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Photodynamic inactivation of microorganisms sensitized by cationic BODIPY derivatives potentiated by potassium iodide

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ABSTRACT

The photodynamic inactivation mediated by 1,3,5,7-tetramethyl-8-[4-(*N*,*N*,*N*-trimethylamino)phenyl]-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene **3** and 8-[4-(3-(*N*,*N*,*N*-trimethylamino)propoxy)phenyl]-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene **4** were investigated on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. *In vitro* experiments indicated that BODIPYs **3** and **4** were rapidly bound to microbial cells at short incubation periods. Also, fluorescence microscopy images showed the green emission of the BODIPYs bound to microbial cells. Photosensitized inactivation improved with an increase of the irradiation times. Similar photoinactivation activity was found for both BODIPYs in bacteria. The photoinactivation induced by these BODIPYs was effective for both bacteria. However, the Gram-positive bacterium was inactivated sooner and with a lower concentration of photosensitizer than Gram-negative. After 15 min irradiation, a complete eradication of *S. aureus* was obtained with 1 μM photosensitizer. A reduction of 4.5 log in the *E. coli* viability was found using 5 μM photosensitizer and 30 min irradiation. Also, the last conditions produced a decrease of 4.5 log in *C. albicans* cells treated with BODIPY **3**, while **4** was poorly effective. On the other hand, the addition of KI was investigated on photoinactivation at different irradiation periods and salt concentrations. A smaller effect was observed in *S. aureus* because the photosensitizers alone were already very effective. In *E. coli*, photokilling potentiation was mainly found a longer irradiation periods. Moreover, the photoinactivation of *C. albicans* mediated by these BODIPYs was increased in presence of KI. In solution, an increase in the formation of the BODIPY triplet states was observed with the addition of the salt, due to the effect of external heavy atom. The greater intersystem crossing together with the formation of reactive iodine species induced by BODIPYs may be contributing to enhance the inactivation of microorganisms. Therefore, these BODIPYs represent interesting photosensitizers to inactivate microorganisms. In particular, BODIPY **3** in combination with KI was highly effective as a broad spectrum antimicrobial photosensitizer.

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Keywords: BODIPY; photodynamic inactivation; potassium iodide, microorganisms; photosensitizer.

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Introduction

Across the world, bacterial resistance to antimicrobial therapy has increased dramatically to such a high level that it endangers public health.[1] In this sense, *Staphylococcus aureus* is an important cause of health care-associated infections and it is endemic in many health care facilities worldwide.[2] Also, the global emergence of multidrug-resistant Gram-negative bacteria is a growing threat to antibiotic therapy.[3] In particular, *Escherichia coli* have developed resistance to most available antibiotics. In addition, fungal infections have mainly increased in the critical care setting.[4] Even though fungal infections are rarer than bacterial ones, the mortality can be higher. Commensal fungal pathogens like *Candida albicans* can cause disease under facilitating circumstances that range from superficial to life- threatening systemic.[5] Moreover, resistant *C. albicans* strains have emerged with frequent exposure to antifungal drugs.[6] Therefore, it is imperative that new actions are carried out both at the community level and hospitals, in order to change the way in which microbial infections can be treated. Photodynamic inactivation (PDI) of microorganisms has been proposed as an alternative therapy to eradication of microbes.[7] PDI is founded in the administration of a photosensitizer, which is preferentially bound to microbial cells. The subsequent aerobic irradiation of the affected area with visible light produces cell damages that inactivate the microorganisms. Two photodynamic processes can occur after activation of the photosensitizer.[8] In the type I pathway, the excited photosensitizers can interact with different substrates to produce radicals in a hydrogen atom or electron transfer reaction. These radicals rapidly react with oxygen to produce a mixture of highly reactive oxygen intermediates that can oxidize a wide diversity of biomolecules. In contrast, in the type II mechanism, singlet molecular oxygen, $O_2(^1\Delta_g)$, is produced by triplet energy transfer reaction from the photosensitizer to the ground state of molecular oxygen. These reactive oxygen species (ROS) generate irreversible damage in major cell components leading to microbial inactivation. The contribution of each process depends on the photosensitizer, the presence of substrates and the polarity of surrounding medium.[9]

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Several tetrapyrrole macrocycles with appropriate functionalization have been investigated in PDI.[7] Most of these structures have pronounced cationic charges, especially for targeting Gram-negative bacteria. However, few studies of antimicrobial photoinactivation have been performed with 4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) derivatives. Addition of heavy halogen atoms in the *s*-indacene ring pyrrole rings increases the triplet excited state formation allows acting as potential photosensitizers for photodynamic therapy.[10] Previous investigation demonstrate that 2,6-diiodo-1,3,5,7-tetramethyl-8-(*N*-methyl-4-pyridyl)-4,4'-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene was effective to eradicate several bacteria, yeast and viruses.[11,] Also, two conjugates of a zinc(II)-dipicolylamine targeting unit and a BODIPY chromophore were prepared.[13] The oxygen photosensitizing analogue containing 2,6-diiodo was effective to photoinactivate bacterial samples. Also, a BODIPY derivative was designed to obtain a dormant $O_2(^1\Delta_g)$ photosensitizer that can be activated upon its reaction with reactive ROS.[14] This structure was successfully tested via the PDI of a ROS stressed *E. coli* strain. Recently, the spectroscopic properties and photodynamic activity of two cationic BODIPYs **3** and **4** (Scheme 1) were investigated in different biomimetic media.[15] These photosensitizers showed a low production of $O_2(^1\Delta_g)$. However, the addition of KI increased the photoinduced decomposition of the amino acid L-tryptophan. Iodide anions can increase the formation of triplet excited state by external heavy-atom effect.[16,17] Moreover, the formation of reactive iodine species can be formed in this system. It was also demonstrated that the addition of KI enhanced the photokilling of microorganisms mediated by different photosensitizers.[18,19]

In the present investigation, we are interested in evaluating the ability of BODIPYs **3** and **4** to photoinactivate a Gram-positive *S. aureus*, a Gram-negative *E. coli* and a yeast *C. albicans*. First, the uptake of photosensitizers by microbial cells was studied because this is an important feature for the efficacy of photoinactivation.[20] Thus, the binding of these BODIPYs to microbial cells were determined after different incubation periods. Photokilling of cells sensitized by BODIPYs **3** and **4** was compared in presence of KI varying the irradiation periods and inorganic salt concentrations. Laser flash photolysis experiments were performed to determine the effect of KI on the transient

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absorption spectrum of these BODIPYs. The results were used to establish conditions for the eradication of microorganisms in presence of iodide anions.

Materials and methods

General

Absorption spectra were performed on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). A Turner SP-830 spectrophotometer (Dubuque, IA, USA) was used to determine cell growth. Fluorescence spectra were carried out on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc, Edison, NJ, USA). Fluence rates were measured with a Radiometer Laser Mate-Q (Coherent, Santa Clara, CA, USA). The visible light source was a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector equipped with a 150 W lamp. The heat from the lamp was removed using a 2.5 cm glass cuvette filled with water. A wavelength range between 350 and 800 nm was selected by optical filters with a fluence rate of 70 mW/cm^2 at 500 nm. Chemicals from Sigma-Aldrich (Milwaukee, WI, USA) were used without further purification.

