

## Rapid Control of Wound Infections by Targeted Photodynamic Therapy Monitored by *In Vivo* Bioluminescence Imaging<sup>¶</sup>

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### ABSTRACT

The worldwide rise in antibiotic resistance necessitates the development of novel antimicrobial strategies. In this study we report on the first use of a photochemical approach to destroy bacteria infecting a wound in an animal model. Following topical application, a targeted polycationic photosensitizer conjugate between poly-L-lysine and chlorin<sub>e6</sub> penetrated the Gram (–) outer bacterial membrane, and subsequent activation with 660 nm laser light rapidly killed *Escherichia coli* infecting excisional wounds in mice. To facilitate real-time monitoring of infection, we used bacteria that expressed the lux operon from *Photorhabdus luminescens*; these cells emitted a bioluminescent signal that allowed the infection to be rapidly quantified, using a low-light imaging system. There was a light-dose dependent loss of luminescence in the wound treated with conjugate and light, not seen in untreated wounds. Treated wounds healed as well as control wounds, showing that the photodynamic treatment did not damage the host tissue. Our study points to the possible use of this methodology in the rapid control of wounds and other localized infections.

### INTRODUCTION

The worldwide rise in multiantibiotic resistance amongst bacteria that infect wounds and burns (1) has led to the search for alternative methods of selectively destroying bacteria without harming the host tissue (2). Because the infection is initially localized to the wound, one method of selectively killing bacteria may be the combination of dyes and visible light, known as photodynamic therapy (PDT). PDT

is a therapy for cancer and other diseases and has received regulatory approvals for several indications in many countries (3). It uses certain nontoxic dyes known as photosensitizers (PS) that should preferentially accumulate at the target tissue or cell-type. Subsequent illumination with visible light of the appropriate wavelength excites the PS molecule to the excited singlet state. This excited state may then undergo intersystem crossing to the slightly lower-energy triplet state, which may then react further by one or both of two pathways known as Type I and Type II photoprocesses, both of which require oxygen (4). The Type I pathway involves electron transfer reactions from the PS triplet state, with the participation of a substrate, to produce radical ions, which can then react with oxygen to produce cytotoxic species, such as superoxide, hydroxyl and lipid-derived radicals (5). The Type II pathway involves energy transfer from the PS triplet state to ground state molecular oxygen (triplet) to produce the excited state singlet oxygen, which can then oxidize many biological molecules, including proteins, nucleic acids and lipids, leading to cytotoxicity (6).

One of the first demonstrations of the effect that later formed the basis of PDT was reported in the early years of the last century when German workers noticed that many microorganisms died after being incubated with certain dyes and exposed to sunlight (7). Since these early experiments, many workers have studied the photodynamic inactivation (PDI) of bacteria *in vitro* with the view that the process may be applied to the treatment of various localized infectious diseases (8,9). In the 1990s it was observed that there was a fundamental difference in susceptibility to PDI between Gram (+) and Gram (–) bacteria (10–12). In general, neutral or anionic PS molecules were found to bind efficiently to and photodynamically inactivate Gram (+) bacteria, whereas they may bind to the outer membrane only of Gram (–) bacterial cells, but these cells are not inactivated. PS with an overall cationic charge, however, can efficiently kill both Gram (+) and Gram (–) species. This has been shown for cationic porphyrins (13), phthalocyanines (11) and phenothiazines (8). Alternative approaches have been discovered for allowing noncationic PS to kill Gram (–) bacteria, such as the use of a small polycationic peptide known as polymyxin-B nonapeptide that was reported to stimulate the translocation of deuteroporphyry through the outer mem-

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Abbreviations: c<sub>e6</sub>, chlorin<sub>e6</sub>; cfu, colony-forming units; DMSO, dimethyl sulfoxide; LED, light-emitting diode; PBS, phosphate-buffered saline; PDI, photodynamic inactivation; PDT, photodynamic therapy; pL, poly-L-lysine; pL–c<sub>e6</sub>, poly-L-lysine chlorin<sub>e6</sub> conjugate; PS, photosensitizer; SDS, sodium dodecyl sulfate.

