it could be that a few lesions in the DNA were not sufficient to produce a detectable damage by electrophoresis. Moreover, an unmodified pattern of DNA was found for the cells treated with $ZnEPc^{4+}$ in dark with respect to the control assay (Fig. 6, line 2).

3.7. Photodynamic effect on cellular structure of S. mitis

In order to investigate at morphological level the photocytotoxic effect of $ZnEPc^{4+}$, the cell structure of *S. mitis* was analyzed by TEM. Thus, *S. mitis* cells ($\sim 2 \times 10^8$ CFU/mL) were treated with 2 μM of $ZnEPc^{4+}$ for 30 min in dark. Cultures were irradiated with visible light for different times before the TEM images. Representative results are shown in Fig. 7. The normal coccoid shape structure could be observed in control cells without photosensitizer (Fig. 7A) and in cells treated with $ZnEPc^{4+}$ and kept in dark (Fig. 7B). The cell wall and the plasmatic membrane can be well differentiated in these electron micrographs. Also, ribosomes can be observed like dark particles in the cytoplasm and the nucleotide as a clear zone. Treated *S. mitis* cells with $ZnEPc^{4+}$ and irradiated for 30 min showed alterations at ultrastructural level (Fig. 7C), which were not detected in the controls (Fig. 7A and B). After PDI treatment, *S. mitis* cells contained multilamellar, mesosome-like structures (Fig. 7C). Laminar mesosomes were also seen arising from the septa and cell wall. An increase of affected zones was observed after 2 h irradiation (Fig. 7 D). In addition, it was found cells that lost their normal structure with decreased electrodensity in the cytoplasm (Fig. 7 D). After 2 h, the cytoplasm also showed segregation patterns. There were also structural changes at the plasmatic membrane and cell wall (Fig. 7D). Thus, PDI appeared to have effects on the cell envelope, including variability in wall thickness.

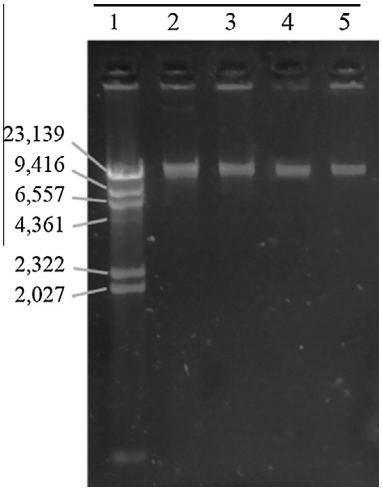

Fig. 6. Agarose gel electrophoresis of genomic DNA sample extracted from *S. mitis* cells after PDI treatment. The cultures ($\sim 2 \times 10^8$ CFU/mL) were incubated with 2 μM $ZnEPc^{4+}$ for 30 min at 37 °C in dark and irradiated with visible light (30 mW/cm^2) for 0.5; 1 and 2 h (lines 3–5, respectively) in presence of 2 μM $ZnEPc^{4+}$. Line 1: Lamda *Hin*d III DNA weight maker, line 2: control with 2 μM $ZnEPc^{4+}$ in dark.

