Photodiagnosis and Photodynamic Therapy (2015) 12, 67—75

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/pdpdt

Effects of divalent cations, EDTA and chitosan on the uptake and photoinactivation of *Escherichia coli* mediated by cationic and anionic porphyrins

Natalia S. Gsponer, Mariana B. Spesia, Edgardo N. Durantini PhD*

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

Available online 2 January 2015

KEYWORDS
Porphyrin;
Uptake;
Photodynamic inactivation;
Chitosan;
Escherichia coli

Summary The effect of divalent cations, EDTA and chitosan (CS) on the uptake and photoinactivation of *Escherichia coli* produced by 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$), 5,10-di(4-methylphenyl)-15,20-di(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($MPAP^{2+}$) and 5,10,15,20-tetra(4-sulphonatophenyl)-porphyrin ($TPPS^{4-}$) were examined under different conditions. These porphyrins were rapidly bound to *E. coli* cells (<2.5 min) and the uptake of photosensitizers was not dependent on incubation temperature, reaching values of 0.61, 0.18 and 0.08 nmol/10^8 cells for $TMAP^{4+}$, $MPAP^{2+}$ and $TPPS^{4-}$, respectively. The addition of Ca^{2+} or Mg^{2+} to the cultures enhanced the uptake of $MPAP^{2+}$ and $TPPS^{4-}$ by cells. In contrast, the amount of $TMAP^{4+}$ bound to cells was decreased. The presence of EDTA produced an increase in the uptake of porphyrins by cells, while CS mainly enhanced the amount of $TPPS^{4-}$ bound to *E. coli*. The photoinactivation of *E. coli* cells mediated by $TMAP^{4+}$ was highly effective even at low concentration (1 μM) and short irradiation period (5 min). However, a reduction in the phototoxicity was found for $TMAP^{4+}$ in presence of Ca^{2+} and Mg^{2+}. In contrast, the phototoxic activity mediated by $MPAP^{2+}$ and $TPPS^{4-}$ was increased. Addition of EDTA did not show effect on the photoinactivation induced by cationic porphyrins, while a small enhance was found for $TPPS^{4-}$. Moreover, inactivation of *E. coli* cells was achieved in the presence CS. This cationic polymer was antimicrobial by itself in the dark. Using a slightly toxic CS concentration, the phototoxic activity induced by $TMAP^{4+}$ was diminished. This effect was mainly observed at lower concentration of $TMAP^{4+}$

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

http://dx.doi.org/10.1016/j.pdpdt.2014.12.004

(0.5—1 μM). In contrast, an increase in *E. coli* photoinactivation was obtained for $MPAP^{2+}$ and $TPPS^{4-}$ in presence of CS. Thus, this natural polymeric destabilizer agent mainly benefited the photoinactivation mediated by $TPPS^{4-}$.

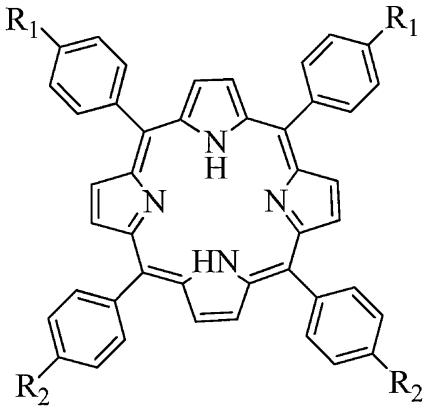
Introduction

The emergence of antibiotic resistance amongst pathogenic bacteria has led to a major research effort to find alternative antibacterial therapies [1]. In this sense, photodynamic inactivation (PDI) of microorganisms has been proposed as an alternative to controlling bacterial infections [2]. This methodology involves a photosensitizer, visible light and oxygen to yield highly reactive oxygen species (ROS), which specifically produces cell damages that inactivate the microorganisms [3].

The studies have shown that Gram-positive bacteria are efficiently photoinactivated by a variety of photosensitizers, whereas Gram-negative bacteria are unaffected by the action of negatively charged or neutral agents [2]. The resistance of Gram-negative bacteria to the action of photosensitizers has been ascribed to the presence of highly organized outer membrane (OM), which hinders the interaction of the photosensitizer with the cytoplasmic membrane and intercepts the ROS [4,5]. The bacterial envelope is a highly complex multilayered structure that consists of a planar peptidoglycan sheet to which the OM is anchored [6]. The additional membrane layer in the cell wall architecture, which is located outside the peptidoglycan layer, shows an asymmetric lipid structure composed by negatively charged lipopolysaccharides (LPS), lipoproteins and proteins with porin function. Therefore, LPS molecules provide a polyanionic external surface that is partly neutralized by divalent cations Mg^{2+} and Ca^{2+} [7].

Photosensitization of Gram-negative bacteria can be increased by the addition of biological or chemical molecules, which modify the native consistence of the OM [4,8]. These changes enhance its permeability facilitating the penetration of photosensitizers to the cytoplasmic membrane. Photodynamic studies were performed destabilizing the bacterial OM by the addition of Tris—EDTA [9] or polymyxin B nonapeptide (PMNP) [10]. EDTA removes Mg^{2+} and Ca^{2+} avoiding the neutralization of negative charges and producing electrostatic repulsion between the LPS. Thus, the molecules can penetrate into the inner cytoplasmic membrane. Photoinactivation of *Escherichia coli* and *Klebsiella pneumoniae* by hematoporphyrin or zinc phthalocyanine was effective using Tris—EDTA before treatment [9]. Furthermore, PMNP displaces cationic counterions reducing the barrier properties of the OM [11]. It was found that deuteroporphyrin works much better in concert with PMNP than other photosensitizers, including porphyrins, phthalocyanines and merocyanine 540, to photoinactive a multi-antibiotic resistant of *Acinetobacter baumannii* [12]. Also, PDI of bacteria was performed using chitosan (CS), a natural linear polycationic biopolymer [13,14]. CS has a wide range of applications in the fields of biomedicine and food industries, agriculture and environmental science [15,16]. CS exhibits a broad range of antibacterial and antifungal activity [17,18]. The key considerations that justify this interest are that CS is biocompatible and biodegradable [15].