Cationic BODIPY derivatives

1,3,5,7-Tetramethyl-8-[4-(*N*,*N*,*N*-trimethylamino)phenyl]-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene **3** and 8-[4-(3-(*N*,*N*,*N*-trimethylamino)propoxy)phenyl]-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene **4** were synthetized from 1,3,5,7-tetramethyl-8-[4-(*N*,*N*-dimethylamino)phenyl]-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene **1** and 8-[4-(3-(*N*,*N*-dimethylamino)propoxy)phenyl]-4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene **2**, respectively, as previously described.[15] A stock solution 0.5 mM BODIPY was prepared in 1 mL of *N*,*N*-dimethylformamide (DMF). BODIPY concentrations were established by absorption taking into account the molar extinction coefficient, ε = 8.51 x 10^4 M^{-1} cm^{-1} at 502 for **3** and ε = 9.49 x 10^4 M^{-1} cm^{-1} at 498 nm for **4** in DMF.[15]

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Microorganisms and growth conditions

The microorganisms used in this study were the strains of *S. aureus* ATCC 25923, *E. coli* (EC7) and *C. albicans* (PC31), which were previously characterized and identified.[21,22] Microbial cells were grown aerobically in sterile condition overnight at 37 ºC in 4 mL tryptic soy or Sabouraud (Britania, Buenos Aires, Argentina) broths for cultures of bacteria or yeast, respectively. An aliquot (60 μL) of the bacterial culture was aseptically transferred to 4 mL of fresh tryptic soy broth and incubated at 37 ºC to exponential phase of growth (absorbance 0.6 at 660 nm). Cells were centrifuged (3000 rpm for 15 min) and re-suspended in equal amount of 10 mM phosphate-buffered saline (PBS, pH = 7.4) solution. Then the cells were diluted 1/10, corresponding to ~10^7 colony forming units (CFU)/mL. After overnight cultures of *C. albicans*, cells were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in PBS. Yeast cells (absorbance 0.5 at 650 nm) were diluted 1:4 in PBS to obtain ~10^6 CFU/mL. After each assay, cell suspensions were serially diluted 10-fold in PBS. After each experiment, cell suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable microbial cells were monitored and the number of CFU was determined after ~24 h (bacteria) or ~48 h (yeast) incubation at 37 ºC in the dark.

Photosensitizer binding to microbial cells and fluorescence images

Microbial cell suspensions (2 mL, ~10^7 CFU/mL bacteria and ~10^6 CFU/mL yeast) in PBS were incubated with 1 μM (*S. aureus*) or 5 μM photosensitizer (*E. coli* and *C. albicans*) for different times in dark at 37 ºC in Pyrex culture tubes (13x100 mm). Photosensitizer was added from a stock solution 0.5 mM in DMF. After each incubation time, aliquot of cell suspension (1 mL) were transferred to an eppendorf tube (1.5 mL) and then centrifuged at 14000 rpm for 1 min. Pellets were re-suspended in 1 mL of 2 % aqueous SDS, incubated overnight at 4 ºC and sonicated for 15 min. The concentration of photosensitizers in the supernatant was determined by spectrofluorimetry (λ_{exc} = 470 nm, λ_{em} = 512 nm). The fluorescence intensities of each sample were

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referred to the total number of cells. The concentration of the photosensitizer in the solution was calculated by comparison with a calibration curve obtained with standard solutions (0.05-0.2 μM) of the photosensitizer in 2 % SDS.

Microscopic observations were performed using a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) fluorescence microscope equipped with a HBO 100 W mercury lamp. Images were captured using an AxioCam HRc camera and subsequently processed using AxioVision Rel. 4.3 software. Fluorescence images of BODIPYs in microbial cells were observed using a Filter Set 25 (488025-0000-000, Carl Zeiss, excitation TBP 400 + 495 + 570, beam splitter TFT 410 + 505 + 585, emission TBP 460 + 530 + 625). The images were recorded from cell cultures treated with photosensitizer for 30 min in the dark as described above in binding experiments. Confocal images were acquired in an Olympus FluoView FV1000 confocal microscope (Olympus Latin America, Miami, FL). Merging of the images was done with FV10-ASW 3.0 software. For the intracellular localization of BODIPYs **3** and **4**, *C. albicans* cells were labeled with fluorescence markers. The nuclei were labeled with a DNA-specific fluorescent dye, Hoechst 33258 (Sigma-Aldrich), at a concentration of 1 μg/mL in PBS at room temperature. The lumen of the vacuole was labeled with the specific fluorescent dye 7-amino-4-chloromethylcoumarin (CellTracker™ Blue CMAC, Invitrogen, Carlsbad, CA, USA) at a concentration of 100 μM in PBS at room temperature. *C. albicans* suspensions cells in PBS were treated with 5 μM of the photosensitizers and incubated for 15 min at 37ºC, then the suspensions were harvested by centrifugation at 3000 rpm for 15 min. Hoechst and CMAC were added on the cell pellets and the images were observed in the 10 min after the addition of the markers. For BODIPYs, excitation was carried out with the 488 nm line. Detection of Hoechst and CMAC was performed with excitation at 351 nm.

Photosensitized inactivation of microorganisms

Cell suspensions were incubated with 1 μM (*S. aureus*) or 5 μM photosensitizer (*E. coli* and *C. albicans*) for 30 min in dark at 37 °C as mentioned in 2.4. Then, 200 μL of each cell suspension

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were transferred to 96-well microtiter plates (Deltalab, Barcelona, Spain). Cells were exposed for different time intervals to visible light. The addition of KI was carried out using a stock solution (1 M) in water. Microbial cells were incubated with different concentrations of KI (10, 25 and 50 mM) for 10 min in dark at 37°C. After that, photosensitizer was added and cells were incubated for 30 min in dark at 37°C. Cell suspensions were immediately irradiated with visible light. The number of viable cells was determined as previously described.

Laser flash photolysis experiment

Transient absorption spectra of BODIPYs **3** and **4** were determined in Argon-saturated DMF solution by laser flash photolysis. A Spectron SL400 Nd:YAG laser generating 532 nm for BODIPY **3** or 355 nm for BODIPY **4** laser pulses (20 mJ per pulse, ca. 18 ns FWHM) was the excitation source. The experiments were performed with rectangular quartz cells with right angle geometry. The laser beam was defocused in order to cover all the path length (10 mm) of the analyzing beam from a 150 W Xe lamp. The detection system comprised a PTI monochromator coupled to a Hamamatsu R666 PM photomultiplier. The signals were acquired and averaged by a digital oscilloscope (Hewlett-Packard 54504) and then transferred to a computer.

Controls and statistical analysis

Control experiments were performed in presence and absence of photosensitizer in the dark and in the absence of photosensitizer with cells irradiated. The amount of DMF (<1% v/v) used in each experiment was not toxic to microbial cells. Three values were obtained per each condition and each experiment was repeated separately three times. Normal distributions were assessed by Kolmogorov-Smirnov test and homogeneity of variances was assessed by Levene test. The significance of the PDI effect of each photosensitizer and of the irradiation time on microbial cells viability was assessed by one-way analysis of variance (ANOVA). A *p*-value below 0.05 was

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considered statistically significant. Data were represented as the mean ± standard deviation of each group.

Results

Binding of BODIPYs to microbial cells

The ability of BODIPYs **3** and **4** to bind to microbial cells was determined in *S. aureus*, *E. coli* and *C. albicans*. The amount of photosensitizer recovered from cells after each incubation time are showed in Figure 1. These BODIPYs were rapidly bound to microbial cells, reaching high binding values after 5 min incubation. Moreover, the amount of cell-bound photosensitizer was not appreciably changed incubating the yeast cells for longer times. For the three microorganisms studied, it was found that the amount of recovered molecules was higher for BODIPY **3** than **4**. Thus, in *S. aureus*, a value of 0.06 nmol/10^7 cells was obtained for BODIPY **3** after 30 min incubation, while the uptake was 0.03 nmol/10^7 for BODIPY **4** (Figure 1A). In the case of *E. coli*, the binding of BODIPY **3** was 0.05 nmol/10^7. The uptake of BODIPY **4** by *E. coli* cells reached a value of ~0.03 nmol/10^7 cells. These values were similar to those obtained in *S. aureus* but using 5 μM photosensitizer. (Figure 1B). When *C. albicans* cells were incubated with 5 μM photosensitizer, the quantity of BODIPY **3** recovery was 0.6 nmol/10^6 cells, whereas BODIPY **4** achieved a value of 0.3 nmol/10^6 cells (Figure 1C).