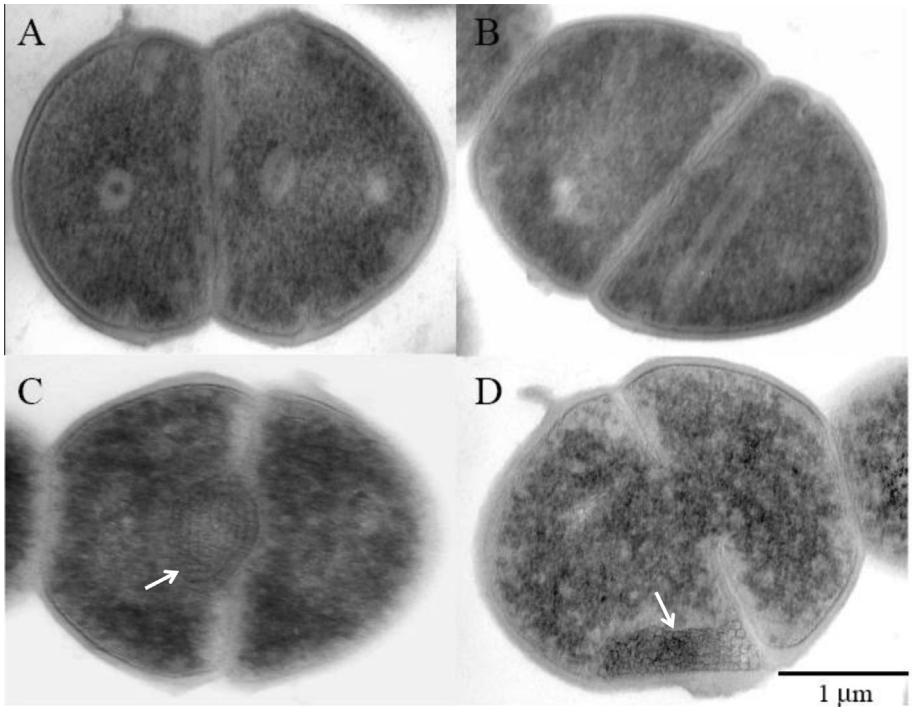

Fig. 7. Transmission electron microscopy (TEM) of *S. mitis* cells (~2×10^8 CFU/mL) treated with 2 μM $ZnEPc^{4+}$ for 30 min at 37 °C in dark. (A) Control of untreated cells; (B) treated cells kept in dark; (C) treated cells irradiated for 30 min and (D) treated cells irradiated for 2 h with visible light (30 mW/cm^2). Arrows indicate the mesosome-like structures. Magnification: 60,000×.

Moreover, the morphology and surface characteristics of *S. mitis* were analyzed by SEM. Untreated *S. mitis* cells appeared ellipsoidal and pyknotic (Fig. 8A). After incubation with 2 μM $ZnEPc^{4+}$, they were arranged in chains, similarly to untreated *S. mitis* (Fig. 8B). As can be observed in Fig. 8, no significant changes on cellular surface were found between the untreated cells control (Fig. 8A) and treated cells with 2 μM $ZnPPc^{4+}$ and irradiated for 30 min (Fig. 8C). Fig. 8 D shows a representative microscope image after cells incubated with 2 μM $ZnPPc^{4+}$ and irradiated for 2 h. Under these conditions, the protrusion of small bubbles of various shapes was appreciated on the surface of a cell in twenty. Similar results were also found in the analysis of ten SEM micrographs containing about twenty cells each. The presence of these bubbles was not detected in the untreated controls. After this PDI treatment, cell inactivation was not complete and the appearance of significant damage visualized by SEM on the surface was only found in a few cells. Moreover, no lysed bacterial cells were observed after 2 h irradiation.

3.8. Photosensitized effect on the leakage of intracellular DNA and proteins of S. mitis cells

The release of biopolymers was monitored by UV absorption at 260 nm as an indication of membrane damage. Thus, *S. mitis* cell suspensions (~2×10^8 CFU/mL) were incubated with 2 μM $ZnPPc^{4+}$ for 30 min in dark and irradiated with visible light for 30 min and 120 min. The cultures were centrifuged and the supernatants analyzed by UV spectroscopy as shown in Fig. 9. In all these cases, the absorbance at 260 nm was almost unchanged. Therefore, this PDI treatment of *S. mitis* cells did not induce fast release of $\lambda \sim 260$ nm absorbing materials from cells. This suggests that although the functionality of the membrane can be damaged, it was not a sufficient amount to produce release of higher molecular weight species.

4. Discussion

The phthalocyanine $ZnEPc^{4+}$ contains four cationic centers attached to the macrocycle periphery by an aliphatic chain, which provides a greater mobility to the charges. This structure could facilitate the interaction with the bacterial cell envelope. The ability of $ZnEPc^{4+}$ to bind to bacterial cells was previously analyzed in *E. coli* and *S. mitis* [12]. For *S. mitis* cells (~5×10^5 CFU/mL) treated with 1 μM, the amount of $ZnEPc^{4+}$ bound to bacterial cells was estimated on 0.21 ± 0.05 nmol/10^6 cells after 30 min of incubation. This affinity is consequently accompanied by an effective photocytotoxic activity. Thus, photosensitization of *S. mitis* by $ZnEPc^{4+}$ produced a diminishing in the cellular viability of ~99.993% (~4 log decrease) for cultures irradiated for 30 min with visible light. In the present study, the photodynamic effect induced by this phthalocyanine was investigated in *S. mitis* cultures using ~2×10^8 CFU/mL. The photoinactivation of *S. mitis* incubated with 2 μM $ZnEPc^{4+}$ was evaluated after different irradiation periods. An irradiation of 30 min induced a ~99.95% (3 log decrease) of *S. mitis* photoinactivation. Under these conditions, an enhancement in the cell inactivation was found increasing the irradiation times. Thus, an exposure to visible light of 2 h was required to produce a photoinactivation of 4 log decrease (99.995% of cellular inactivation). When cell suspensions of ~2×10^8 CFU/mL were used instead of ~5×10^5 CFU/mL, the amount of $ZnEPc^{4+}$ molecule by each microbial cell is reduced by thousand factors. Therefore, the effectiveness of PDI diminishes significantly with an increase in the cell density. The effect of cell density on PDI was previously observed for different microorganisms [21,22]. The decrease in cell inactivation is mainly produced by a major distribution of photosensitizer between the number of cells. Thus, the amount of phthalocyanine per cell is smaller at high densities and consequently it is accompanied by a minor cytotoxicity. For this reason, the concentration of phthalocyanine was duplicated to 2 μM when experiments were

