Effect of Cell-Photosensitizer Binding and Cell Density on Microbial Photoinactivation

Tatiana N. Demidova[1,2] and Michael R. Hamblin[1,3,4]*

*Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, Massachusetts 02114[1]; Department
of Molecular, Cellular and Developmental Biology, Tufts University, Boston, Massachusetts 02111[2];
Department of Dermatology, Harvard Medical School, Boston, Massachusetts 02115[3]; and
Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts 02139[4]*

Photodynamic therapy involves the use of nontoxic dyes called photosensitizers and visible light to produce reactive oxygen species and cell killing. It is being studied as an alternative method of killing pathogens in localized infections due to the increasing problem of multiantibiotic resistance. Although much has been learned about the mechanisms of microbial killing, there is still uncertainty about whether dyes must bind to and penetrate various classes of microbe in order to produce effective killing after illumination. In this report, we compare the interactions of three antimicrobial photosensitizers: rose bengal (RB), toluidine blue O (TBO), and a poly-L-lysine chlorin(e6) conjugate (pL-ce6) with representative members of three classes of pathogens; *Escherichia coli* (gram-negative bacteria), *Staphylococcus aureus* (gram-positive bacteria), *Candida albicans* (yeast). We compared fluence-dependent cell survival after illumination with the appropriate wavelengths of light before and after extracellular dye had been washed out and used three 10-fold dilutions of cell concentration. pL-ce6 was overall the most powerful photosensitizer, was equally effective with and without washing, and showed a strong dependence on cell concentration. TBO was less effective in all cases after washing, and the dependence on cell concentration was less pronounced. RB was ineffective after washing (except for *S. aureus*) but still showed a dependence on cell concentration. The overall order of susceptibility was *S. aureus* > *E. coli* > *C. albicans*, but *C. albicans* cells were 10 to 50 times bigger than the bacteria. We conclude that the number and mass of the cells compete both for available dye binding and for extracellularly generated reactive oxygen species.

Considerable progress has been made in treatment of infections; however, the increasing worldwide occurrence of antibiotic-resistant bacteria is a considerable concern. Therefore, there is a significant need for new antimicrobial techniques. Photodynamic therapy (PDT) uses light-activatable dyes termed photosensitizers (PS) and visible light that, when combined in the presence of oxygen, produce cytotoxic species and tissue destruction (6, 8). It has been approved for treatment of age-related macular degeneration and certain types of cancer and premalignant lesions (7). Antimicrobial therapy is one of the possible future applications of this technique. PDT is known to be effective against viruses, bacteria, and fungi and therefore has been proposed to be used as a therapy for localized infections (10). The development of microbial resistance to PDT is not known and is thought unlikely to be developed (15). Although antimicrobial PDT has been known for about a century (22), the underlying mechanisms of its action are not completely understood. Nonetheless, some parameters important for bacterial inactivation are established.

It is known that gram-positive bacteria are generally more susceptible to PDT as compared to gram-negative species (17, 24). This difference is explained by the structural differences in the cell walls. Gram-negative cells have a complex outer barrier structure including two lipid bilayers, while gram-positive cells have only one lipid bilayer and a relatively permeable outer coat. The yeasts such as *Candida albicans* are even more resistant to photodynamic inactivation (PDI) due to the presence of a nuclear membrane that may present an additional barrier for PS penetration (29); therefore, higher doses of PS and light have to be used. It was shown previously (12, 26) that a positive charge on the PS molecule (such as a poly-L-lysine–PS conjugate or a cationic substitution-containing porphyrin) allows it to bind to, and in some cases penetrate, the microbial permeability barrier. Therefore, positively charged PS are generally more efficient and can act at lower concentrations than neutral and anionic PS molecules. Negatively charged PS are not able to penetrate this gram-negative barrier but may still be effective (although at higher concentrations); in this case, singlet oxygen generated during the irradiation at the outer surface or in solution in close proximity to the cell is thought to diffuse into bacteria and produce fatal damage to lipids and proteins in the inner membrane (4, 5).

We formed the hypothesis that there are three groups of antimicrobial PS: those that are tightly bound and penetrate into microorganisms, those that are only loosely bound, and those that do not demonstrate binding. In order to confirm this hypothesis, we employed three PS thought to be representative of these PS classes—a poly-L-lysine chlorin(e6) conjugate (pL-ce6), toluidine blue O (TBO), and rose bengal (RB)—with representative species from different microbial groups: gram-negative bacterium *Escherichia coli*, gram-positive bacterium *Staphylococcus aureus*, and yeast *Candida albicans*. Fluence-dependent cell survival after illumination with the appropriate wavelength of light before and after extracellular PS had been washed out from the cell suspension was compared. Using the