On the other hand, the cellular localization of BODIPYs in microbial cells was investigated by fluorescence microscopy. Images show that *S. aureus* and *E. coli* cells incubated with 5 μM photosensitizers in PBS for 30 min in the dark exhibited green fluorescence typical of BODIPYs (Figure 2). Also, fluorescence images were analyzed in *C. albicans* cells. No significant difference was observed in yeast cells incubated in dark with BODIPY **3** or **4** in presence or absence of 50 mM KI (Figure 3). Similar results were obtained after irradiation without KI or containing the inorganic salt (Figure 4). Fluorescence confocal microscopy images of *C. albicans* were performed in cells incubated with 5μM BODIPY in the dark. Moreover, *C. albicans* cells that were stained with the

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nuclear stain Hoechst or with the vacuole stain CMAC.[23] Merging of the fluorescence confocal microscopy images showed blue nucleus stained with Hoechst or blue vacuole stained with CMAC, which contrast with the green emission of the BODIPYs (Figure 5).

Photosensitized inactivation of microorganisms

Photosensitized inactivation of *S. aureus*, *E. coli* and *C. albicans* induced by BODIPY **3** and **4** was investigated after different irradiation periods with visible light. Survival curves are summarized in Figure 6. No toxicity was found for the cells treated with BODIPYs for 30 min in dark (results not shown). Moreover, the viability of microbial cells was not affected by irradiation without photosensitizer (Figure 6). Photoinactivation of microorganisms was dependent on irradiation times. *S. aureus* was the most susceptible strain (Figure 6A). For all irradiated times, survival was significantly reduced when compared with the control ($p < 0.05$). After a short irradiation time of 5 min, both BODIPYs induced a 4.5 log decrease in cell survival. Also, they achieved a complete eradication after 15 min irradiation. BODIPY **3** produced a photoinactivation of *S. aureus* similar to those found for BODIPY **4**. In *E. coli*, the photokilling effectiveness mediated by BODIPYs **3** and **4** was very similar (Figure 6B) and the cell survival was diminished when compared with the control ($p < 0.05$). After 5 min irradiation, a reduction of 1.5 log in the *E. coli* survival was determined, while a 2.5 log decrease was observed using an irradiation of 15 min. It was necessary 30 min irradiation to obtain a 4.5 log decrease in *E. coli* survival. Photodynamic activity induced by these BODIPYs was also evaluated in *C. albicans* cell (Figure 6C). For cells treated with BODIPY **3**, significant ($p < 0.05$) decreases were obtained respect to the control at all irradiated times. The photodynamic effect yielded a 2.5 log decrease in the cell viability after 20 min irradiation. A high decrease of *C. albicans* survival (4.5 log) was detected after 30 min irradiation. In contrast, BODIPY **4** was poorly effective. The differences between all tested combinations were not statistically significant ($p > 0.05$) at 10 and 20 min irradiation. After 30 min irradiation, BODIPY **4** induced a 1 log reduction ($p < 0.05$) in the survival of the yeast cells.

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Effect of KI on photoinactivation of microorganisms

Microbial cells killing mediated by BODIPYs was evaluated in the presence of KI after different irradiation periods. The results are shown in Figure 6. The addition of 50 mM KI alone or combined with photosensitizers were not toxic for microorganisms in dark for 30 min incubation (results not shown). Also, no toxicity was observed for microbial cells incubated with 50 mM KI and irradiated for 30 min in absence of BODIPYs (Figure 6). In *S. aureus*, the addition of KI produced a slight increase in the photoinactivation after 5 min irradiation. However, the effect of KI was not significant respect to PDI without this inorganic salt ($p > 0.05$) to the longer irradiation times because both BODIPYs were effective without the addition of the inorganic salt (Figure 6A). In *E. coli*, two behavior were observed in presence of KI (Figure 6B). After 5 min irradiation, photoinactivation decreased in presence of 50 mM KI. However, a potentiation in photokilling of *E. coli* was observed to the longer irradiation times (15 and 30 min) when compared with PDI without KI ($p < 0.05$). After 15 min irradiation, addition of KI produced a 1 log and 4 log increase in bacterial inactivation for BODIPY **3** and **4**, respectively. Moreover, *E. coli* cell survival was not detected after 30 min irradiation when PDI was carried out in the presence of KI. Under the last conditions, both BODIPYs produced a decrease greater than 7 log in cell viability. Photoinactivation of *C. albicans* was potentiated by the presence of KI (Figure 6C) in comparison with PDI in absence of KI ($p < 0.05$). After 10 min irradiation, a potentiated photoinactivation of 2.5 log was found for BODIPY **3**. Also, a complete eradication of yeast cells was obtained after 20 min irradiation. A lower effect of iodide anions was observed for BODIPY **4**. Cell survival decreases 2.5 log respects to the cell suspension without KI after 30 min irradiation.

On the other hand, photoinactivation of *E. coli* and *C. albicans* was evaluated using different concentrations of KI (0-50 mM) (Figure 7). These microorganisms were chosen because KI effect was greater in them. In particular, the addition of KI to *E. coli* cells was analyzed after 5 min and 15 min irradiation (Figure 7A and B). At short irradiation times, the presence of iodide anions had little

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impact on photokilling of bacteria (Figure 7A). Inclusively, a small protection in inactivation was observed as the KI concentration increased. After 15 min irradiation, photokilling was increased with the KI concentration (Figure 7B). The effect of KI was mainly observed for BODIPY **4**. A lower potentiation by KI was found using BODIPY **3**. In *C. albicans* a progressive increase of inactivation was produced with KI concentration, after 30 min irradiation (Figure 7C). A higher potentiation induced by KI was obtained in the photoinactivation of yeast sensitized by BODIPY **4** than **3**. However, BODIPY **3** already induced a complete eradication of *C. albicans* with 25 mM KI.

Transient absorption spectra determination

The transient absorption spectra of BODIPY **3** taken at different times after the laser pulse are shown in Figure 8A. Laser flash excitation of BODIPY **3** in argon-saturated DMF solution at 532 nm results in the formation of transient species, which absorb from 350 nm to 450 nm with a maximum near to 420 nm and a minor band at a longer wavelength centered near to 620 nm. A bleaching band of about 500 nm represents the ground state depletion upon photoexcitation. Previous studies with other BODIPYs, showed transient absorption spectra with similar characteristics.[24,25] These results, strongly suggest that the observed absorption spectrum correspond to a single transient species, which is assigned to the triplet excited state of BODIPY **3**. In the presence of different concentration of KI a relevant increment in the absorption of triplet specie at zero time was observed at both wavelengths (Figure 8B and 1C). This increase was directly proportional to the concentration of KI in solution (Figure 8B and 1C insets). From the first-order exponential adjustment of the triplet decay profiles, the triplet state lifetimes (τ_T) of BODIPY **3** were obtained at both wavelengths of absorption (420 and 620 nm). The values of τ_T in the absence and in the presence of KI are shown in Table 1. In absence of KI, the lifetime of the excited triplet state obtained for BODIPY **3** was about 50 μs. In the presence of KI, these values decreased in proportion to the concentration of the inorganic salt in solution. Upon 355 nm pulsed laser excitation of BODIPY **4**, the band around 420 nm with a low differential absorption was observed

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(Figure 9A). In this case, it was observed a long-lifetime transient (Figure 9B). Also, an increase in the absorption at zero time was found at a concentration of 50 mM KI (Figure 9B).