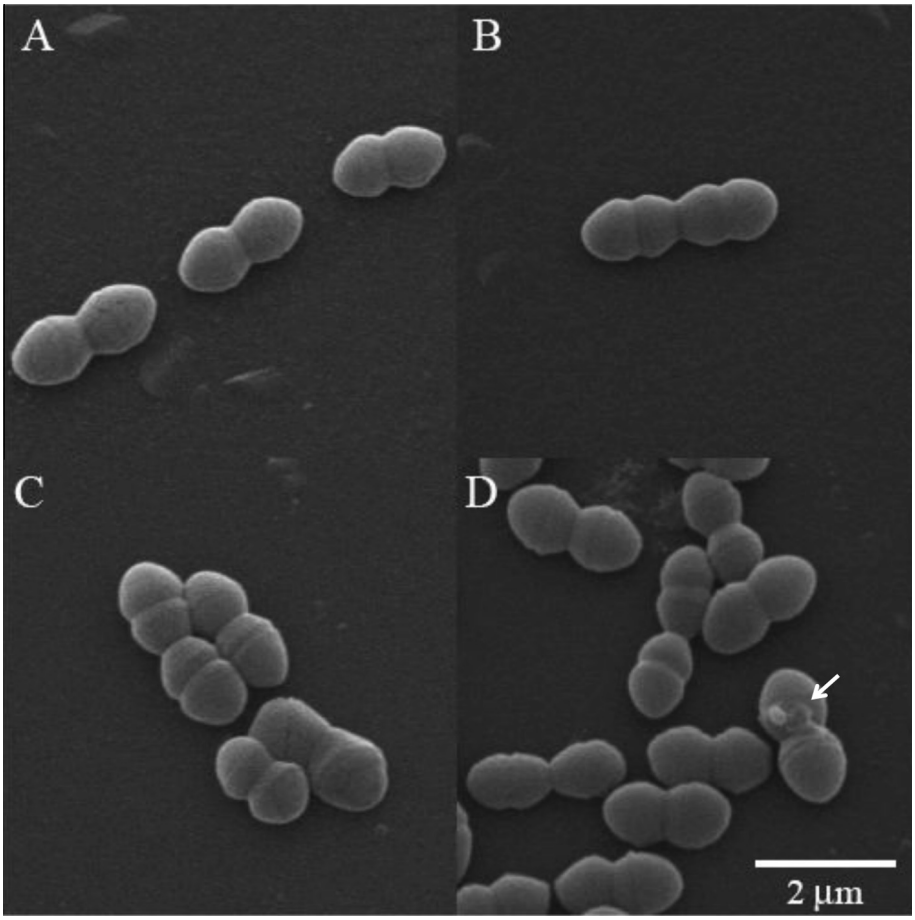

Fig. 8. Scanning electron microscopy of *S. mitis* cells (~2 × 10^8 CFU/mL) treated with 2 μM $ZnEPc^{4+}$ for 30 min at 37 °C in dark. (A) Control of untreated cells; (B) treated cells kept in dark; (C) treated cells irradiated for 30 min and (D) treated cells irradiated for 2 h with visible light (30 mW/cm^2). Arrow indicates the protrusion of small bubbles. Magnification: 10,000×.