In the present study, we investigated the cellular uptake and photodynamic activity of 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($T\text{-}MAP^{4+}$), 5,10-di(4-methylphenyl)-15,20-di(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($MPAP^{2+}$) and 5,10,15,20-tetra(4-sulphonatophenyl)porphyrin ($TPPS^{4-}$) (Scheme 1) in *E. coli* cells. In particular, $TMAP^{4+}$ and $TPPS^{4-}$ were selected as recognized cationic and anionic photosensitizers evaluated in PDI [19]. In contrast to $TMAP^{4+}$, it is known that $TPPS^{4-}$ is not an effective photosensitizer to eradicate Gram negative bacteria, although both porphyrins exhibit a similar subcellular distribution pattern, being mainly localized in the protoplasts or spheroplasts [20]. Therefore, the goal of this study was to evaluate experimental conditions that favoring the inactivation mainly mediated by $TPPS^{4-}$. Thus, the amount of porphyrin bound to *E. coli* cells was examined under different conditions, such as incubation times, effect of temperature, addition of divalent cations, Ca^{2+} and Mg^{2+}, influence of EDTA and CS. Also, eradication of *E. coli* mediated by these photosensitizers was compared under different treatments to establish the best conditions of PDI.



$MPAP^{2+}$ R_1: $-N^+(CH_3)_3\ I^-$ R_2: $-CH_3$

$TMAP^{4+}$ $R_1=R_2$: $-N^+(CH_3)_3$ *p*-Tos^-

$TPPS^{4-}$ $R_1=R_2$: $-SO_3^-Na^+$

Scheme 1 Structure of the cationic and anionic porphyrins.

Materials and methods

General

Absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Fluorescence measurements were performed on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). Quartz cells of 1 cm path length were used at room temperature. The cell suspensions were irradiated with a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. A wavelength range between 350 and 800 nm was selected by optical filters. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. Experiments were performed at room temperature with a fluence rate of 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. CS from shrimp shells with a deacetylated degree $\geq$85% was purchased from Sigma (St. Louis, MO, USA). Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

Photosensitizers

5,10,15,20-Tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin *p*-tosylate ($TMAP^{4+}$) and 5,10,15,20-tetra(4-sulphonatophenyl)porphyrin ($TPPS^{4-}$) sodium salt were purchased from Aldrich. 5,10-di(4-Methylphenyl)-15,20-di(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin iodide ($MPAP^{2+}$) was synthesized as previously described [21]. Stock solutions (0.5 mM) of porphyrins were prepared by dissolution in 1 mL of *N*,*N*-dimethylformamide (DMF).

Bacterial culture conditions

The strain of *E. coli* (EC7) was previously characterized and identified [19]. Microbial cells were grown aerobically overnight at 37 °C in tryptic soy (TS, Britania, Buenos Aires, Argentina) broth. Aliquots ($\sim$40 μL) of this culture was aseptically transferred to 4 mL of fresh TS broth and incubated at 37 °C to mid logarithmic phase (absorbance $\sim$0.6 at 660 nm). Cell growth was measured with a Turner SP-830 spectrophotometer (Dubuque, IA, USA). Cells were centrifuged (3000 rpm for 15 min) and re-suspended in equal amount of 10 mM phosphate-buffered saline (PBS, pH = 7.0) solution, corresponding to $\sim10^8$ colony forming units (CFU)/mL. After treatments, cell suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable bacteria were monitored and the number of CFU was determined on TS agar plates after $\sim$24 h incubation at 37 °C.

Porphyrin binding to *E. coli* cells

E. coli cells suspensions (2 mL, $\sim10^8$ CFU/mL) in PBS were incubated with 5 μM porphyrin for different times (2.5—15 min) in Pyrex culture tubes (13 × 100 mm). Photosensitizer was added from a stock solution ($\sim$0.5 mM) in DMF. Cell suspensions were kept at 4 °C or 37 °C for 10 min in dark and then centrifuged (3000 rpm for 10 min). Pellets were re-suspended in 2 mL of 2% aqueous SDS, incubated overnight at 4 °C and sonicated for 30 min. The concentration of porphyrins in the supernatant was determined by spectrofluorimetry ($TMAP^{4+}$: λ_{exc} = 415 nm, λ_{em} = 651 nm; $MPAP^{2+}$: λ_{exc} = 417 nm, λ_{em} = 654 nm; $TPPS^{4-}$: λ_{exc} = 412 nm, λ_{em} = 644 nm). The fluorescence intensities of each sample were referred to the total number of cells. The concentration of the porphyrin in the solution was calculated by comparison with a calibration curve obtained with standard solutions of the photosensitizer in 2% SDS, varying [porphyrin] between 0.05 and 1.0 μM. Binding experiments were achieved incubating the cells with the appropriated concentration of the agent for 10 min in dark and then treating the cell suspensions with the porphyrin as described below at 37 °C. Divalent cations, $CaCl_2$ and $MgCl_2$, and the chelating agent, EDTA, were added from 2 M stock solutions in water to obtain 50 mM in the cell suspensions. CS stock solution (10 mg/mL) was prepared in 1% (v/v) acetic acid aqueous solution. From this solution, different concentrations of CS (0.12—1 mg/mL) were added to the cells.

Photosensitized inactivation of *E. coli* cells

E. coli cells were previously treated as described above. In all the experiments, 200 μL of the cell suspensions ($\sim10^8$ CFU/mL) were transferred to 96-well microtiter plates (Deltalab, Barcelona, Spain). Then the cells were exposed for different time intervals (5 and 10 min) to visible light. Photodynamic experiments in presence of divalent cations, EDTA or chitosan were achieved incubating the cells with the appropriated concentration of the agent for 10 min in dark and then treating the cell suspensions with the porphyrin as described above at 37 °C.