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brane of *Escherichia coli* (10). It has been shown that bacteria that have developed antibiotic resistance exhibit equal susceptibility to PDI as naïve strains (14).

Our approach to carrying out PDI of bacteria involves covalently linking the PS to a polycationic molecule to produce a species that will bind to and penetrate both Gram (+) and Gram (-) species and allow efficient killing after illumination. We previously showed that attaching the PS chlorin<sub>e6</sub> (*c<sub>e6</sub>*) to a poly-L-lysine (pL) chain of 20 amino acids to give a conjugate with an approximate 1:1 substitution ratio was effective in PDI of both the Gram (+) *Actinomyces viscosus* and the Gram (-) *Porphyromonas gingivalis* (15). With the relatively short incubation times used in this study, mammalian epithelial cells were essentially unharmed by the treatment. Other workers have since used similar conjugates of pL with PS to inactivate various bacteria *in vitro* (16,17).

Despite considerable research assessing the effects of PDI of bacteria *in vitro*, there is a paucity of data pertaining to effective treatment of localized infection in animal models (18). This lack of success may be because of inefficient PS targeting and the inability to selectively kill bacteria compared to eukaryotic cells, but another reason may be the lack of accurate and reproducible *in vivo* assays for assessing the progress of laboratory animal models of infectious disease. Genetically engineered bacteria that emit bioluminescence can be detected *in vivo* using a sensitive imaging camera (19). In this manner the extent of infection can be determined in real time in living animals, providing both temporal and spatial information about the labeled pathogen (20,21). We report here on a demonstration of proof-of-principle of PDT for infection, using a topical application of PS followed by illumination with red light to destroy bacteria infecting excisional wounds in mice, without harming the host tissue.

## MATERIALS AND METHODS

**Preparation of poly-L-lysine chlorin<sub>e6</sub> conjugate.** All reactions were carried out in the dark at room temperature. Two hundred and twenty milligrams (10  $\mu\text{mol}$ ) of pL-HBr (average molecular weight = 22 000, degree of polymerization = 110, Sigma Chemical Co., St. Louis, MO) were dissolved in 3 mL dry dimethyl sulfoxide (DMSO), to which was added 67 mg (112  $\mu\text{mol}$ ) *c<sub>e6</sub>* (Porphyrin Products, Logan, UT) and 61 mg (320  $\mu\text{mol}$ ) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma). Triethylamine (20  $\mu\text{L}$ , Sigma) was then added, and the mixture was stirred for 24 h. The progress of the reaction was checked by thin layer chromatography (Polygram SIL G/UV254 plates, Macherey Nagel, Duren, Germany) in methylene chloride-methanol-ammonium hydroxide (8:2:0.5). After 24 h when the reaction was complete, methanol (1 mL) and water (1 mL) were added, and the mixture was evaporated to dryness under vacuum. The residue was dissolved in sodium acetate buffer (10 mM, pH = 5.5, 3 mL), applied to a column of Sephadex G25 (60 cm  $\times$  1 cm) and eluted with the same buffer at a flow rate of 3.3 mL h<sup>-1</sup>. Three-milliliter fractions were collected, and fractions 14–21 were combined and evaporated to give the product poly-L-lysine chlorin<sub>e6</sub> conjugate (pL-*c<sub>e6</sub>*). The substitution ratio was calculated from the absorption spectrum of the conjugate to be 7.4 *c<sub>e6</sub>* per pL chain, assuming that the absorption coefficient of conjugated *c<sub>e6</sub>* was the same as that of free *c<sub>e6</sub>* ( $\epsilon_{400\text{ nm}} = 150\,000\text{ M}^{-1}\text{ cm}^{-1}$ ).