Discussion

The main structural differences between BODIPYs **3** and **4** are the position of the cationic group and the substitution of the *s*-indacene ring. In BODIPY **3**, the cationic ammonium group is directly attached to the *meso*-phenyl ring, while positive charge is isolated from the BODIPY structure by an aliphatic spacer in BODIPY **4**. Moreover, compound **3** contains methyl groups at the 1,3,5 and 7 positions of the *s*-indacene ring. Both compounds exhibited typical spectral characteristics of the BODIPY core with a narrow intense absorption band at ~500 nm ($\varepsilon \sim 10^5$ M^{-1} cm^{-1}).[15] However, BODIPY **3** exhibited a higher fluorescence quantum yield ($\Phi_F = 0.29$) than **4** ($\Phi_F = 0.030$) in DMF, according with its sterically hindered rotation of the phenylene ring. Photodynamic studies indicated a low generation of photosensitized by BODIPYs **3** ($\Phi_\Delta = 0.07$) and **4** ($\Phi_\Delta = 0.03$) in DMF. However, localization of BODIPYs in microheterogenic organized system formed by reverse micelles decrease the vibrational decay, favoring the photosensitization of $O_2(^1\Delta_g)$. Also, these BODIPYs efficiently sensitized the photodecomposition of Trp, possibly with a contribution of type I photoprocess.[15]

In vitro experiments with microorganisms showed that the binding of cationic BODIPY **3** and **4** to cells occurred in short times (<5 min) and the amount of cell-bound BODIPYs was not dependent on the incubation periods between 5 and 30 min at 37 °C. In several cases, the cell envelope of microorganisms represents a significant barrier to the binding of the photosensitizer and the number and distribution of charges may play a predominant role.[22] However, these results reflect the high affinity between these two BODIPYs and the microbial cells. The cationic nature of both structures is reflected in fast cellular uptake in the first 5 min of treatment. As already shown, cationic derivative better interact with negatively charged components present in the cell envelopes facilitating the subsequent incorporation. Similar results were previously found with cationic

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porphyrin derivatives bound to *E. coli* and *C. albicans* cells.[26,27] Moreover, this behavior was previously observed in the binding of a porphyrin and its chlorin derivate to *S. aureus*, *E. coli* and *C. albicans*.[28] For the three microorganisms studied, the cell-bound photosensitizer was higher for BODIPY **3** than **4**. The uptake of photosensitizers by microbial cells is an important feature for the efficacy of photoinactivation. In particular, the outer membrane (OM) of *E. coli* acts as a barrier to prevent the interaction of PDI-induced cytotoxic agents with vital targets, such as the membrane or cytoplasmic components. Also, lipopolysaccharides (LPS) on the surface of the OM provide a density of negative charges, which avoid the uptake by cells of neutral and anionic compounds. Therefore, it is recognized that cationic photosensitizers are required for effective inactivation of Gram-negative bacteria.[7] This condition indicates a charge-dependent interaction between the photosensitizer and the cells. Thus, it was necessary five times higher concentration of BODIPYs to obtain similar amount of photosensitized bound to *E. coli* than *S. aureus*. The higher binding of BODIPYs to *C. albicans* than bacteria may be due to the larger cell size of the yeast and the lower cell density of the culture.[20] The uptake value found for BODIPY **3** was about half that obtained for tetracationic porphyrins in *C. albicans*, such as 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin (1.4 nmol/10^6 cells) and 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin (1.7 nmol/10^6 cells).[22,27] In general, the photosensitizer affinity is consequently accompanied by an increase in the photocytotoxic activity. The results obtain in the incorporation experiments although are associated with the effectiveness of treatment, also provide information about the dark incubation time required in the presence of the photosensitizer.[29]

Fluorescence microscopic images provided additional insight about the uptake of BODIPYs by cells. Green fluorescence in microbial cells shows that these BODIPYs have particularly high binding affinity for these microorganisms. Similar results were previously found for porphyrin derivatives bound to microbial cells.[28,30] In solution, the Φ_F value of BODIPY **4** was about one order of magnitude lower than BODIPY **3** due to free rotation of the *meso*-phenyl group.[15] However, the fluorescence intensity of BODIPYs **3** and **4** bound to the cells does not show

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differences between both BODIPYs. This behavior may occurs because the reduction of the rotation substituent group in environment more viscous, as surrounding the microbial cell.[31] In previous studies, two structurally related optical probes that are conjugates of a zinc(II)-dipicolylamine targeting unit and a BODIPY chromophore was studied.[32] One probe was a microbial targeted fluorescent imaging agent, mSeek, and the other was an oxygen photosensitizing analogue, mDestroy. Fluorescence imaging and detection studies of mSeek were examined in bacterial strains: *E. coli*, *S. aureus*, *K. pneumonia*, and *B. thuringiensis* vegetative cells and purified spores. Fluorescence studies proved that the highly fluorescent and non-phototoxic mSeek stains multiple strains of bacteria. In most of cases, strong BODIPY fluorescence was observed in the entire cell. However, green emission was not detected in a circular organelle, which for their size in relation to cell could correspond to vacuole. Merging of the fluorescence confocal microscopy images of *C. albicans* cells incubated with BODIPY **3** or **4** and stained with Hoechst or CMAC indicated that the photosensitizers did not interacted with these organelles.[23]

Photoinactivation of microorganisms mediated by BODIPYs **3** and **4** was compared using different irradiation periods. The structural differences between the BODIPY **3** and **4** did not influence in the photodynamic inactivation of bacterial cells. Both molecules were similarly efficient, increasing their activity with the irradiation time. *S. aureus* cell suspensions treated with 1 μM BODIPY produced an over 4.5 log decrease in the viability after 5 min irradiation. Under these conditions, complete eradication was found after 15 min irradiation with both BODIPYs. When the *E. coli* cells were incubated with 5 μM BODIPY, an enhancement in the cell inactivation was found increasing the irradiation times, reaching a decrease of 4.5 log survival after 30 min irradiation. As expected due to the nature of the envelope of Gram-negative bacteria, it was more difficult to inactivate *E. coli* than *S. aureus*.[7] Comparison of these results with other photosensitizers already described is difficult because different experimental conditions and microorganisms. BODIPY substituted by *N*-methyl-4-pyridyl group containing and two iodine atoms at the dipyrrolylmethene structure was able to eradicate *Staphylococcus xylosus* and *E. coli*.[11] A high degree of phototoxicity

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(>6 log units) was observed with the photosensitizer 0.5 μM against *S. xylosus* and 5.0 μM against *E. coli*, following 5 min irradiation with a green LED device. Moreover, BODIPY was demonstrated to be efficient against planktonic cultures of *Pseudomonas aeruginosa*, causing a 7 log unit reduction of viable cells when administered at 2.5 μM.[33] It was also suggested that the treatment has a biocidal effect against bacterial biofilm cells. This BODIPY was able to mediate the photodynamic inactivation of clinically-relevant microbes, including Gram-positive, Gram-negative, and drug-resistant bacteria, as well as pathogenic yeast and model viruses, at nanomolar concentrations and short illumination times using visible light.[12] Furthermore, treatment of *C. albicans* with BODIPY **3** was significantly more effective than **4**. Under these conditions, the loss of survival was dependent on the irradiation time. Yeast cell suspensions incubated with 5 μM BODIPY **3** produced a photoinactivation of 99.997% (~4.5 log decrease) after 30 min irradiation. In contrast, using 5 μM BODIPY **4** the photoinactivation remained low (< 1 log) even at the time of longer irradiation. Although both BODIPYs have an amino cationic group attached to the phenyl ring, the uptake of BODIPY **3** by *C. albicans* cells was greater than **4**. Also, BODIPY **3** presents a higher photodynamic activity than **4**. These characteristics of BODIPY **3** may contribute to increase the photokilling of yeast cells.