* Corresponding author. Mailing address: Massachusetts General Hospital, BAR314B, 40 Blossom Street, Boston, MA 02114-2698. Phone: (617) 726-6182. Fax: (617) 726-8566. E-mail: hamblin@helix.mgh.harvard.edu.
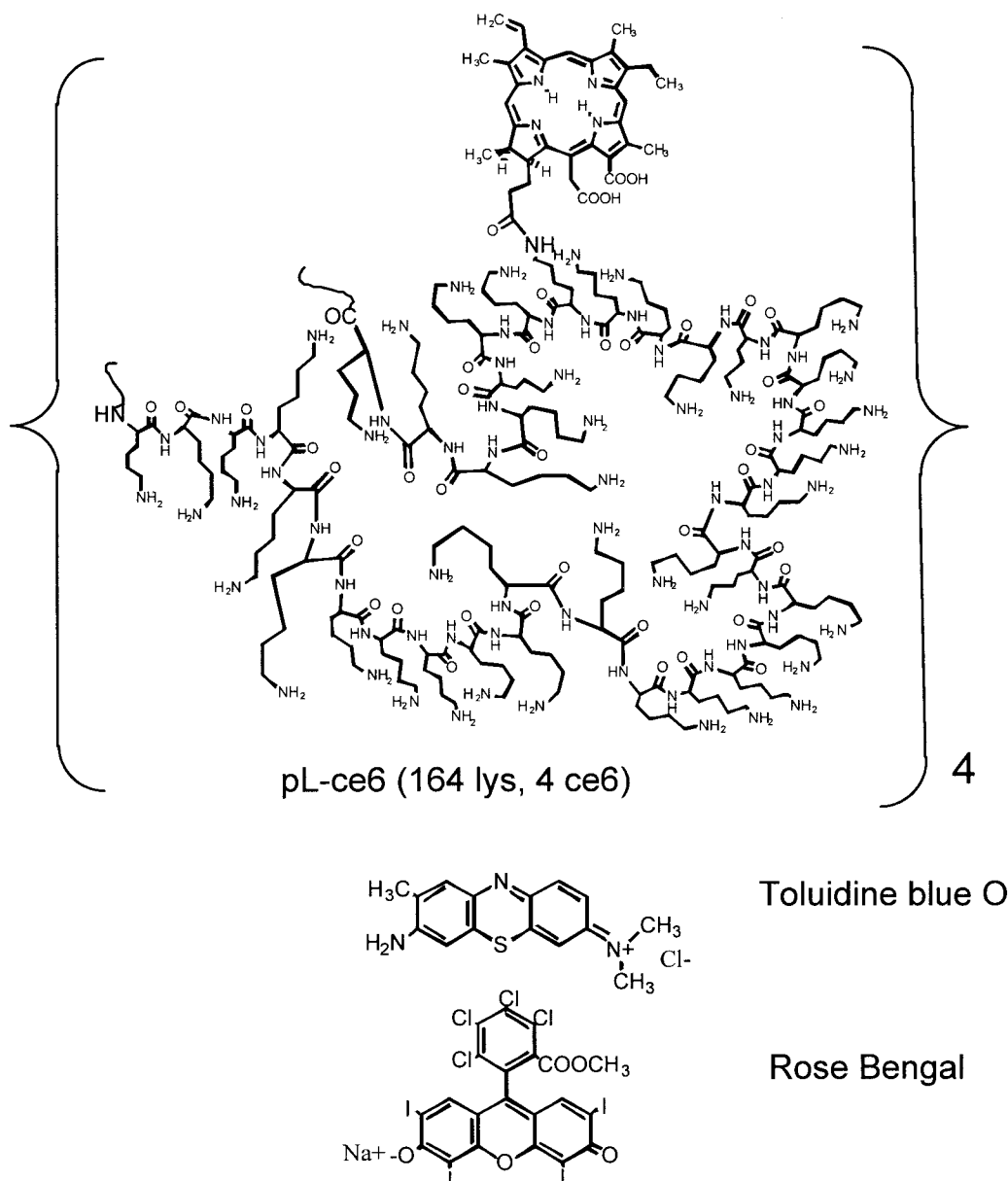
FIG. 1. Chemical structures of the PS used in this study.

same microbial strains, we also tested the hypothesis that microbial cells compete for available dye and reactive oxygen, and therefore the cell density is an important parameter for defining the effectiveness of antimicrobial PDT.

MATERIALS AND METHODS

Bacteria. The microorganisms studied were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (SA113) (9), and *Candida albicans* (ATCC 18804). Cells were grown at 37°C in aerobic conditions in a shaker at 150 rpm. Brain heart infusion broth (Difco, BD Diagnostic Systems, Sparks, MD) was used for *E. coli* and *S. aureus*; YM medium (Difco) was used for *C. albicans*.