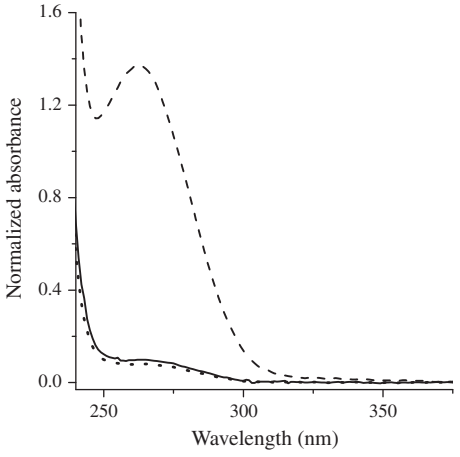

Fig. 9. Release of absorbing material from *S. mitis* cells (~2 × 10^8 CFU/mL) treated with 2 μM $ZnEPc^{4+}$ for 30 min in dark and irradiated for 2 h (solid line) with visible light. Dark control with 2 μM $ZnEPc^{4+}$ (dotted line) and cultures sonicated for 30 min without $ZnEPc^{4+}$ (dashed line).

carried out with ~2 × 10^8 CFU/mL, increasing the availability of photosensitizer in the medium. Also, the photodynamic activity of $ZnEPc^{4+}$ was evaluated on growth curves of *S. mitis*. Under these conditions, the growth of bacterial cultures was completely detained in the presence of 5 μM phthalocyanine, indicating that the photoinactivation of *S. mitis* cells was still possible when the cultures were not under starvation conditions.

On the other hand, after activation of the photosensitizer two oxidative mechanisms can mainly take place under PDI conditions, which are considered to be principally implicated in the cell photodamage [23]. In the type I photoprocess, the photosensitizer interacts with substrates to yield free radicals. The majority of these radicals instantaneously reacts with oxygen and generates a complicated mixture of highly ROS. In contrast, $O_2(^1\Delta_g)$ is produced in the type II mechanism as the main species responsible for cell inactivation. These intermediates are capable to oxidize a wide variety of biomolecules leading to a loss of appropriate biological functionality. Thus, depending on the experimental conditions, these mechanisms can occur simultaneously and the ratio between the two processes is influenced by the photosensitizer, substrate and the nature of the medium. The photodynamic activity of $ZnEPc^{4+}$ indicated a value of 0.47 in the quantum yield of $O_2(^1\Delta_g)$ in DMF [12]. However, the value of Φ_Δ determined in solution can significantly change in a biological medium. To evaluate the participation of $O_2(^1\Delta_g)$, PDI experiments were carried out in the presence of sodium azide, a known quencher of $O_2(^1\Delta_g)$ in cells [18]. The presence of 50 mM sodium azide produced practically a complete photoprotection of *S. mitis*. Therefore, azide ions cause significant lowering of the photodynamic activity of $ZnEPc^{4+}$ by quenching $O_2(^1\Delta_g)$. Also, mannitol was used as a type I scavenger to evaluate the main mechanisms of action [19].However, the phototoxicity efficacy to *S. mitis* cells induced by $ZnEPc^{4+}$ was not significantly affected in presence of 50 mM mannitol. Also, the photoinactivation of *S. mitis* was negligible under an anoxic atmosphere. Although oxygen is needed for the generation of $O_2(^1\Delta_g)$, it also plays a major

role in the type I mechanism by adding to biochemical radicals. In a type I process, the light-excited photosensitizers directly interact with substrate to yield radical ions in a hydrogen atom or electron transfer reaction. The majority of these radicals instantaneously reacts with oxygen and generates a complicated mixture of highly reactive oxygen intermediates, which can oxidize a wide variety of biomolecules. Oxygen is also required for the formation of superoxide anion radical that can occur as the result of the reaction of molecular oxygen with the radical anion of the photosensitizer. Therefore, the studies under anoxic conditions indicated that oxygen is necessary for the photoinactivation of *S. mitis*, but this experiment did not allow establishing the predominant photoreaction process. To evaluate the $O_2(^1\Delta_g)$-mediated photoinactivation of *S. mitis*, the PDI was performed in saline D_2O. Under this condition, the photocytotoxic effect was higher in D_2O than in aqueous solution. However, the phototoxicity enhanced less than 1 log with the addition of D_2O. This effect can be mainly due to that the intracellular water was not completely replaced by deuterium water in the cells during an incubation period of 30 min [24]. In general, the involvement of $O_2(^1\Delta_g)$ in several photosensitized processes *in vivo* is accepted by the observed D_2O enhancement and azide inhibition of diverse oxidative reaction rates [20,25]. Thus, the results reveal a contribution of type II photosensitization in the *S. mitis* cells inactivation induced by $ZnEPc^{4+}$.