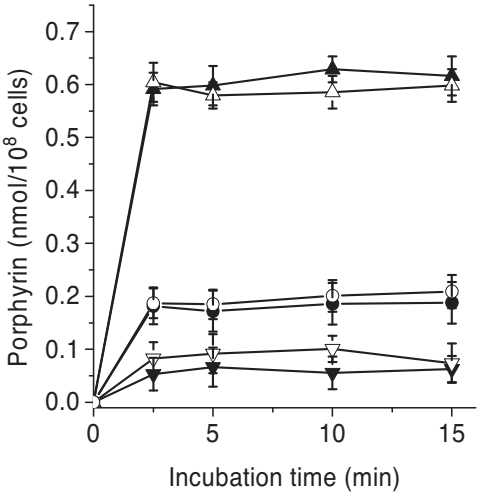


Figure 1 Amount of porphyrins recovered from *E. coli* cells ($\sim10^8$ CFU/mL) in PBS treated with 5 μM $TMAP^{4+}$ (▲), $MPAP^{2+}$ (●) and $TPPS^{4-}$ (▼) at 37 °C and with 5 μM $TMAP^{4+}$ (△), $MPAP^{2+}$ (◯) and $TPPS^{4-}$ (▽) at 4 °C in dark for different incubation times. Values represent mean ± standard deviation of two separate experiments.

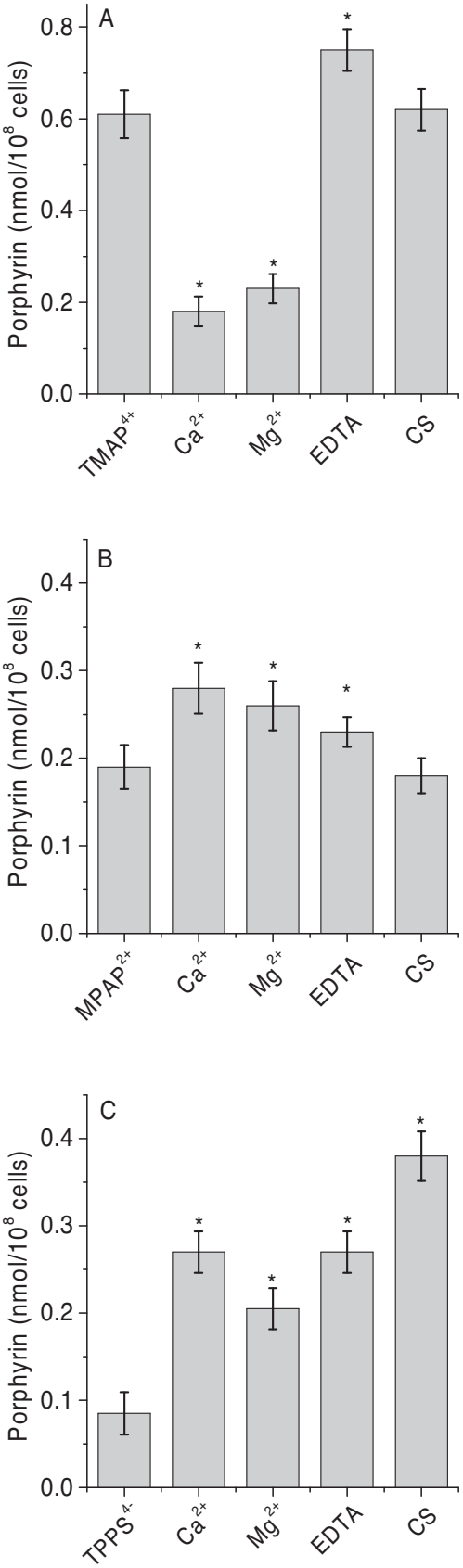


Figure 2 Amount of (A) $TMAP^{4+}$, (B) $MPAP^{2+}$ and (C) $TPPS^{4-}$ recovered from *E. coli* cells ($\sim 10^8$ CFU/mL) in PBS treated with $CaCl_2$ (50 mM), $MgCl_2$ (50 mM), EDTA (50 mM) or CS (0.25 mg/mL) for 10 min at 37 °C in dark and then incubated with 5 μM photosensitizer for 10 min at 37 °C in dark. Values represent mean ± standard deviation of three separate experiments (*$p < 0.05$, compared to photosensitizer alone).

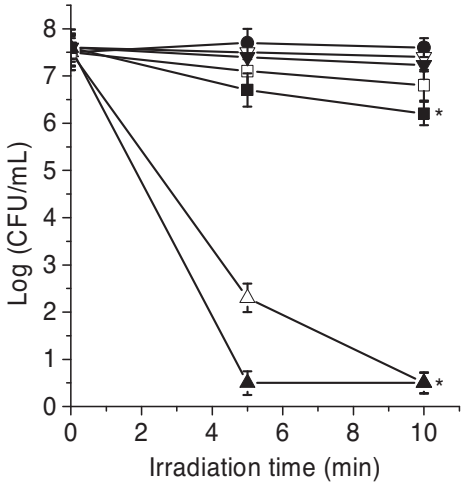


Figure 3 Survival curves of *E. coli* cells ($\sim 10^8$ CFU/mL) treated with 1 μM $TMAP^{4+}$ (△), $MPAP^{2+}$ (□) and $TPPS^{4-}$ (▽) and 5 μM $TMAP^{4+}$ (▲), $MPAP^{2+}$ (■) and $TPPS^{4-}$ (▼) for 10 min at 37 °C in dark and exposed to visible light (90 mW/cm^2) for different irradiation periods. Control culture untreated with photosensitizer and irradiated (●). Values represent mean ± standard deviation of three separate experiments (*$p < 0.05$, compared to $t = 0$).