Exponential cultures obtained by reculturing stationary overnight precultures were used for all experiments. *E. coli* and *S. aureus* were grown in fresh medium for approximately 1 h to a density of 10^8 cells/ml; the optical density (OD) values at 650 nm were 0.6 and 0.8, respectively. *C. albicans* was grown for approximately 4 h to an approximate density of 10^7 cells/ml, corresponding to an OD of 6 at 650 nm (10-fold dilution measured). Cells of all microorganisms then were diluted

with phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} or concentrated 10-fold by centrifugation at 1,550 g for 6 min and then resuspension in PBS. Concentrations of 10^9, 10^8, and 10^7 cells/ml for *E. coli* and *S. aureus* and 10^8, 10^7, and 10^6 cells/ml for *C. albicans* were used for each experiment. Dry weights of microbial pellets were measured. Microbial suspensions in distilled water (10 ml of 10^8 cells/ml for *E. coli* and *S. aureus* and 10^7/ml for *C. albicans*) were centrifuged in preweighed tubes and freeze-dried (Freezone 4.5; Labconco, Kansas City, MO). Exact cell numbers were confirmed by counting CFU obtained after serial dilutions were streaked horizontally on square brain heart infusion agar plates as described by Jett et al. (14). Gram-stained slides were prepared and examined by bright-field microscopy and a digital camera. This allowed a determination of the relative size of microorganisms studied.

Photosensitizers and light sources. The structures of the three PS used (RB, TBO, and pL-ce6) are shown in Fig. 1, and their optical properties are given in Table 1. RB (89% purity) and TBO (84%) were purchased from Sigma (St. Louis, MO); pL-ce6 was prepared as described previously (11). The conjugate had an average length of 164 lysine residues, to which were attached an average of six (range, four to eight) chlorin(e6) molecules. Stock solutions (TBO and RB

TABLE 1. Optical properties of photosensitizers used in this study

PS	Absorption maximum (nm)	Extinction coefficient	Illumination wavelength (nm)
TBO	630	59,665	620–650
RB	544	90,400	525–555
pL-ce6	664	40,000[a]	645–675

[a] Based on ce6 equivalent.

at 2 mM, pL-ce6 at 3.3 mM ce6 equivalent) were prepared in water and stored at 4°C in the dark for no longer than 2 weeks before use. Spectra of stock solutions diluted 140-fold in methanol were recorded on a UV-visible spectroscopy system (model 8453; Agilent Technologies Deutschland GmbH, Waldbronn, Germany). A noncoherent light source with interchangeable fiber bundles (LumaCare, London, United Kingdom) was employed. Thirty-nanometer bandpass filters at ranges given in Table 1 allowed total powers of roughly 1 W to be obtained.

PS uptake by microbial cells. Suspensions of microorganisms with different cell densities were incubated for 20 min with photosensitizers in the dark at room temperature. Unbound PS was washed out by centrifugation of the mixture of dye and microorganisms for 6 min at 1,550 g followed by resuspension of washed pellets in 10 ml PBS without Ca^{2+} or Mg^{2+}. Aliquots (200 μl) of these suspensions were used for PDI experiments, the remaining suspensions were centrifuged again, and the pellets were dissolved in 6 ml 0.1 M NaOH–1% sodium dodecyl sulfate for at least 24 h. These suspensions were used for PS uptake measurement. The fluorescence of dissolved pellets was measured on a spectrofluorimeter (FluoroMax3; SPEX Industries, Edison, NJ). For pL-ce6, the excitation wavelength was 400 nm and the emission spectra of the solution were recorded from 580 to 700 nm. For TBO, the excitation wavelength was 620 nm and the range for emission was 627 to 720 nm. For RB, the excitation wavelength was 552 nm and the emission was recorded in the range from 555 to 620 nm. The fluorescence was calculated from the height of the peaks recorded. Calibration curves were made from pure PS dissolved in NaOH-sodium dodecyl sulfate and used for determination of PS concentration in the suspension. Uptake values were obtained by dividing the number of nmol of PS in the dissolved pellet by the number of CFU obtained by serial dilutions and the number of PS molecules/cell calculated by using Avogadro's number.

PDI studies. Illumination was performed either after or before excess dye was washed out. Wash allowed the adjustment of the cell densities to 10^8 (for *E. coli* and *S. aureus*) and 10^7 (for *C. albicans*) before illumination; then aliquots of 200 μl were placed in 96-well plates and illuminated with the appropriate light at room temperature. Fluences ranged from 0 to 200 J cm^{-2} at an irradiance of 50 to 400 mW cm^{-2}. During illumination after defined fluences had been delivered, aliquots of 20 μl were taken to determine the CFU. The contents of the wells were mixed before sampling. The aliquots were serially diluted 10-fold in PBS without Ca^{2+} or Mg^{2+} to give dilutions of 10^{-1} to 10^{-6} times the original concentrations, and colonies were grown overnight at 37°C as described. Colo-

nies were counted, and the survival fraction was determined compared to that in the untreated control. Dye in the absence of light and light alone were also used as controls. PS were generally not toxic for microorganisms in the dark (except for pL-ce6 and *S. aureus* at 10^7/ml), and light alone did not cause cell destruction. All experiments were performed in triplicate. Effective killing was taken to be >99.9% or 3 logs.