The studies in presence of calf thymus DNA showed that $ZnEPc^{4+}$ strongly interacts with the double-stranded DNA in solution. Similar tight binding was also found for a tetracationic phthalocyanine, zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)] phthalocyanine ($ZnPPc^{4+}$) [17]. Moreover, DNA samples were exposed to photoexcited $ZnEPc^{4+}$. In this study a very high light dose (432 J/cm^2) was applied to demonstrate that DNA photocleavage occurred after a long period of irradiation. However, this light dose was much higher than that used in the photoinactivation of *S. mitis*. The photocleavage ability of this tetracationic phthalocyanine was considerable smaller than that found for a tetracationic porphyrin, such as 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) [16]. DNA photodamage photosensitized by $TMAP^{4+}$ was observed by the appearance of a remarkable smearing after electrophoresis. Even though $ZnEPc^{4+}$ has a large K_{DNA}, it was not as effective as this tetracationic porphyrin to photocleavage DNA. Only an increasing smearing appears after long irradiation periods due to the modification and photocleavage of DNA. Moreover, genomic DNA isolated from *S. mitis* after PDI was not cleaved even after an irradiation period of 2 h. In previous studies, it was found that $ZnPPc^{4+}$ produced the inactivation of the *E. coli* cells, while the chromosomal DNA remained intact [17]. The results of the present study about DNA integrity extracted from *S. mitis* indicated that genomic DNA remained practically undamaged during PDI treatments.

TEM studies showed alterations in the ultrastruture of the *S. mitis* cells after PDI treatment with $ZnEPc^{4+}$. Also, irregularities in the cell envelope were observed along the bacterium. Multilamellar membrane structures that are connected directly to the cytoplasmatic membrane appear in the cells after PDI treatment. These mesosome structures must be regarded as being indicative of cytoplasmic membrane modification, in this case induced by PDI treatments, since untreated cells did not contain them. Furthermore, as the cytoplasmic membrane is instrumental in cell wall synthesis, a perturbation of this membrane may also affect cell wall integrity. Thus, mesosome-like structures in most PDI treated cells are indicative of cytoplasmic-membrane alteration. The formation of mesosome-like structures was previously found in terpinen-4-ol-treated *Staphylococcus aureus* cells suggesting cytoplasmic membrane damage [26]. Also, mesosomes developing structures were observed in Gram-positive bacteria treated with diverse cationic peptides, indicating cytoplasmic-membrane alteration [27,28].Therefore, the ultrastructural changes demonstrated by TEM supported the view that photoactivated $ZnEPc^{4+}$ 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. Differences on the cellular surface were also observed via SEM for *S. mitis* following PDI with $ZnEPc^{4+}$. The presence of bubbles was detected on the cellular surface after 2 h irradiation. Despite this, the results were consistent with the absence of a direct observation of cell-wall rupture and cell lysis after PDI treatment. This result was consistent with the absence of intracellular biopolymers release upon photodynamic action. Therefore, this investigation points out that structural changes and alterations in the membrane induced by the photodynamic action of $ZnEPc^{4+}$ play a major role in *S. mitis* damage.

5. Conclusions

In vitro studies showed that *S. mitis* cell suspensions ($\sim 2 \times 10^8$ cells/mL) in PBS treated with $ZnEPc^{4+}$ were efficiently photoinactivated by this tetracationic phthalocyanine. The photodamage induced by $ZnEPc^{4+}$ was also confirmed by the growth delay studies, indicating a faster inactivation of the *S. mitis* cells. To elucidate the oxidative processes that occur during the killing of *S. mitis*, the effect of additives was analyzed on cell photoinactivation. Photoprotection was found using azide ion as type II scavenger, while phototoxicity efficacy was not affected when mannitol was used as a type I scavenger. The effect of the media showed that an oxygen atmosphere is necessary for an efficient cell photodamage. Also, photoinactivation was further enhanced in D_2O due to a prolonged lifetime of $O_2(^1\Delta_g)$. Therefore, the PDI of *S. mitis* cells by $ZnEPc^{4+}$ appears to be mainly mediated by $O_2(^1\Delta_g)$. On the other hand, experiments were performed to obtain insight about the mechanism of cellular damage. Although $ZnEPc^{4+}$ strongly interacts with DNA, photocleavage of genomic DNA was not observed after PDI. Therefore, the photodynamic activity induced by $ZnEPc^{4+}$ produces alteration in the cytoplasmatic components and modifications in the cell envelope causing the inactivation of *S. mitis*.

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