Controls and statistical analysis

Control experiments were performed in presence and absence of porphyrin in the dark and in the absence of porphyrin with cells irradiated. The amount of DMF (<1%, v/v) used in each experiment was not toxic to *E. coli* cells. Three values were obtained per each condition and each experiment was repeated separately three times. The unpaired *t*-test was used to establish the significance of differences between groups. Differences were considered statistically significant with a confidence level of 95% ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

Results

Binding of porphyrins to *E. coli* cells

The capacity of $TMAP^{4+}$, $MPAP^{2+}$ and $TPPS^{4-}$ porphyrins to bind to *E. coli* was evaluated in cell suspensions ($\sim 10^8$ cells/mL) in PBS. Thus, *E. coli* cells were treated with 5 μM porphyrin for different times (2.5, 5, 10 and 15 min) in the dark. Also, to determine the effect of temperature, the experiments were compared at 37 °C and 4 °C. Fig. 1 shows the amount of porphyrins recovered after each incubation period. The results indicated that binding of $TMAP^{4+}$ to microbial cells was the most effective of the studied porphyrins at both temperatures. This porphyrin was rapidly bound to *E. coli* cells (2.5 min), reaching the highest value

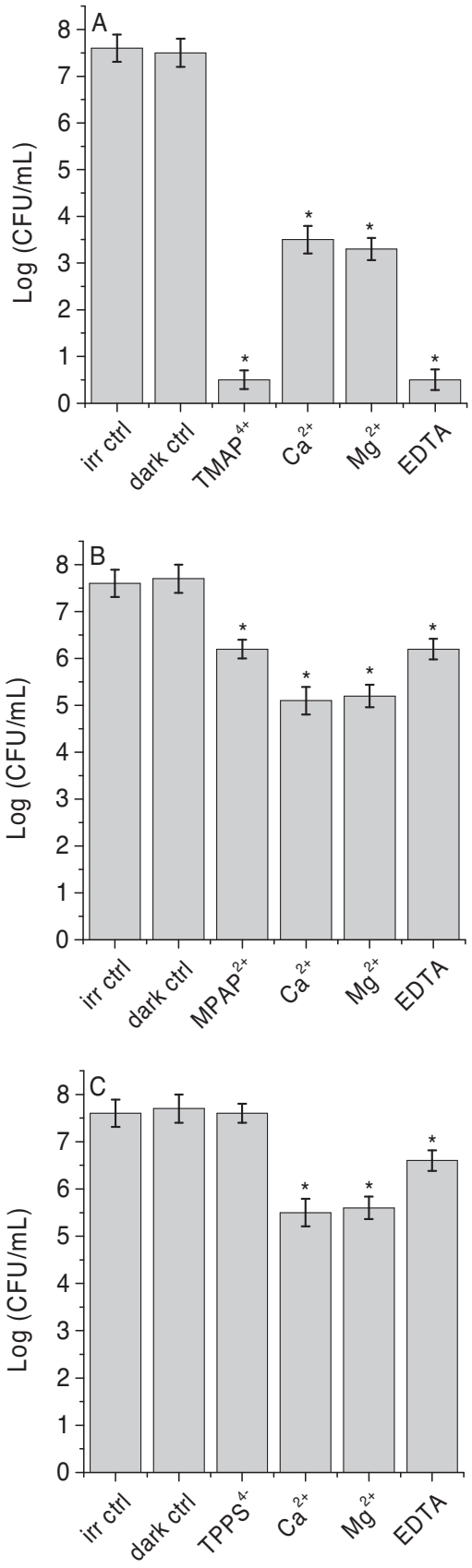


Figure 4 Survival of *E. coli* cells mediated by (A) $TMAP^{4+}$, (B) $MPAP^{2+}$ and (C) $TPPS^{4-}$. The cell suspensions ($\sim 10^8$ CFU/mL) were treated with 50 mM $CaCl_2$, $MgCl_2$ or EDTA for 10 min at 37 °C in dark and then incubated with 5 μM photosensitizer for 10 min at 37 °C in dark and exposed to visible light (90 mW/cm^2) for 10 min. Values represent mean ± standard deviation of three separate experiments (*$p < 0.05$, compared to control).

of cell-bound photosensitizer. An addition in the incubation time (15 min) did not produce changes in the amount of photosensitizer. Also, no significant difference was found incubating the cells at 37 °C or 4 °C. Therefore, binding of porphyrins reached values of ~0.61, 0.18 and 0.08 nmol/10^8 cells for $TMAP^{4+}$, $MPAP^{2+}$ and $TPPS^{4-}$, respectively.

Cell suspensions were incubated with 50 mM Ca^{2+}, Mg^{2+} or EDTA for 10 min at 37 °C in dark and then treated with 5 μM porphyrin. Fig. 2 shows the effect of these agents on the uptake of porphyrins by *E. coli*. As can be observed, a decrease in the recovered amount of $TMAP^{4+}$ was obtained in presence of Ca^{2+} and Mg^{2+} (Fig. 2A). In contrast, these divalent cations produced an enhancement in cell-bound $MPAP^{2+}$ (Fig. 2B) and $TPPS^{4-}$ (Fig. 2C). Also, the presence of EDTA facilitated the uptake of the anionic porphyrin. Moreover, *E. coli* cells were treated with 0.25 mg/mL CS for 10 min in dark at 37 °C previous to the incubation with 5 μM porphyrin. This procedure did not yield significant effect on the recovered amount of cationic porphyrins. However, the quantity of cell-bound $TPPS^{4-}$ was about four times higher in cells preincubated with CS than those untreated.

Photosensitized inactivation of *E. coli*

Photodynamic activity mediated by $TMAP^{4+}$, $MPAP^{2+}$ and $TPPS^{4-}$ was investigated in cell suspensions of *E. coli* in PBS. Cells were treated with different photosensitizer concentrations (1 and 5 μM) for 10 min in the dark at 37 °C. These concentrations of porphyrins were not toxic in dark. Also, control experiments indicated that the viability of *E. coli* was not modified by irradiation with visible light for 10 min (Fig. 3), demonstrating that the cell inactivation was due to the photosensitization effect of the porphyrin.

After irradiation, the viability of *E. coli* cells was dependent on porphyrin structure and concentrations used in the treatment (Fig. 3). An increase in the porphyrin concentration produced an enhancement in the PDI efficiency. *E. coli* cells were rapidly photoinactivated in presence of $TMAP^{4+}$. This porphyrin produced ~7 log decrease of cell survival, when cells were incubated with 5 μM $TMAP^{4+}$ and irradiated for 5 min. The photoinactivation was similar using 1 or 5 μM $TMAP^{4+}$ at longer irradiation time used (10 min). Photosensitization of cells mediated by dicationic $MPAP^{2+}$ induced a low diminishing in the cell viability (~1.5 log) using 5 μM after 10 min irradiation. Moreover, no significant inactivation activity was observed for *E. coli* treated with 5 μM $TPPS^{4-}$ under the same conditions.