**Bacteria.** The bioluminescent strain of *E. coli* DH5 $\alpha$  was constructed by transformation with the plasmid pCGLS1, an expression vector that contains a complete bacterial luciferase operon as described (22). Bacteria were grown in a brain-heart infusion medium containing ampicillin (100  $\mu\text{g mL}^{-1}$  to select for resistance encoded by the plasmid) to an O.D.<sub>650 nm</sub> of 0.6 corresponding to 10<sup>8</sup> organisms per milliliter. This suspension was centrifuged, washed with

phosphate-buffered saline (PBS) and resuspended in PBS at the same density. Luminescence was routinely measured on 100  $\mu\text{L}$  aliquots of bacterial suspensions in 96-well black-sided plates, using a Victor-2 1420 Multilabel Plate Reader (EG&G Wallac, Gaithersburg, MD).

***In vitro* studies.** Suspensions of the bacteria (10<sup>8</sup> cells mL<sup>-1</sup>) were incubated in the dark at room temperature for 30 min with varying concentrations of conjugate, measured as  $\mu\text{M}$  *c<sub>e6</sub>* equivalent (final concentration in PBS). Incubations were carried out in triplicate. The cell suspensions were centrifuged (9000 g, 1 min), the PS solution was aspirated and bacteria were washed twice by resuspending the cell pellet in 1 mL sterile PBS followed by centrifugation. Finally, the cell pellet was dissolved by digesting it in 1.5 mL 0.1 M NaOH–1% sodium dodecyl sulfate (SDS) for at least 24 h to give a homogenous solution. Cellular uptake of *c<sub>e6</sub>* was measured by fluorescence assay as previously described (15,23), using a calibration curve constructed with known amounts of conjugate dissolved in 0.1 M NaOH–1% SDS. The protein content of the entire cell extract was then determined by a modified Lowry method (24), using bovine serum albumin dissolved in 0.1 M NaOH–1% SDS to obtain a calibration curve. Results were expressed as moles of *c<sub>e6</sub>* per milligram of cell protein.

Suspensions of bacteria (10<sup>8</sup> mL<sup>-1</sup>) were incubated in the dark at room temperature for 30 min with 1–12  $\mu\text{M}$  *c<sub>e6</sub>* equivalent of the conjugate in PBS as described earlier. Cell suspensions were centrifuged, the cells were washed twice with sterile PBS and 1 mL fresh PBS was added. The bacterial suspensions (1 mL) were then placed in 24-well plates. The wells were illuminated from below, in the dark, at room temperature. A 660 nm, 300 mW diode laser (SDL Inc., San Jose, CA) was coupled with a 1 mm optical fiber that delivered light into a lens, which formed a uniform circular spot, 2 cm in diameter, on the base of the 24-well plate. Fluences ranged from 0 to 40 J cm<sup>-2</sup> at an irradiance of 100 mW cm<sup>-2</sup>. The plates were kept covered during the illumination in order to maintain the sterility of the culture. At intervals during the illumination, when the requisite fluences had been delivered, two aliquots (100  $\mu\text{L}$  each) were taken from each well to determine colony-forming units (cfu) and luminescence. Care was taken to ensure that the contents of the wells were thoroughly mixed before sampling, as bacteria can settle to the bottom. One aliquot was serially diluted for cfu on nutrient agar plates containing ampicillin as described by Jett *et al.* (25). Plates were streaked in triplicate and incubated for 24 h at 37°C in the dark. The remaining aliquot was used for luminescence measurement as described. Plates were inserted into the luminometer 15 min after the completion of the illumination.

**Bioluminescence imaging.** The low-light imaging system consisted of an ICCD camera (model C2400 Hamamatsu Photonics KK, Bridgewater, NJ) fitted with a f1.2 50 mm lens (NIKOR) and mounted in a light-tight specimen chamber (model A4178, Hamamatsu Photonics KK). The light-tight box was fitted with a light-emitting diode (LED), allowing a background grayscale image of the entire mouse to be captured with a minimum setting on the image-intensifier control module (model M4134, Hamamatsu Photonics KK). In the photon-counting mode, an image of the emitted light was captured, using an integration time of 2 min at a maximum setting, on the image-intensifier control module. Using ARGUS software (Hamamatsu Photonics KK), the luminescence image was presented as a false-color image superimposed on top of the grayscale reference image. The image-processing component of the software gave mean pixel values on defined areas within each wound on a 256 grayscale. The same analysis area of 1200 pixels was used for all the wounds at all timepoints.