Heavy atoms can be incorporated into the structure of photosensitizers with low triplet quantum yield as a strategy to enhance spin-orbit coupling leading to facilitate intersystem crossing.[34] This effect enables a greater of ROS generation in the presence of oxygen.[35] However, the covalent attachment of heavy atoms further hinders synthetic work and often leads to an increase in dark toxicity.[36,37] Also, the presence of heavy atoms covalent binding to BODIPY inhibits its emission of fluorescence. On the other hand, by external addition of the heavy atoms, it is possible to use these compounds in dual form, as photosensitizers and also as fluorophores for cellular imaging.[38,39] For these reasons, in the present work the heavy atom iodine was externally incorporated as a salt, KI, in the reaction system. In *S. aureus* treated with 50 mM KI, a slight increase was only detected after 5 min irradiation, because both BODIPYs are highly effective

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using longer irradiation periods. In *E. coli*, the effect of KI was dependent on the irradiation times. At short times, the effect was rather protective, while inactivation is potentiated at longer times. Photoinactivation induced by BODIPY **4** in presence of 50 mM KI was considerably increased reaching over 7 log units after 15 min irradiation, while the photokilling activity of BODIPY **3** was potentiated by 1 log decrease. Under these conditions, BODIPY **4** was significantly more effective than **3** for long time treatments. This effect was only observed at the highest concentration of KI used. Apparently, BODIPY **4** can be confined in a cellular microenvironment that allows increasing the photodynamic activity potentiated by the presence of KI. For both BODIPYs, complete eradication of *E. coli* was found after 30 min irradiation. Also, addition of KI potentiated the photoinactivation of *C. albicans*. Photoinactivation of yeast cells treated with KI and BODIPY **3** produced over log decrease after 20 min irradiation. These results represent a value greater than 99.9999% of cell inactivation. Also, the cytotoxic activity remained elevated during a shorter irradiation time of 10 min that produced 4 log decrease. Also, KI potentiation was observed with BODIPY **4** in *C. albicans* cells, leading to a photosensitizing activity of 3.5 log units after 30 min irradiation.

Furthermore, PDI of microorganisms sensitized by BODIPY **3** and **4** was investigated in cell suspensions varying the concentration of KI (10-50 mM). In *E. coli*, little effect was found using lower KI concentrations after 5 min PDI treatment. Even some protection was observed at 50 mM KI and 5 min irradiation. The salt effect can possibly be influencing the characteristics of the membrane of the bacteria. However, potentiation of microbial photokilling was found for *E. coli* and *C. albicans*. This inert salt produced a dose-dependent increase of light-mediated bacterial killing of *E. coli* and *C. albicans* after 15 min and 30 min irradiation, respectively. This potentiation in the presence of the inorganic salt may be mainly due to the increase in the formation of excited triplet states of BODIPYs by intersystem crossing through the effect of the external heavy atom. This effect was observed for BODIPYs in solution after the addition of different concentration of KI, as evidenced by laser flash photolysis studies. This inorganic salt is known to enhance the rate

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of intersystem crossing of many photosensitizers.[16] The addition of KI generates an increase of the population of the triplet states as indicated by the increment in the absorption of triplet specie at zero time. KI was also found to decrease of triplet state lifetime. Apart from a heavy atom effect promoting intersystem crossing to the triplet state, KI can also enhance the triplet state deactivation. It was previously found that KI enhances the triplet-state decay rate by a charge-coupled deactivation.[17] Thus, an increase in ROS production may be expected and also generation of reactive iodine species, which enhance cell damage. Previously, the efficacy of PDI mediated by the phenothiazinium dye methylene blue (MB) was investigated in *S. aureus* and *E. coli* by addition of KI.[18] A consistent increase of red light-mediated bacterial killing was found in presence of 10 mM KI. Moreover, it was found that PDI of *Acinetobacter baumannii*, *S. aureus* and *C. albicans* with cationic fullerenes and some derivative could be potentiated by iodide both *in vitro* and *in vivo*.[19] It is known that MB and fullerene derivatives are effective photosensitizers to produce ROS. Thus, in the present investigation we demonstrated that KI can be also used to enhance the photokilling of microorganisms induced by BODIPYs that have a low production of $O_2(^1\Delta_g)$.

In aqueous media, the reaction of iodide anions and $O_2(^1\Delta_g)$ results in the production of triiodide anions (I_3^-) (equations 1-2).[40,41] This reaction has even been proposed as a method for indirect detection of $O_2(^1\Delta_g)$. In this process hydrogen peroxide (H_2O_2) is formed (equation 3), which can react further with iodide anions to generate I_2 (equation 4). In presence of an excess of iodide anions, the iodine atoms produces I_3^- (equation 2).

$$O_2(^1\Delta_g) + I^- + H_2O \xrightarrow[-OH^-]{} IOOH \xrightarrow{I^-} I_2 + HO_2^- \quad (1)$$

$$I_2 + I^- \longrightarrow I_3^- \quad (2)$$

$$HO_2^- + H_2O \longrightarrow H_2O_2 + OH^- \quad (3)$$

$$H_2O_2 + 2I^- + 2H^+ \longrightarrow I_2 + 2H_2O \quad (4)$$

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The characteristic spectrum of the I_3^- was obtained after aerobic irradiation of BODIPYs solutions containing KI.[15] In contrast, I_3^- was not obtained under an argon atmosphere, indicating that it is formed by the reaction with $O_2(^1\Delta_g)$. In this system, other short-lived reactive iodine species, such as iodine atoms ($I^{\cdot}$) and iodine radical anions ($I_2^{\cdot-}$) could also be generated.[42,43] The generated $I^{\cdot}$ is assumed to react with I^- to yield $I_2^{\cdot-}$. The $I_2^{\cdot-}$ intermediates are unstable with respect to disproportionation, yielding I_3^- and $I^{\cdot}$.[44] In the present study, photoinactivation of *S. aureus* was slight affected by the presence of KI due to this bacterium was the most susceptible strain. However, an increase of microbial inactivation of *E. coli* and *C. albicans* was observed by adding KI. The effect of iodide was mainly observed for BODIPY **4**. This effect was also previously observed in the photodecomposition de Trp in solution.[15] Thus, the interaction of the ROS and KI during light exposure, biocidal molecular iodine (I_2) or tri-iodide (I_3^-) can be formed improving bacterial inactivation.[45] Therefore, this complementary pathway of photodamage can be used to improve the photodynamic activity produced by BODIPYs in the microorganisms studied.

Conclusions

The photodynamic activity of two cationic BODIPYs **3** and **4** were investigated *in vitro* on *S. aureus*, *E. coli* and *C. albicans* cells to obtain conditions that allow the eradication of microorganism. Both agents showed high affinity for microbial cells. Further fluorescence imaging studies revealed that these BODIPYs efficiently stain bacteria and yeast cells. Fluorescence microscopy of *C. albicans* cells treated with BODIPYs showed fluorescence throughout the cytoplasm. However, they did not show colocalized stainig of the nuclei or vacuole. Both BODIPYs were effective to eradicate *S. aureus* using a low photosensitizer concentration and short irradiation period. As expected, the Gram-negative strain of *E. coli* cells were the most difficult to eradicate and similar photocytotoxic effect was found for both BODIPYs in bacteria. In contrast, BODIPY **3** was more effective than **4** in *C. albicans*. The addition of the nontoxic salt KI, significantly increases the effect of PDI in prolonged treatment periods due to the effect of the external heavy

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atom and the formation of reactive iodine species induced by photosensitizers. Therefore, these BODIPYs may be used as fluorescent probe to the detection of microbial cells. Moreover, potentiation of photokilling may be obtained by the addition of KI.