Effect of divalent cations on PDI of *E. coli*

PDI of *E. coli* was investigated in presence of 50 mM of $CaCl_2$ or $MgCl_2$. This concentration of cations was not toxic in the dark or under irradiation without porphyrin (result not shown). A similar behavior was found for Ca^{2+} or Mg^{2+}. The addition of divalent cations produced a reduction in the

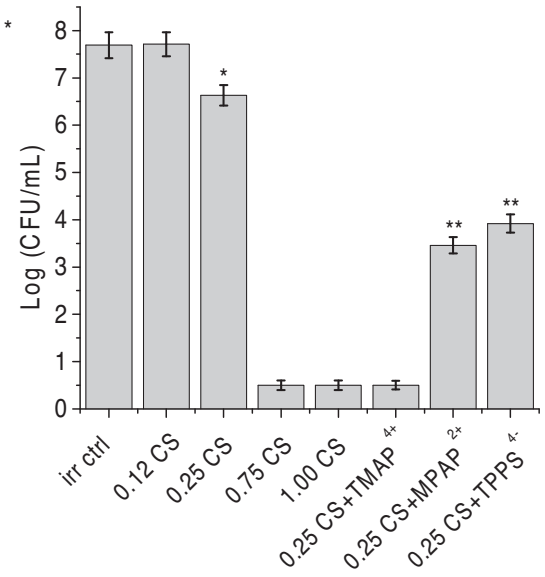


Figure 5 Survival of *E. coli* cells (~10^8 CFU/mL) treated with different concentration (0.12, 0.25, 0.75, 1.00 mg/mL) of CS for 10 min at 37 °C in dark and then incubated with 5 μM porphyrin for 10 min at 37 °C in dark and exposed to visible light (90 mW/cm^2) for 10 min. Values represent mean ± standard deviation of three separate experiments (*$p < 0.05$, compared to control, **$p < 0.05$, compared to 0.25 mg/mL CS alone).

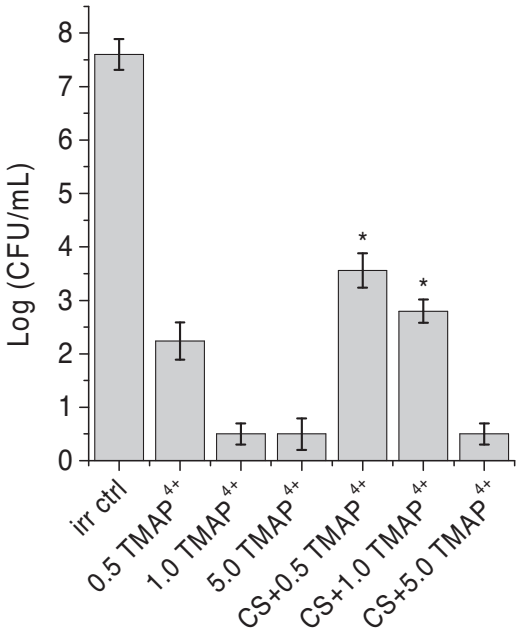


Figure 6 Survival of *E. coli* cells (~10^8 CFU/mL) treated with 0.25 mg/mL CS for 10 min at 37 °C in dark and then incubated with different concentration (0.5, 1.0 and 5.0 μM) of $TMAP^{4+}$ for 10 min at 37 °C in dark and exposed to visible light (90 mW/cm^2) for 10 min. Values represent mean ± standard deviation of three separate experiments (*$p < 0.05$, compared to $TMAP^{4+}$ alone).

inactivation of *E. coli* induced by $TMAP^{4+}$ (Fig. 4A). Under this condition, no more than a 4 log decrease was observed in the survival of the bacteria treated with 5 μM $TMAP^{4+}$ after 10 min irradiation. However, the presence of Ca^{2+} or Mg^{2+} produced an increase of 1 log and 2.5 log in the PDI of *E. coli* mediated by $MPAP^{2+}$ (Fig. 4B) and $TPPS^{4-}$ (Fig. 4C), respectively.

Effect of EDTA on PDI of *E. coli*

Photoinactivation of *E. coli* treated with 5 μM porphyrin was evaluated in the presence of 50 mM EDTA. The addition of this amount of EDTA was not cytotoxic in dark or irradiated without porphyrin (result not shown). Under this condition, no significant difference in the photoinactivation was observed for cells incubated with $TMAP^{4+}$ and $MPAP^{2+}$ (Fig. 4A and B). However, the presence of EDTA produced a decrease of 1 log in cell viability of *E. coli* treated with $TPPS^{4-}$ with respect to that without the chelating agent (Fig. 4C).

Effect of CS on PDI of *E. coli*

Inactivation of *E. coli* cells was first analyzed in the presence of different CS concentrations (0.12—1.00 mg/mL). In all cases, cells were incubated with CS for 10 min in dark. As shown in Fig. 5, 0.75 and 1.00 mg/mL CS were toxic to the cell cultures. Instead, no toxicity was observed using 0.12 mg/mL CS, while 0.25 mg/mL showed 1 log decrease in cell viability. Therefore, the last concentration was used in PDI studies due to it produced a partial destabilization in the cell structure.

After treatment with 0.25 mg/mL CS and incubation with 5 μM porphyrin, the cells were irradiated with visible light for 10 min. PDI mediated by $TMAP^{4+}$ was similar to that found without CS, reducing 7 log of cell survival (Fig. 5). Moreover, the photoinactivation was examined using different $TMAP^{4+}$ concentrations (0.5—5 μM). Fig. 6 shows the same result 1 and 5 μM $TMAP^{4+}$ without CS. At the lowest $TMAP^{4+}$ concentration used (0.5 μM), the eradication of *E. coli* was also effective, producing 5 log decrease in cell survival. The effect of 0.25 mg/mL CS on the photocytotoxic activity was mainly observed in cells treated with 0.5 and 1 μM $TMAP^{4+}$ (Fig. 6). In both cases, the presence of CS produced a reduction in the photoinactivation of *E. coli* induced by this tetracationic photosensitizer.