***In vivo* studies.** All animal experiments were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital. Male Balb/c mice (n = 6) weighing 20–25 g were shaved on the back and depilated with Nair (Carter-Wallace Inc., New York, NY). Mice were anesthetized with an intraperitoneal injection of ketamine-xylazine cocktail. Four full-thickness excisional wounds were made in a line along the dorsal surface (numbered from 1 nearest the head to 4 nearest the tail), using surgical scissors and forceps. Wounds measured 8  $\times$  12.5 mm and had at least 5 mm of unbroken skin between them. The bottom of the wound was panniculus carnosus, with no visible bleeding. A suspension (50  $\mu\text{L}$  PBS) containing 5  $\times$  10<sup>6</sup> cells of midlog phase *E. coli* (10<sup>8</sup> cells

mL<sup>-2</sup>) was inoculated into each wound, and the mouse was imaged with the luminescence camera to ensure equal bacterial loading into each wound. After 30 min, to allow the bacteria to attach to the tissue, 50  $\mu$ L of a solution of the conjugate in PBS (100  $\mu$ M  $c_{66}$  equivalent) was added to wound numbers 1 and 4. After a further 30 min under subdued room lighting, to allow the conjugate to bind to and penetrate the bacteria, the mice were again imaged to quantify any dark toxicity of the conjugate to the bacteria. Mice were then illuminated with 665 nm light delivered by a 1 W diode laser (Model BWF-665-1, B&W Tek, Newark, DE) coupled with a 200  $\mu$ m fiber that gave a circular spot of 3 cm diameter on the mice and equally illuminated wounds 3 and 4 with an irradiance of 100 mW cm<sup>-2</sup>. A shaped vertical piece of cardboard between wounds 2 and 3 acted as a light shield to ensure that no light reached wounds 1 and 2. Mice were given a total fluence of 160 J cm<sup>-2</sup> in four 40 J cm<sup>-2</sup> aliquots, with imaging taking place after each aliquot of light. The total illumination time was 27 min. At the conclusion of the experiment, mice were allowed to recover from anesthesia in an animal warmer and resume their normal activity. There were no visible differences between any of the wounds at the completion of illumination or, indeed, at any time during the healing process. Wounds were not dressed, as preliminary tests with transparent dressings showed that the bacteria tended to grow on the moist undersurface of the dressing. Mice were kept in their cages in the dark for the next 2 days. On each of the next 2 days, the mice were anesthetized with a small dose of ketamine–xylazine and were imaged under the same conditions. The wounds were measured in two dimensions each day for 2 weeks until the wounds were healed, and the areas were calculated.

## RESULTS AND DISCUSSION

### Preparation of conjugates

We devised a new synthetic scheme using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride as the coupling agent with free  $c_{66}$  in DMSO to covalently attach  $c_{66}$  to the pL backbone. The products of the synthesis could be easily purified by column chromatography and could be stored in the dark at 4°C without aggregation. In previous studies (15,23) we described a synthetic scheme to give pL- $c_{66}$ , using dicyclohexylcarbodiimide to prepare an *N*-hydroxysuccinimide ester from  $c_{66}$  and its subsequent reaction with pL dissolved in DMSO. However, although these conjugates performed relatively well, they suffered from a tendency to aggregate over time (several days), which made purification and storage difficult.