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Table 1. Triplet state lifetimes (τ_T) of **3** in the absence and presence of KI at the two wavelengths of maximum absorption in DMF.

KI (mM)	τ_T (μs) λ = 420 nm	τ_T (μs) λ = 620 nm
0	50.3 ± 0.8	48.9 ± 0.8
10	46.1 ± 0.7	45.0 ± 0.8
25	41.2 ± 0.7	39.8 ± 0.7
50	31.2 ± 0.6	32.4 ± 0.6

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Figures and Schemes captions

Figure 1. Amount of BODIPYs recovered from (A) *S. aureus*, (B) *E. coli* and (C) *C. albicans* treated with 1 μM for *S. aureus* and 5 μM for last two, of **3** (▲) and **4** (▼) for different incubation times at 37ºC in dark.

Figure 2. Fluorescence microscopic observation of *S. aureus* and *E. coli* incubated with 5 μM BODIPY **3** or **4** for 30 min at 37 °C in the dark. Inset: cells under bright field (100×microscope objective, scala bar 2 μm).

Figure 3. Fluorescence microscopic observation of *C. albicans* incubated with 5 μM BODIPY **3** or **4** for 30 min in the dark and with 5 μM BODIPY **3** or **4** for 30 min followed by 50 mM KI for 10 min in dark at 37 °C. Inset: cells under bright field (100×microscope objective, scala bar 4 μm).

Figure 4. Fluorescence microscopic observation of after 30 min irradiation of *C. albicans* treated with 5 μM BODIPY **3** or **4** and with 5 μM BODIPY **3** or **4** followed by 50 mM KI. Inset: cells under bright field (100×microscope objective, scala bar 4 μm).

Figure 5. Merging of the fluorescence confocal microscopy images of *C. albicans* cells incubated with 5 μM BODIPY **3** or **4** in dark and stained with Hoechst or CMAC. Inset: cells under bright field (100×microscope objective, scala bar 4 μm).

Figure 6. Survival curves of (A) *S. aureus* (~10^7 CFU/mL) treated with 1 μM photosensitizer, (B) *E. coli* (~10^7 CFU/mL) treated with 5 μM photosensitizer and (C) *C. albicans* (~10^6 CFU/mL) treated with 5 μM photosensitizer for 30 min in dark and irradiated with visible light for different periods. Effect of **3** in absence (▲) and in the presence (△) of 50 mM KI and **4** in absence (▼) and in the presence (▽) of 50 mM IK. Irradiated control culture without photosensitizer (●); irradiated

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control culture treated with 50 mM KI without photosensitizer (o). *p < 0.05, compared with untreated cells.

Figure 7. Survival curves of (A) *E. coli* (~10^7 CFU/mL) 5 min irradiation (B) *E. coli* 15 min irradiation and (B) *C. albicans* (~10^6 CFU/mL) 30 min irradiation treated with 5 μM **3** (▲) or **4** (▼) and different KI concentrations. Irradiated control culture without photosensitizer (●).

Figure 8. (A) Transient absorption spectra of BODIPY **3** in argon-saturated DMF after pulsed laser excitation at 532 nm. (B) Absorption decay profiles of BODIPY **3** at 420 nm in presence of 0, 10, 25 and 50 mM KI, inset: changes in the initial absorbance difference (ΔA_i) with the concentration of KI at 420 nm. (C) Absorption decay profiles of BODIPY **3** at 620 nm in presence of 0, 10, 25 and 50 mM KI, inset: changes in the initial absorbance difference (ΔA_i) with the concentration of KI at 620 nm.

Figure 9. (A) Transient absorption spectra of BODIPY **4** in argon-saturated DMF after laser flash excitation at 355 nm. (B) Absorption decay profiles of BODIPY **4** at 420 nm without KI and in presence of 50 mM KI.

Scheme 1. Structure of cationic BODIPYs **3** and **4**.

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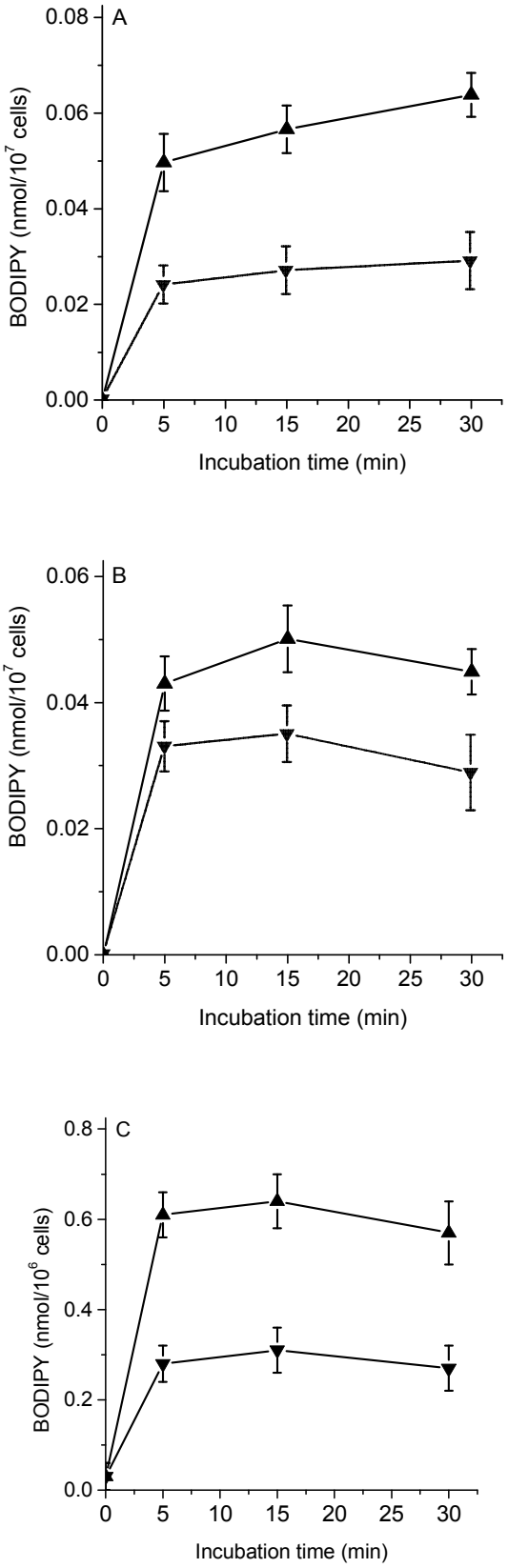

Figure 1

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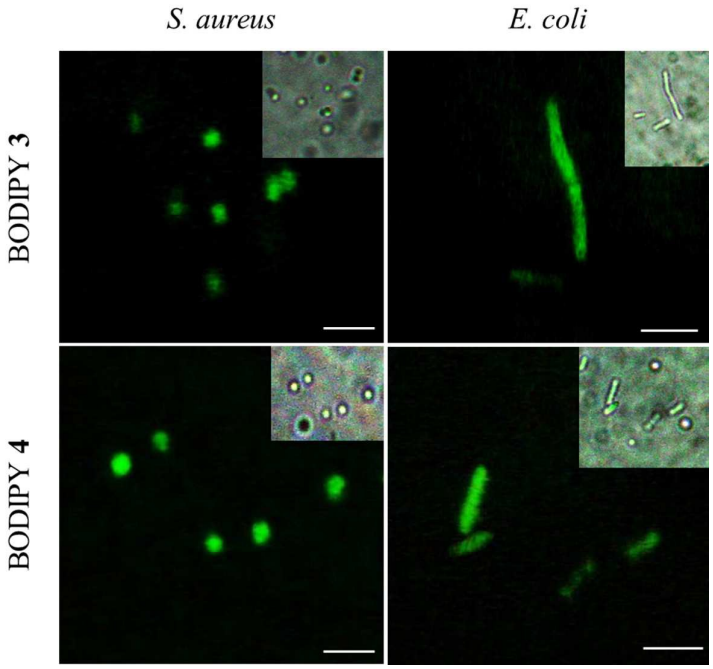