On the other hand, the photoinactivation of *E. coli* using $MPAP^{2+}$ in presence of 0.25 mg/mL CS was more effective than without the polymer, producing a reduction of 4 log in cell survival (Fig. 5). Also, $TPPS^{4-}$ induced 3.5 log decrease in cell survival in the presence of CS, while photoinactivation did not take place in the absence of polymer. This results indicate that this destabilizer agent affect the cell viability favoring the photosensitizer action of $TPPS^{4-}$ against a Gram-negative bacterium (Fig. 5). Moreover, PDI experiments were performed incubating the cells with a previously prepared mixture of 0.25 mg/mL CS and 5 μM $TPPS^{4-}$ for 10 min in dark. After 10 min irradiation, 4 log cell survivals was obtained, which was slightly more effective than separated incubations of CS and anionic porphyrin.

Discussion

The uptake of photosensitizers by microbial cells is an important feature for the efficacy of photoinactivation [8]. The OM acts as a barrier to prevent the interaction of PDI-induced cytotoxic agents with vital targets, such as the membrane or cytoplasmic components. Also, LPS on the surface of the OM provide a density of negative charges, which avoid the uptake by cells of neutral and anionic compounds [6]. Therefore, it is recognized that cationic photosensitizers are required for effective inactivation of Gram-negative bacteria [19,20]. This condition indicates a charge-dependent interaction between the photosensitizer and the cells. According with that, the photoinactivation mediated by $TMAP^{4+}$, $MPAP^{2+}$ and $TPPS^{4-}$ could be predicted in *E. coli* cell. Thus, $TMAP^{4+}$ was used as a tetracationic active photosensitizer to eradicate Gram-negative bacteria, while a very low activity was expected to $TPPS^{4-}$ [19]. Also, $MPAP^{2+}$ represented an amphiphilic photosensitizer that contains two cationic charges and two lipophilic phenyl groups in the macrocycle periphery. Also, these porphyrins showed high quantum yields of singlet molecular oxygen, $O_2(^1\Delta_g)$, production, with values of 0.77 for $TMAP^{4+}$ in water, 0.74 for $TPPS^{4-}$ in water and 0.36 for $MPAP^{2+}$ in DMF [22—24]. 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 [25].

The results in cell suspensions of *E. coli* (10^8 cells/mL) showed that the amount of cell-bound porphyrin was not dependent on the incubation time between 2.5 and 15 min (Fig. 1). As expected by the positive charges, the uptake of $TMAP^{4+}$ was 7.6 times higher than that for $TPPS^{4-}$. A similar behavior was previously found for $TMAP^{4+}$ and $TPPS^{4-}$, using 10^6 cells/mL *E. coli* [19]. Also, the recovered amount of $TMAP^{4+}$ was higher than for $MPAP^{2+}$, indicating that the number of cationic groups is an important factor in the binding of porphyrin to cells [26]. The experiments at 4 and 37 °C showed that the uptake of these porphyrins was unaffected by temperature. It was suggested that the uptake of anionic photosensitizers by bacterial cells may be mediated through a combination of electrostatic charge interaction and by protein transporters, while the uptake of cationic ones is mediated by electrostatic interactions and self-promoted uptake pathways [8].

Photoinactivation of *E. coli* treated with 5 μM $TMAP^{4+}$ produced over 7 log decrease after 5 min irradiation (Fig. 3). Using a lower $TMAP^{4+}$ concentration (1 μM) no colony formation was detected after 10 min irradiation. These results represent a value greater than 99.9999% of cell inactivation. Moreover, the cytotoxic activity remained elevated during a shorter irradiation time of 5 min that produced 5 log decrease. When the cells were incubated with 1 μM $TMAP^{4+}$, an enhancement in the cell inactivation was found increasing the irradiation times. Under this condition, the photodynamic effect was mainly associated with $TMAP^{4+}$ that was tighter bound to cells. The photocytotoxic effect for cells treated with 5 μM $MPAP^{2+}$ diminished about 1.5 log after 10 min irradiation. Also, this dicationic porphyrin was not a highly effective photosensitizer to inactivate a lower *E. coli* cell density of 1×10^6 cells/mL [26]. As expected due to its low binding to *E. coli* cells, no inactivation effect was found for cultures treated with 5 μM of anionic $TPPS^{4-}$ still after 10 min irradiation. This result is in agreement with that reported before for $TPPS^{4-}$, indicating that these noncationic porphyrins are unsuccessful photosensitizers for Gram-negative bacteria under these conditions [19]. However, the photosensitizing activity can be influenced by the presence of ions in the suspending medium that change the electrolyte composition of the cell envelope.

To evaluate the effect of OM destabilization produced by divalent cations on the photoinactivation of *E. coli*, the PDI was performed in presence of Ca^{2+} and Mg^{2+}. Both cations produced a decrease in the uptake of $TMAP^{4+}$ by cells (Fig. 2), which was accompanied by a lower photoinactivation (Fig. 4A). In previous studies the effect of cations Na^+ and Ca^{2+} on the efficacy of the photoinactivation was investigated in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, using *meso*-tetra(*N*-methyl-4-pyridyl)porphyrin or *meso*-mono-phenyl-tri(*N*-methyl-4-pyridyl)porphyrin as photosensitizer [4]. Addition of cations strongly decreased the sensitivity of both bacteria to photoinactivation. In these cases, the decreased sensitivity was accompanied by a reduced binding of the photosensitizers to the bacteria. Also, the effect of divalent cations was examined on the uptake of a cationic photosensitizer, methylene blue (MB), and two anionic photosensitizers, rose bengal (RB) and indocyanine green (ICG), by *Enterococcus faecalis* and *Actinobacillus actinomycetemcomitans* [8]. Divalent cations enhanced the uptake and photodynamic inactivation potential of both RB and ICG in *E. faecalis* and *A. actinomycetemcomitans*, while they decreased the uptake and bacterial killing by MB. In this work an enhancement in the PDI induced by $MPAP^{2+}$ and $TPPS^{4-}$ was observed in presence of Ca^{2+} or Mg^{2+}. These divalent cations produced an increase in the uptake of these porphyrins by *E. coli* cells. The excessive binding of divalent cations to LPS can produce OM cracks, through which macromolecules, such as porphyrins, can diffuse.