### Bioluminescent bacteria

The luminescence signal was linearly proportional to bacterial cfu from 10<sup>3</sup>–10<sup>7</sup> organisms (data not shown). The method of monitoring bacterial numbers and viability in real time in living animals, using genetically engineered bacteria that emit luminescence together with ultrasensitive photon-counting cameras, has been demonstrated in several models (19–22). Because the entire lux gene operon encoding both the bacterial luciferase and the biosynthetic enzymes for substrate synthesis is transfected, the resulting bacteria are bioluminescent without the need for exogenous administration of luciferin to animals *in vivo* (20). Prior to the introduction of this novel methodology, response to therapy in animal models of infection had been monitored either by survival if the infection developed into sepsis, or by tissue or body fluid sampling and subsequent cfu determination. The first method suffers from the disadvantage of requiring large numbers of animals, raises animal welfare issues and gives no information on bacterial load at sites of interest. The sec-

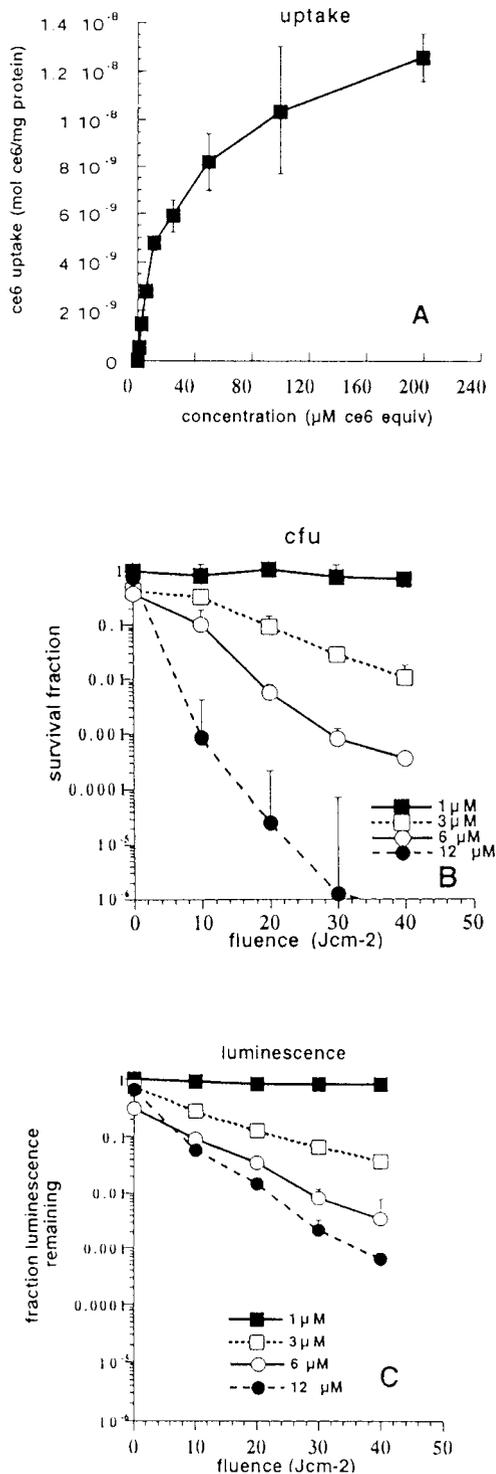
ond method is labor-intensive and suffers from sampling limitations, which may be another potential source of experimental error. In addition, the number of biopsies able to be taken from any site of infection may be limited, and extraction of bacteria from solid tissue may present difficulties in accurate quantitation (19,26). Neither of these assays provide data in real time.