Figure 2

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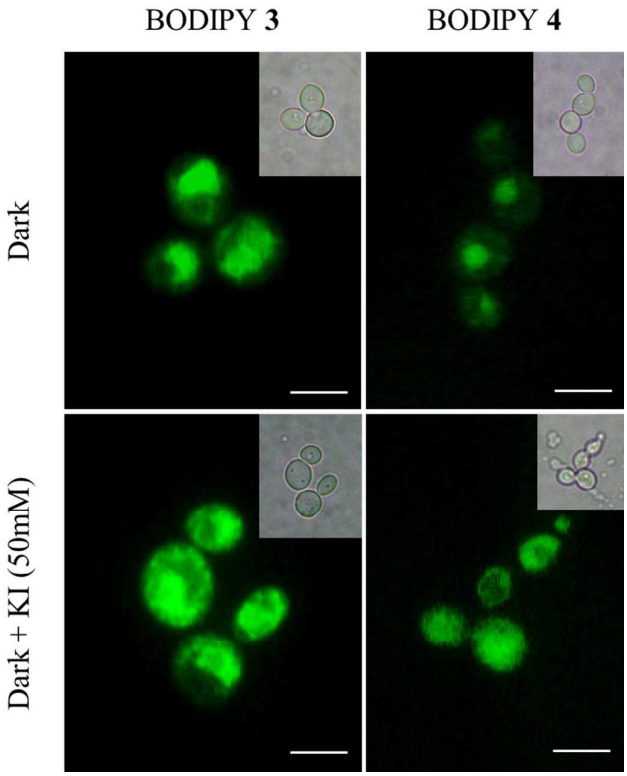

Figure 3

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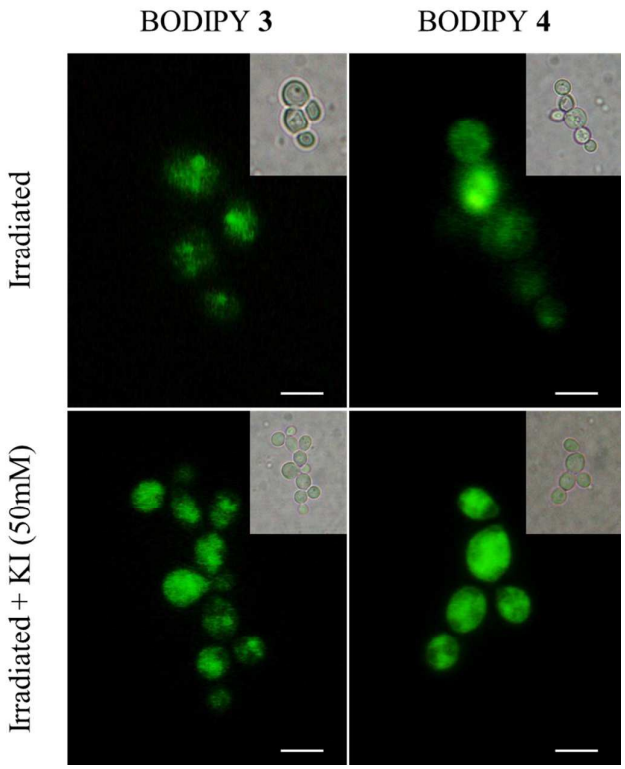

Figure 4

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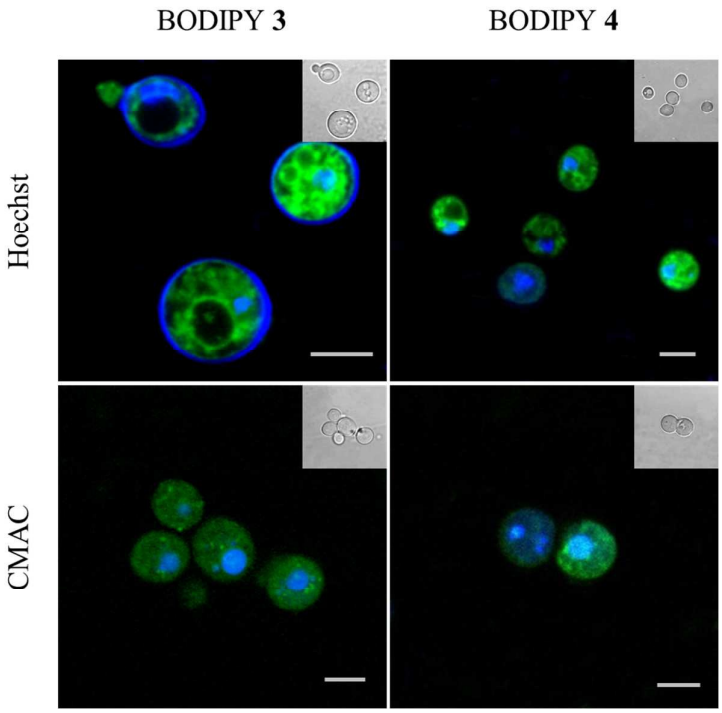

Figure 5

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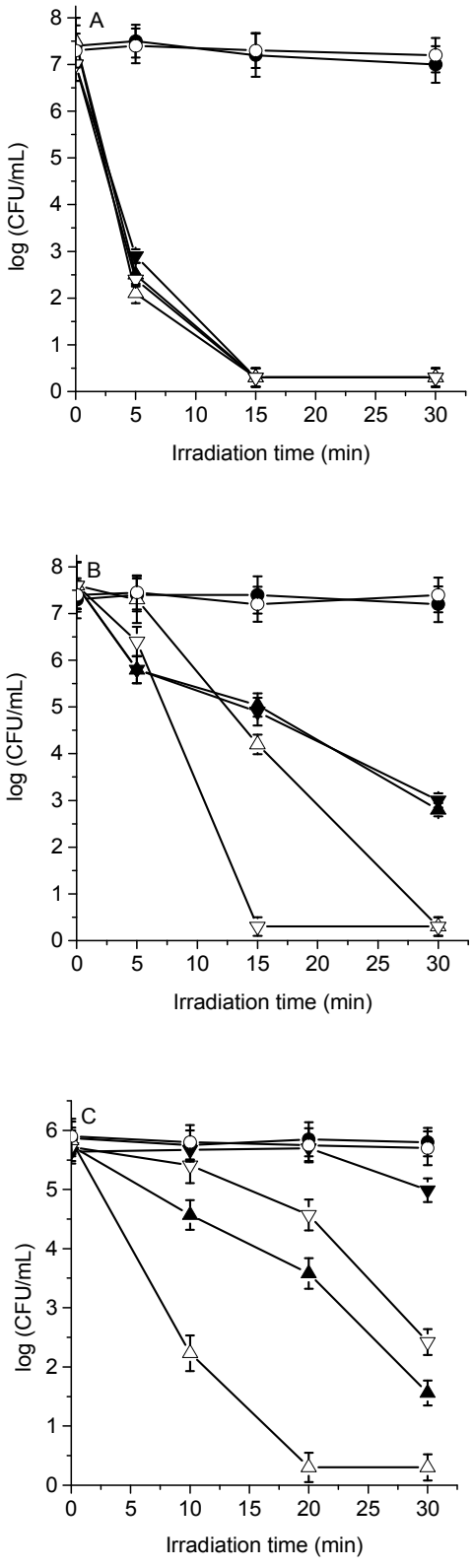