Moreover, experiments were carried out in the presence of EDTA to evaluate the effect of a chelating agent on the PDI of *E. coli*. No significant changes were found in the photoinactivation mediated by cationic porphyrins (Fig. 4A and B), while in presence of $TPPS^{4-}$ only a slight increase in the cytotoxic activity was observed (Fig. 4C). The uptake of $TMAP^{4+}$ and $MPAP^{2+}$ was slightly increased by EDTA, however, it was improved about three times for $TPPS^{4-}$ producing a higher PDI. Also, it was previously observed that *E. coli* was insensitive to the photosensitizing action of both lipid-soluble Zinc-phthalocyanine (Zn-Pc) and water-soluble Zinc-mono/disulfonated phthalocyanine (Zn-PcS) [9]. However, photosensitivity was obtained by alteration of the OM by either induction of competence or treatment with Tris—EDTA. Both phthalocyanines largely bind at the level of the cytoplasmic membrane; however, Zn-PcS showed a superior photosensitizing activity as compared with Zn-Pc. Thus, EDTA can produce effect on the OM permeability barrier of Gram-negative bacteria. The chelating agent can remove stabilizing divalent cations from their binding sites in LPS [7]. Therefore, a very short treatment can be used to introduce macromolecules and hydrophobic compounds through the OM without affecting cell viability. A nonlethal short treatment with EDTA sensitizes *E. coli* to a number of

hydrophobic antibiotics. However, the effect of EDTA and other chelators can be poor in growth media which contain divalent cations.

On the other hand, antibacterial activity of CS was assessed for a wide range of Gram-negative and Gram-positive bacteria. It is known that CS can be used as an antimicrobial agent [27]. The actual mechanism of its antimicrobial activity is not yet fully understood, but Gram-negative bacteria are less susceptible to its action. However, previous investigations provide evidence that CS disrupts the barrier properties of the OM of Gram-negative bacteria [17]. It was proposed that the inhibition activity of CS against *E. coli* was produced by increased the permeability of the OM and inner membrane (IM) of *E. coli*, and ultimately disrupted the cell membrane with the release of cellular contents. The damage of bacterial cell membranes was caused by the electrostatic interaction between $-NH_3^+$ groups of CS and carbonyl and phosphoryl groups of the phospholipid components of the cell membrane [18,28]. In this work, CS demonstrated strong inhibition activity against *E. coli* (Fig. 5). No colony formation was detected for *E. coli* cells incubated with 0.75 or 1.00 mg/mL CS. A treatment of the cells with a 0.25 mg/mL CS produced a cytotoxic activity of 1 log decrease in *E. coli* viability in dark. This concentration of CS did not affect the PDI induced by 5 μM $TMAP^{4+}$. Thus, the electrostatic repulsion caused by the cationic polymer is not sufficient to avoid the effect of this photosensitizer. However, at lower concentrations of $TMAP^{4+}$ (0.5 and 1 μM) a reduction in the photoinactivation of *E. coli* was observed probably due to a decrease in the interaction with the OM. Moreover, the addition of CS increased the photoinactivation mediated by $MPAP^{2+}$. The asymmetric charge distribution at the peripheral position of this porphyrin can produce an increase in the amphiphilic character of the structure, which can help a better accumulation in cell membrane [29]. On the other hand, this concentration of CS enhanced the amount of $TPPS^{4-}$ bound to *E. coli* cells by about four times (Fig. 2C). This effect was accompanied by an increase in the photoinactivation induced by the anionic porphyrin. This amount of CS alone produced a low antimicrobial activity probably by a slight disruption of the cell envelope, while the PDI—CS combination induced by $TPPS^{4-}$ produced a potentiated PDI effect. CS interacts with the membrane of the *E. coli* cells to alter cell permeability, increasing the photocytotoxic activity of the anionic porphyrin. It was demonstrated that CS can potentiate the PDI efficacy of hematoporphyrin (Hp) against Gram-positive (*S. aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*) and Gram-negative (*P. aeruginosa*, *A. baumannii*) bacteria, including antibiotic-resistant strains [13]. Moreover, CS was used as a carrier of chlorophyllin increasing its concentration at a close vicinity to the cells. High local concentration of chlorophyllin around cells could be a crucial factor significantly enhancing antibacterial activity of photoactivated chlorophyllin—CS complex [14,16]. These results indicate that combined with CS, an anionic porphyrin and light can efficiently function as a microbial killing agent. Thus, the combination of PDI mediated by $TPPS^{4-}$ and CS was shown to be a promising antimicrobial approach against infectious disease.

Conclusions

In these studies, we demonstrated that Ca^{2+} or Mg^{2+} mainly produced an increase in the amount of $TPPS^{4-}$ bound to cells, enhancing the phototoxic activity against *E. coli*. In contrast, high concentrations of these ions produced electrostatic repulsion of the tetracationic porphyrin leading to a decrease in the photoinactivating activity. The addition of EDTA did not affect the photoinactivation mediated by cationic porphyrins and induced a slight enhance in the PDI induced by $TPPS^{4-}$. On the other hand, the higher CS concentrations used was toxic to *E. coli* by itself. This natural biopolymer has antimicrobial activity because its positive charges can interfere with the negatively charged *E. coli* cell envelope, resulting in alteration of membrane permeability. Thus, a low CS concentration resulted in a decrease in the phototoxic activity induced by $TMAP^{4+}$ probably due to repulsion between positive charges. However, CS enhanced the photoinactivation of *E. coli* by $TPPS^{4-}$. Therefore, the combined cytotoxic activities of CS and PDI mediated by $TPPS^{4-}$ could be beneficial for the eradication of *E. coli*.