### *In vitro* studies

Bacteria were incubated with increasing concentrations of conjugate to determine the uptake of  $c_{66}$ . A diminution in the rate of increase of uptake was apparent, starting at 20  $\mu$ M  $c_{66}$  equivalent and became more marked at higher concentrations tending toward saturation (Fig. 1A). The light-dose–response curves for bacterial killing after illumination were determined by using both cfu and loss of luminescence as measures of bacterial viability at concentrations of conjugate ranging from 1 to 12  $\mu$ M (Fig. 1B,C). Bacteria were incubated for 30 min in PBS, without Ca<sup>2+</sup> and Mg<sup>2+</sup>, with the indicated concentrations of conjugate. The cfu assay had a limit of sensitivity of six orders of magnitude in reduction of viability, whereas the bioluminescence assay had a limit of three orders of magnitude. Our approach of covalently conjugating a tetrapyrrole PS ( $c_{66}$ ) with an overall anionic charge to a cationic pL chain produces a targeting vehicle that efficiently binds to Gram (–) bacteria and allows the PS to penetrate the outer membrane in a short period of time (15). Because the construct is a macromolecule, it is likely to be taken up by eukaryotic cells by the time-dependent process of endocytosis, thus allowing temporal selectivity for bacteria over the host tissue (23). The *in vitro* experiments showed that conjugate concentrations up to 12  $\mu$ M, together with light doses up to 40 J cm<sup>-2</sup>, killed 6 logs of *E. coli*. Loss of luminescence showed the same dose–response curves as loss of cfu, but the absolute reductions were 1–3 logs less. The reasons for this are two-fold. The limit of sensitivity of the luminescence assay with the plate reader is a 3 log reduction in signal, whereas the cfu assay can measure a 6 log reduction in viability. This accounts for the large absolute difference between the two curves obtained with 12  $\mu$ M conjugate concentration, where the luminescence assay has reached its sensitivity limit. However, in addition, the curves generated with PDI at 3 and 6  $\mu$ M concentration also show an absolute difference of approximately 1 log between cfu and luminescence. This is presumably because the cytotoxic insult to the bacteria causes loss of viability more readily than loss of luminescence. The mechanism by which luminescence decreases after PDI is uncertain, but it may be the result of exhaustion of ATP supplies from the bacteria (needed for the luciferase enzyme to make luminescence), which cannot be replenished if the cells are fatally damaged. It is also possible that cellular targets leading to loss of cfu after PDI are different from the targets leading to loss of luminescence after PDI, and different concentrations of PS may lead to a different percentage distribution of the PS over the various cellular targets.

### *In vivo* studies

We initially sought to establish the animal model of infection by inoculating *E. coli* into an excisional wound on the