Figure 6

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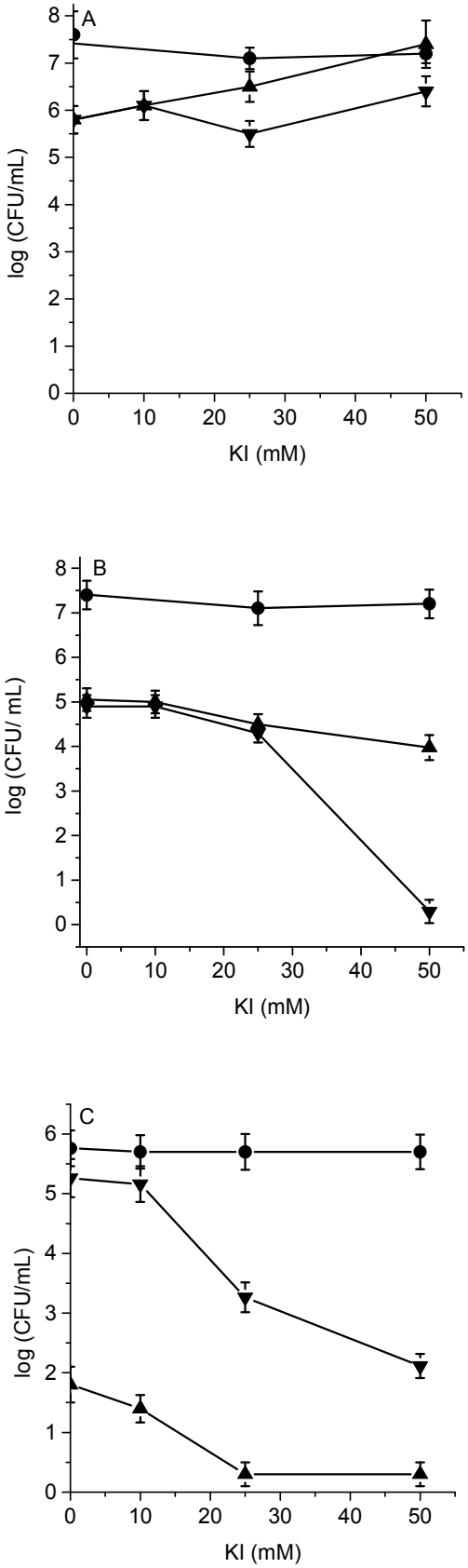

Figure 7

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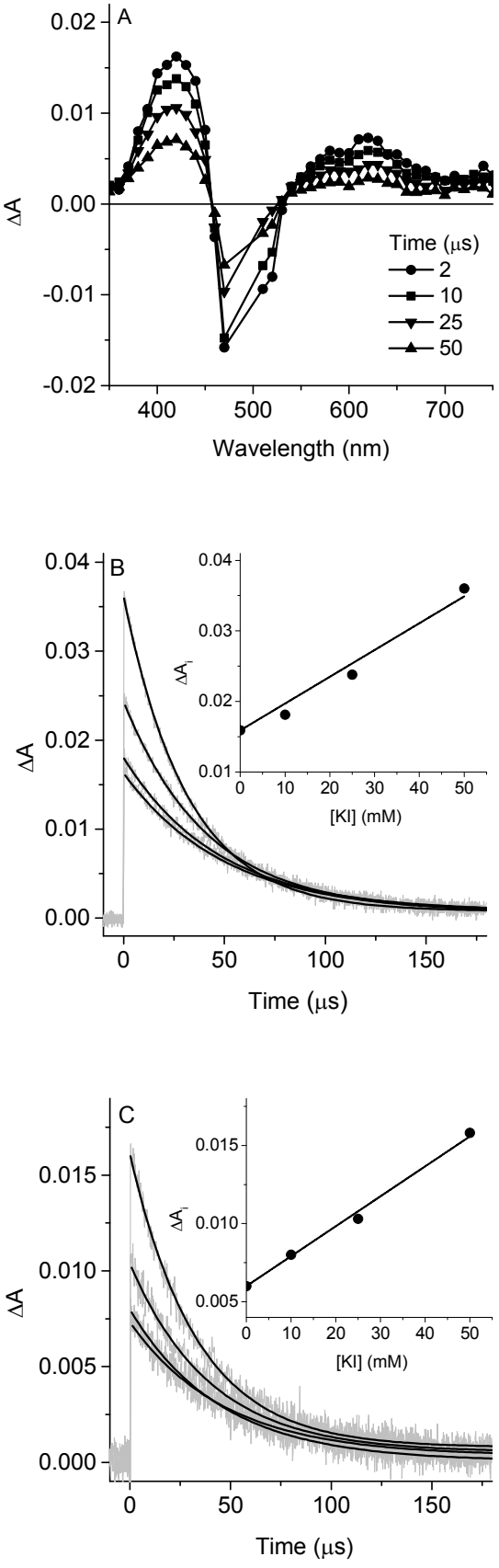

Figure 8

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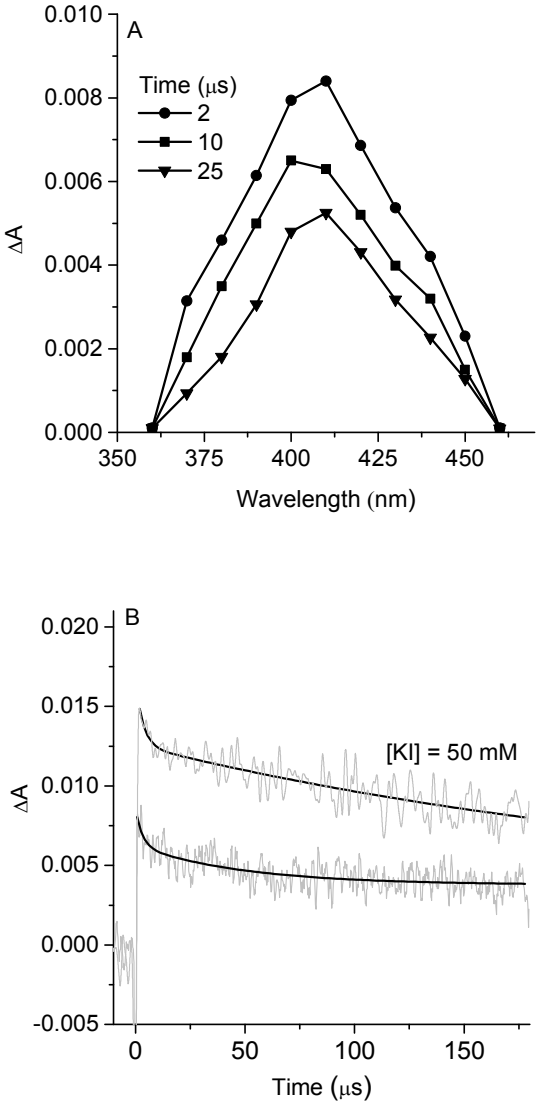

Figure 9

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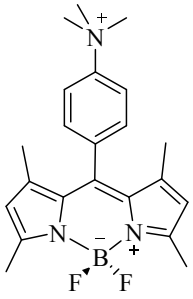
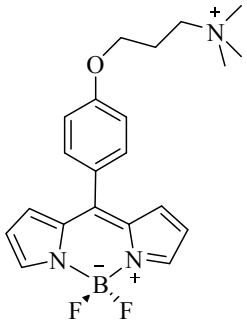


3



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Scheme 1