Statistics. Values are expressed as means ± standard errors. The difference between two means was compared by a two-tailed unpaired Student's t test assuming equal or unequal variances as appropriate. For PDI experiments, statistical significance between killing curves was determined at the highest fluence at which comparisons could be made. P values of <0.05 were considered significant.

RESULTS

Preliminary experiments were carried out in order to determine the appropriate dose ranges (both PS concentration and light fluences) that would allow a light dose response of microbial survival to be achieved with the different microbial species and PS. Once these dose ranges were obtained, PDI experiments were carried out with the chief variable being light fluence and the endpoint survival fraction. The dark toxicity of the PS can be assessed from the survival fraction at 0 J/cm^2. There were three microbial species (*S. aureus* shown in Fig. 2, *E. coli* shown in Fig. 3, and *C. albicans* shown in Fig. 4) and three different PS (pL-ce6, TBO, and RB), giving nine possible combinations. Three 10-fold dilutions of cell concentration were used for each combination of microbe and PS. *C. albicans* is a eukaryotic cell, while *S. aureus* and *E. coli* are prokaryotic cells, and therefore the fungal cell is significantly larger than the bacterial cells. We calculated the dry weight of the three cell types, and the results were as follows (expressed as pg/cell): *S. aureus*, 10.0 ± 4.7; *E. coli*, 1.8 ± 0.8; and *C. albicans*, 99.8 ± 8.9. Due to the large differences in cell size and mass, the cell concentrations of *C. albicans* that were used were 10 times lower (10^8 to 10^6) than those of the two bacterial species (10^9 to 10^7). Finally we compared the PDI of the cells both in the presence of the PS and after the PS had been washed out from the cell suspension. In the latter case, we were able to measure the uptake of the PS by the cells in terms of molecules of PS per cell, and these data are presented in Table 2. Due to the multiple comparisons possible, we will analyze the data set in four sections below.
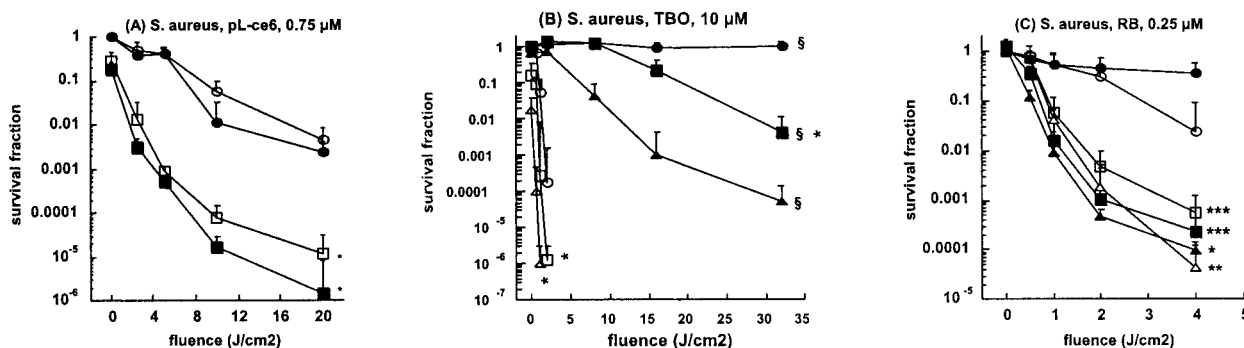