Acknowledgements

Authors are grateful to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 112-201101-00256) of Argentina, SECYT Universidad Nacional de Río Cuarto (PPI 18/C400) and Agencia Nacional de Promoción Científica y Tecnológica (FONCYT, PICT 0714) for financial support. M.B.S. and E.N.D. are Scientific Members of CONICET. N.S.G. thanks to CONICET for the postdoctoral research fellowship.

References

[1] Nigam A, Gupta D, Sharma A. Treatment of infectious disease: beyond antibiotics. Microbiol Res 2014;169:643—51.
[2] Alves E, Faustino MA, Neves MG, Cunha A, Tome J, Almeida A. An insight on bacterial cellular targets of photodynamic inactivation. Future Med Chem 2014;6:141—64.
[3] Durantini EN. Photodynamic inactivation of bacteria. Curr Bioact Compd 2006;2:127—42.
[4] Lambrechts SA, Aalders MC, Langeveld-Klerks DH, Khayali Y, Lagerberg JW. Effect of monovalent and divalent cations on the photoinactivation of bacteria with *meso*-substituted cationic porphyrins. Photochem Photobiol 2004;79:297—302.
[5] Salmon-Divon M, Nitzan Y, Malik Z. Mechanistic aspect of *Escherichia coli* photodynamic inactivation by cationic tetra-*meso*(*N*-methylpyridyl)porphine. Photochem Photobiol Sci 2004;3:423—9.
[6] Hancock REW. The bacterial outer membrane as a drug barrier. Trends Microbiol 1997;5:37—42.
[7] Vaara M. Agents that increase the permeability of the outer membrane. Microbiol Rev 1992;56:395—411.
[8] George S, Hamblin MR, Kishen A. Uptake pathways of anionic and cationic photosensitizers into bacteria. Photochem Photobiol Sci 2009;8:788—95.
[9] Bertoloni G, Rossi F, Valduga G, Jori G, van Lier J. Photosensitizing activity of water- and lipid-soluble phthalocyanines on *Escherichia coli*. FEMS Microbiol Lett 1990;71:149—56.
[10] Nitzan Y, Gutterman M, Malik Z, Ehrenberg B. Inactivation of Gram-negative bacteria by photosensitized porphyrins. Photochem Photobiol 1992;55:89—96.

[11] Vaara M, Vaara T. Polycations as outer membrane disorganizing agents. Antimicrob Agents Chemother 1983;24:114—22.
[12] Nitzan Y, Balzam-Subakevitz A, Ashkenazi H. Eradication of *Acinetobacter baumannii* by photosensitized agents *in vitro*. J Photochem Photobiol B 1998;42:211—8.
[13] Tsai T, Chien H-F, Wang T-H, Huang C-T, Ker Y-B, Chen C-T. Chitosan augments photodynamic inactivation of Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 2011;55:1883—90.
[14] Buchovec I, Kokstaite R, Pamedytyte V, Makuska R, Luksiene Z. Antimicrobial efficiency of photoactivated chlorophyllin—chitosan complex against Gram (—) bacteria *Salmonella enterica*. Chem Technol 2012;3:54—7.
[15] Ravi Kumar MN, Muzzarelli RA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. Chem Rev 2004;104:6017—84.
[16] Luksiene Z, Brovko L. Antibacterial photosensitization-based treatment for food safety. Food Eng Rev 2013;5:185—99.
[17] Helander IM, Nurmiaho-Lassila E-L, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol 2001;71:235—44.
[18] Li X, Feng X, Yang S, Fu G, Wang T, Su Z. Chitosan kills *Escherichia coli* through damage to be of cell membrane mechanism. Carbohydr Polym 2010;79:493—9.
[19] Caminos DA, Spesia MB, Durantini EN. Photodynamic inactivation of *Escherichia coli* by novel *meso*-substituted porphyrins by 4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl and 4-(trifluoromethyl)phenyl groups. Photochem Photobiol Sci 2006;5:56—65.
[20] Merchat M, Bertolini G, Giacomini P, Villanueva A, Jori G. Meso-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. J Photochem Photobiol B 1996;32:153—7.
[21] Lazzeri D, Durantini EN. Synthesis of *meso*-substituted cationic porphyrins as potential photodynamic agents. ARKIVOC 2003;10:227—39.
[22] Verlhac JB, Gaudemer A, Kraljic I. 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 1984;8:401—6.
[23] Gottfried V, Peled D, Winkelman JW, Kimel S. Photosensitizers in organized media: singlet oxygen production and spectral properties. Photochem Photobiol 1988;48:157—63.
[24] Spesia MB, Lazzeri D, Pascual L, Rovera M, Durantini EN. Photoinactivation of *Escherichia coli* using porphyrin derivatives with different number of cationic charges. FEMS Immunol Med Microbiol 2005;44:289—95.
[25] Cormick MP, Quiroga ED, Bertolotti SG, Alvarez MG, Durantini EN. Mechanistic insight of the photodynamic effect induced by tri- and tetra-cationic porphyrins on *Candida albicans* cells. Photochem Photobiol Sci 2011;10:1556—61.
[26] Lazzeri D, Rovera M, Pascual L, Durantini EN. Photodynamic studies and photoinactivation of *Escherichia coli* using *meso*-substituted cationic porphyrin derivatives with asymmetric charge distribution. Photochem Photobiol 2004;80:286—93.
[27] Kong M, Chen XG, Xing K, Park HJ. Antimicrobial properties of chitosan and mode of action: a state of the art review. Int J Food Microbiol 2010;144:51—63.
[28] Choi SS, Lee HK, Chae HS. Synergistic *in vitro* photodynamic antimicrobial activity of methylene blue and chitosan against *Helicobacter pylori* 26695. Photodiagn Photodyn Ther 2014;11:526—32.
[29] Boyle RW, Dolphin D. Structure and biodistribution relationships of photodynamic sensitizers. Photochem Photobiol 1996;64:469—85.