FIG. 2. Photoinactivation of *S. aureus*. Cells were incubated with the specified concentration of PS for 20 min followed by illumination with the appropriate wavelength of light (see text). ● and ○, 10^9 cells/ml; ■ and □, 10^8 cells/ml; ▲ and △, 10^7 cells/ml; ●, ■, and ▲, PS washed from cells before illumination; ○, □, and △, PS left in cell suspension during illumination. The values shown are means of three independent experiments, and bars are the standard error of the mean. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; compared with next highest cell concentration. §, $P < 0.05$, between no wash and corresponding wash.
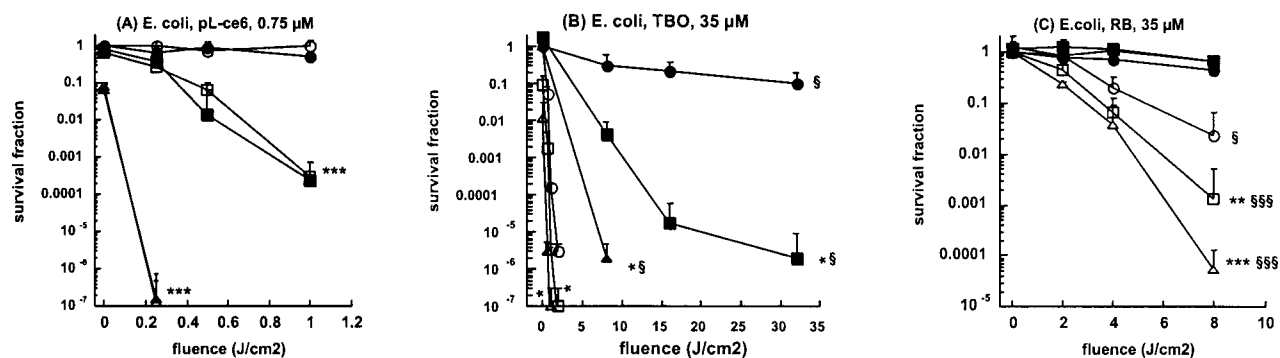
FIG. 3. Photoinactivation of *E. coli*. Cells were incubated with the specified concentration of PS for 20 min followed by illumination with the appropriate wavelength of light (see text). ● and ○, 10^9 cells/ml; ■ and □, 10^8 cells/ml; ▲ and △, 10^7 cells/ml; ●, ■, and ▲, PS washed from cells before illumination; ○, □, and △, PS left in cell suspension during illumination. The values shown are means of three independent experiments, and bars are the standard error of the mean. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, compared with next highest cell concentration. §§§, $P < 0.001$; and §, $P < 0.05$, between no wash and corresponding wash.

Different PS and microbes. The cationic pL-ce6 was by far the most effective at killing all three classes of microbe (compare Fig. 2A, 3A, and 4A). Concentrations of 0.75 μM were sufficient to produce killing of the bacteria, while 5 μM was needed for the fungus. The conjugate was more effective at killing *E. coli* (only 0.2 J/cm² of light) compared to *S. aureus* (20 J/cm² of light). The lowest concentration of *S. aureus* (10^7 cells per ml) was totally killed by pL-ce6 in the dark; hence, there are only two sets of curves in Fig. 2A.

The phototoxicity of TBO was much lower than that of pL-ce6 (compare Fig. 2B, 3B, and 4B). Both the PS concentration and the light dose necessary to kill *S. aureus* (10 μM and 32 J/cm²), *E. coli* (35 μM and 32 J/cm²), and *C. albicans* (50 μM and 40 J/cm²) were all higher than those required for pL-ce6.

The phototoxicity of RB was roughly comparable in degree to that of TBO (compare Fig. 2C, 3C, and 4C). RB was much more phototoxic than TBO toward *S. aureus* (0.25 μM and 4 J/cm²) and somewhat more phototoxic toward *E. coli* (35 μM and 8 J/cm²) but less phototoxic than TBO toward *C. albicans* (200 μM and 40 to 80 J/cm²).

The order of susceptibility of the different species to PDI mediated by pL-ce6 was *E. coli* > *S. aureus* > *C. albicans*. For TBO and RB, it was *S. aureus* > *E. coli* > *C. albicans*.

Effect of washing excess PS from cell suspension. The effect that washing excess PS from the cell suspension had on the PDI of the microbial species was strongly dependent on the chemical nature of the PS. For pL-ce6 (compare Fig. 2A, 3A, and 4A), the killing curves with the pL-ce6 washed from the cell suspension were identical to those with the pL-ce6 still present for all three microbial species. In fact there was a hint that PDI with pL-ce6 could be slightly more effective with a wash than without (Fig. 2A and 4A). For experiments using TBO, the effectiveness of PDI was dramatically reduced when TBO was washed out of the cell suspensions (Fig. 2B, 3B, and 4B). This applied to all three microbial species but was particularly evident for *S. aureus* and *E. coli*. For RB-mediated PDI, there was a very significant drop in the phototoxicity after washing in the case of *E. coli* (Fig. 3C) and *C. albicans* (Fig. 4C) but not in the case of *S. aureus* (Fig. 2C).

PDI of microorganisms depends on cell density. The effectiveness of PDI increased dramatically with decrease of cell density. This effect of cell density on PDI was observed for all microorganisms studied and with all PS used. The dependence
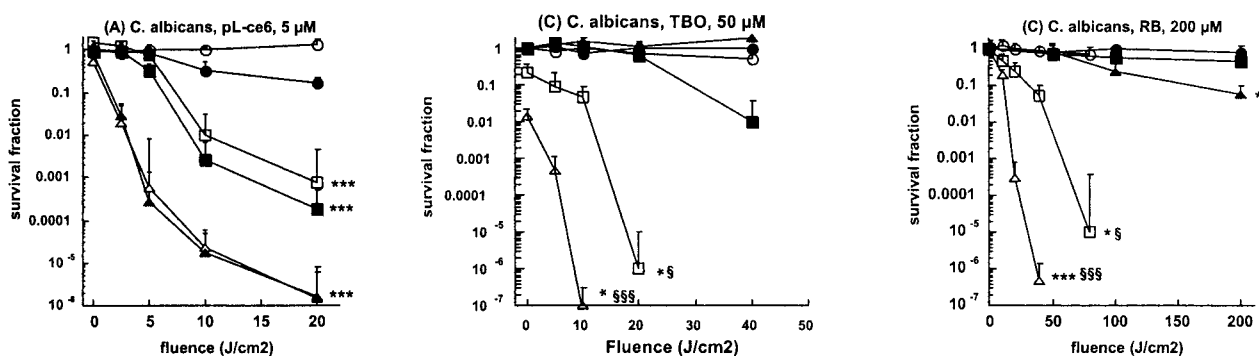

FIG. 4. Photoinactivation of *C. albicans*. Cells were incubated with the specified concentration of PS for 20 min followed by illumination with the appropriate wavelength of light (see text). ● and ○, 10^8 cells/ml; ■ and □, 10^7 cells/ml; ▲ and △, 10^6 cells/ml; ●, ■, and ▲, PS washed from cells before illumination; ○, □, and △, PS left in cell suspension during illumination. Values shown are means of three independent experiments, and bars are the standard error of the mean. ***, $P < 0.001$; *, $P < 0.05$, compared with next highest cell concentration. §§§, $P < 0.001$; §, $P < 0.05$, between no wash and corresponding wash.

TABLE 2. Uptake from bacterial pellets obtained after incubation of cell suspensions with different PS

Species and PS (concn [μM])	Uptake (10^6 molecules/cell) at cell density[a]:			
	10^9/ml	10^8/ml	10^7/ml	10^6/ml
pL-ce6				
S. aureus (0.75)	14.8 ± 1.58	243 ± 49.8***	3,560 ± 220***	
E. coli (0.75)	0.21 ± 0.03	2.52 ± 0.33**	107 ± 1.3*	
C. albicans (5)		13.4 ± 1.1	91 ± 8.2*	1,730 ± 124***
TBO				
S. aureus (10)	0.026 ± 0.013	0.098 ± 0.065	0.562 ± 0.143	
E. coli (35)	0.25 ± 0.018	0.61 ± 0.07*	2.74 ± 0.09*	
C. albicans (50)		0.27 ± 0.11	1.09 ± 0.33	4.9 ± 0.78*
RB				
S. aureus (0.25)	ND[b]	ND	ND	
E. coli (35)	0.81 ± 0.06	1.1 ± 0.15	0.79 ± 0.17	
C. albicans (200)		23.9 ± 0.7	23.8 ± 0.6	24.5 ± 1.0

[a] Values represent the uptake in molecules/cell from bacterial pellets obtained after incubation of the cell suspensions with different PS. Values are the means of three determinations ± standard deviation. Microbial cell densities were 10^9, 10^8, and 10^7 for *S. aureus* and *E. coli* and 10^8, 10^7, and 10^6 for *C. albicans*. PS concentrations were individually chosen to give optimal light-mediated killing of each microbial species. ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$, compared with the next highest cell concentration.
[b] ND, not detectable.

of PDI on cell density was most pronounced with pL-ce6. *S. aureus* (Fig. 2A) showed 2 $\log_{10}$ of killing if 10^9 cells/ml was used; however, killing increased to 6 $\log_{10}$ when 10^8 cells/ml was used; this pL-ce6 concentration was toxic in the dark for cells at 10^7 cells/ml. *E. coli* (Fig. 3A) at a cell density of 10^9 was resistant to PDI with 0.75 μM pL-ce6; 1 $\log_{10}$ of killing was seen under the same conditions when the cell density was reduced to 10^8; finally, killing of 6 $\log_{10}$ was obtained with less light when the cell density was 10^7 cells/ml. This effect was seen if the dye was either left in the solution or washed out. *C. albicans* (Fig. 4A) showed only 0.3 $\log_{10}$ of killing with 10^8 cells/ml, 3 $\log_{10}$ of killing at 10^7 cells/ml, and 6 $\log_{10}$ of killing when the cells were at 10^6/ml.

Experiments with TBO showed less effect of cell density on PDI, and this was mainly apparent when TBO was left in the solution. *S. aureus* (Fig. 2B) cells at 10^9/ml were resistant to PDI, while 2 $\log_{10}$ of killing was observed at 10^8 cells/ml and 4 $\log_{10}$ of killing was observed at 10^7 cells/ml. *E. coli* (Fig. 3B) was resistant at 10^9/ml; however, 5 to 6 $\log_{10}$ of killing was seen after 32 J/cm^2 at 10^8, and similar killing was seen after only 8 J/cm^2 if 10^7 cells per ml was used. In experiments with *C. albicans* (Fig. 4B), the effect of cell density on PDI was seen both if TBO was left in the solution or washed, but it was less prominent in the latter case.

The effect of cell density on PDI was the least in experiments with RB. Experiments with *S. aureus* (Fig. 2C) gave no significant killing at 10^9 cells/ml, while if 10^8 and 10^7 cells/ml were used 4 to 6 $\log_{10}$ of killing was achieved even if excess PS was washed out. With *E. coli* (Fig. 3C) if RB was left in the solution, 2, 3, and 4 $\log_{10}$ of killing was seen at cell densities of 10^9, 10^8, and 10^7 cells/ml, respectively. The dependence of the killing on cell density with *C. albicans* (Fig. 4C) was only seen if RB was left in the solution. A total of 10^8 cells/ml were resistant to PDI, while 4 and 6 $\log_{10}$ of killing were achieved if 10^7 and 10^6 cells/ml were used.

Uptake studies. In experiments where the excess PS was washed out of the cell suspensions, it was possible to measure the uptake of the PS by the microbial cells (except for the case

of *S. aureus* and RB, where the low concentration of RB necessary to achieve killing was below the detection limits of the fluorescence assay). The data are presented in Table 2. The uptake of pL-ce6 by all three microbial cells was much higher than the other PS used by 1 to 3 orders of magnitude, and in addition, the concentration of pL-ce6 used was much lower than that needed for the other PS. The uptake of pL-ce6 showed a marked inverse dependence on cell density. For all three cell types, decreasing the cell density 10-fold gave a corresponding 10-fold increase in cell uptake of pL-ce6. The uptake of TBO by the cells was much lower than that of pL-ce6 despite higher concentrations being used in the incubations. There was again an inverse dependence of uptake on cell density but not as marked as that seen with pL-ce6. When the cell density was reduced 10-fold, the uptake of TBO by the cells increased roughly 3- to 5-fold. The uptake of RB by the cells was of comparable magnitude to that of TBO. However, in marked contrast to TBO, the uptake of RB did not show any dependence on cell density. For *E. coli* and for *C. albicans*, there was the same uptake per cell even though the cell densities were varied 100-fold.

DISCUSSION

This report has compared for the first time the effect of varying the cell concentration over 2 orders of magnitude when incubated with three PS with different degrees of binding to microbes. A previous study had compared porphyrins with various meso substitutions that led to different binding to bacteria (20). We used representative members of the three major classes of human pathogens: gram-positive and gram-negative bacteria and fungi. The pL-ce6 conjugate (28) typifies the class of antimicrobials, PS, that bind well to the negative charged exterior of pathogens and in addition allow penetration of the PS through the permeability barrier of gram-negative bacteria (19). It was overall the most effective PS by a considerable margin, needing much smaller concentrations of PS (0.75 μM for both *E. coli* and *S. aureus* and 5 μM for *C. albicans*) and

smaller fluences of light to accomplish killing. In agreement with data reported previously with a different pL-ce6 conjugate (12), pL-ce6 was more effective in killing the gram-negative *E. coli* than the gram-positive *S. aureus*, although the uptake per cell was 15 to 100 times higher by *S. aureus*. The explanation of this somewhat surprising finding may be that the large molecular weight of the conjugate (in the region of 22,000) reduces its ability to penetrate the outer peptidoglycan of the gram-positive *S. aureus* cell wall despite a high degree of binding to the negative charges available at the surface. In the case of the gram-negative *E. coli* double membrane cell wall structure, the polycationic conjugate can penetrate through the outer membrane by the process known as "self-promoted uptake" (13, 21). In this mechanism, the positive charges on pL-ce6 replace divalent cations in the lipopolysaccharide and also distort the outer membrane structure, allowing channels to form. The high level of dependence on cell density observed with conjugate presumably reflects the strength and avidity of the binding of pL-ce6 to the cells. Since the vast majority of the conjugate in the incubation mixture is bound to the cells, when the cell number is increased the amount of PS bound to each cell decreases. The fact that the same killing is observed whether the pL-ce6 is washed from the suspension or not again shows that it is the cell-bound PS that is effective in mediating PDI. The slight tendency toward increased killing after a wash might be explained by the fact that the PS that remains in solution when there is no wash is able to act as an optical shield, in which the molecules in solution absorb light but have little killing effect and reduce the light which might otherwise be absorbed by PS inside the cell. The pL-ce6 conjugate had the largest dark toxicity of the PS tested toward all three microbes with a particular effect against *S. aureus* so that the most dilute cell suspension failed to grow after incubation with 0.75 μM in the dark. This observation is no surprise as the toxicity of polycationic species such as pL toward various bacteria is well established (27).

TBO showed an intermediate degree of binding toward the microbes in our study but was overall much less effective than the conjugate in killing all three microbial species, requiring 10 to 40 times higher concentrations of PS in the incubation and 2 to 30 times more light. TBO was much more effective in mediating killing when it remained in the suspension than when it was removed by washing for all three microbes. This implies that the TBO in solution is playing a highly active role in the light-mediated killing of the cells. There are two possible explanations for this observation. First, it may be due to the light-mediated extracellular generation of reactive oxygen species (ROS) (particularly singlet oxygen) that can diffuse into the cell producing damage cell death. The second explanation is that the first part of the PDI process produces some damage at the cell surface that allows more TBO molecules present in solution to bind and/or penetrate the cell and therefore increasing the potency of the PDI process. The finding that there was a significant dependence of killing on cell density (although somewhat less than found with pL-ce6) also implies that there is binding or interaction of the cells with a limited concentration of some species and that this occurs whether or not the PS is washed out from the cell suspension.

RB was able to mediate killing of all three microbial species with roughly comparable efficiency to that of TBO. For *S.*

aureus, it was equally effective whether washed out of the cell suspension or left in solution. However for both *E. coli* and *C. albicans*, it was almost ineffective if washed out while mediating significant killing if left in solution. The facts that RB was effective at a very low concentration (0.25 μM) against *S. aureus* together with the equivalence of washing and no washing and the lack of cell density dependence all suggest that RB kills *S. aureus* by entering the cell but that this occurs by a diffusion-controlled process rather than by active binding to the cell as in the case of pL-ce6. Much higher concentrations of RB were needed to kill both *E. coli* and *C. albicans*, and RB was not effective against these species after a wash. There was a cell-density-dependent effect on the killing, which was particularly pronounced with *C. albicans*. These data suggest that it is the extracellular singlet oxygen (or other ROS) that is responsible for killing these species.

This finding is in agreement with a report of experiments in which the bacteria were separated from RB by a layer of moist air and singlet oxygen in the gas phase was generated and allowed to diffuse across the gap before contacting the bacteria (4). Gram-negative species were harder to kill than gram-positive species, and the intracellular content of carotenoids was found to protect the bacteria from photoinactivation. In another study, RB was covalently bound to small polystyrene beads that were allowed to mix with the bacteria in suspension (1).

It is interesting to observe the uptake of RB by the various microbial species at different cell densities. In the two species that could be measured (*E. coli* and *C. albicans*), the uptake was the same at each cell density, although the cell densities varied by 2 orders of magnitude. This is presumably explained by entry into or attachment of the RB molecules to the cells by some diffusion-controlled process rather than affinity-mediated binding. However, this uptake by the cells is apparently insufficient to cause any light-mediated killing after excess RB is washed from the suspension. Presumably this lack of killing reflects the subcellular location of the PS in a relatively superficial layer, as RB lacks any positive charges needed to gain deeper penetration. The cell density dependence of the killing of these two species by RB when still present in the cell suspension implies that the extracellular singlet oxygen is in limited concentration and reacts rapidly with the cells, so that the more cells that compete for available ROS then each cell gets less damage.

The great difference in size between the bacterial and fungal cells means that the amount of singlet oxygen needed to kill a *Candida* cell is much greater than the amount necessary to kill a bacterial cell. This is the case regardless of whether we use the pL-ce6 conjugate that penetrates the cells or TBO or RB, which work mainly from outside the cell. Although many workers have shown that *Candida* species are susceptible to PDI (2, 23, 29), it has not previously been possible to draw comparisons about relative susceptibilities to different PS between fungal and bacterial cells. Now we have shown that *C. albicans* is the hardest to kill with all three PS and that TBO comes closest to having equivalent efficacy for *Candida* as it has for bacteria. In agreement with a report by Polo et al. (25), we have shown that a polycationic PS conjugate with pL is also highly effective against fungal cells as well as bacteria.

The dependence of the effectiveness of PDI on the cell

density in the suspension, which was found to some degree for all species and for all PS, may be intuitively obvious, but this is the first time it has been shown. The finding is important to remember when comparing literature reports of antimicrobial PDI as the cell densities used by various groups can vary. In conclusion, the data reported here will be important in designing experiments concerning antimicrobial PDI and PDT for infections. It should be remembered that both TBO and RB have a long history of medical applications, particularly involving local application to stain various tissues, and are generally accepted as safe (3, 16, 18, 26). Their significant activity in mediating PDI may allow them to progress quickly to clinical trials.

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