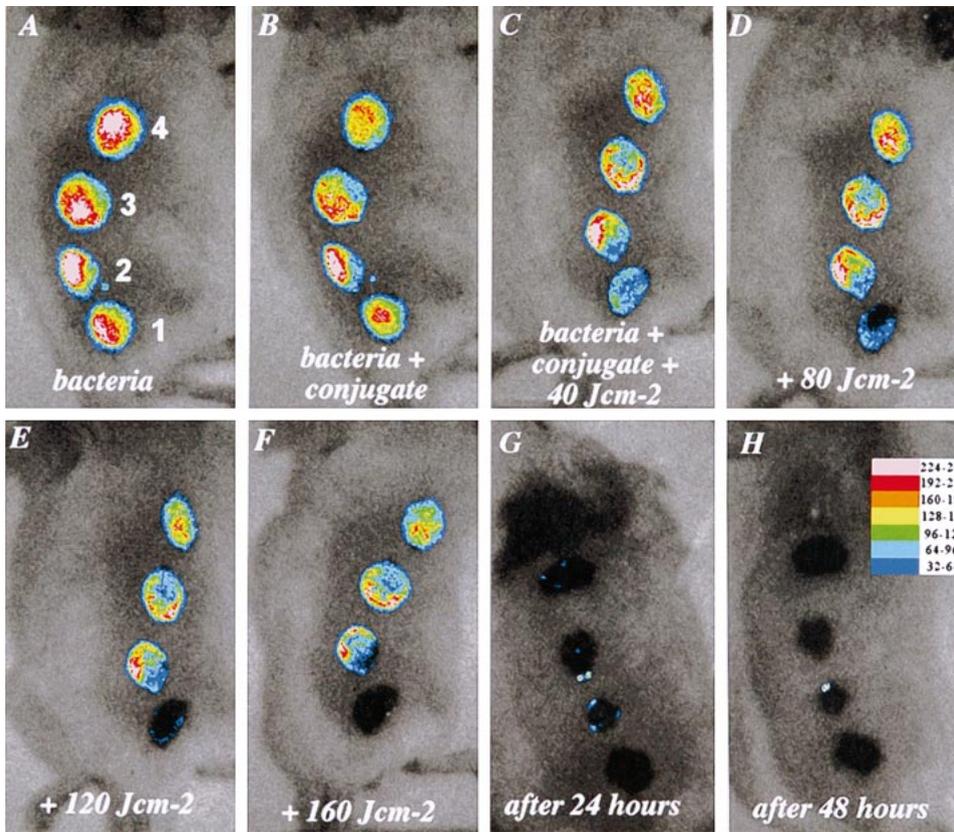


**Figure 1.** Uptake and phototoxicity *in vitro*. (A) Uptake of  $c_{66}$  by bacteria after 30 min incubation in PBS with the stated concentrations of pL- $c_{66}$ , followed by dissolution of the cell pellet in NaOH-SDS, fluorescence and protein assays. Data points are the means of triplicate determinations and two separate experiments, and bars are the SD. Phototoxicity as determined by (B) cfu and (C) luminescence assays. Bacteria were incubated with the stated concentration of conjugate as mentioned earlier, then washed and illuminated with the stated fluence of 660 nm light, with removal of aliquots of bacterial suspension at intervals and serial dilution and plating or luminescence measurement in 96-well plates. Data points are the means of triplicate determinations and two separate experiments, and bars are the SD.

mouse. Five million cfu from a midlog culture in 50  $\mu\text{L}$  gave a sufficiently bright luminescence signal from the wound to allow at least 2 logs of signal reduction to be accurately followed. When this bacterial inoculum was placed into a wound (12.5  $\times$  8 mm) made on the back of a freshly euthanized mouse, the luminescence started to fade rapidly and was totally gone by the time the 50  $\mu\text{L}$  inoculum had dried (<60 min) (data not shown). Untreated infected wounds in living mice showed only a slight loss of luminescence over a period of 4 h. We interpret these findings to mean that the living mouse wound provides nutrients and moisture to the bacteria, and thus is a reasonable model of wound infection. The next day, however, control infected wounds in living mice had lost, on an average, 90% of the original luminescence signal but with considerable interanimal variability (data not shown).

Because the wound infection with *E. coli* DH5 $\alpha$  was found to be self-limiting, *i.e.* this particular strain of *E. coli* is noninvasive (27), it allowed the use of each mouse as its own control to follow wound healing, with four wounds per mouse. The effect of topical application of conjugate and successive applications of light is presented in a series of overlaid luminescence (false-color) and grayscale reference images (Fig. 2). These data were obtained from a mouse in which bacteria were inoculated in all wounds, conjugate was added to wounds 1 and 4, and wounds 3 and 4 were illuminated with red light. Therefore, wound 1 was the dark control with conjugate, wound 2 was the absolute control, wound 3 was the light-alone control and wound 4 was PDT-treated. Topical application of a targeted polycationic PS conjugate followed by illumination led to a 99% reduction in luminescence as measured by imaging software on the luminescence images. There was a semilogarithmic light dose-dependent reduction in luminescence from the PDT-treated wound, not seen in any of the control wounds, as would be expected from a standard PDT experiment (Fig. 3). There was an initial modest decrease in luminescence from wounds that received conjugate without light because of the dark toxicity of the conjugate, but the luminescence did not decrease further during the course of the experiment.

To obtain an equivalent loss of luminescence from the bacteria in the mouse wounds, it was necessary to employ significantly higher amounts of both conjugate and light than that needed *in vitro*. Preliminary experiments with lower conjugate concentrations (10–50  $\mu\text{M}$ ) showed increasing effectiveness with increasing concentrations added to the wound (data not shown). Likewise, increased light doses produced a dose-dependent loss of luminescence. The uptake plot of  $c_{66}$  bound to bacteria *in vitro* (Fig. 1A) demonstrated that there was a tendency toward saturation at high conjugate concentrations (100–200  $\mu\text{M}$ ). Therefore, we hypothesized that the need for much higher concentrations *in vivo* is because of large amounts of conjugate binding to the tissue in the wound, thus being unavailable for binding to bacteria. Rocchetta *et al.* studied the growth of bioluminescent *E. coli* in the neutropenic mouse-thigh abscess model of infection (20). They found that the number of cfu extracted from the thighs of sacrificed animals closely paralleled the luminescence signal

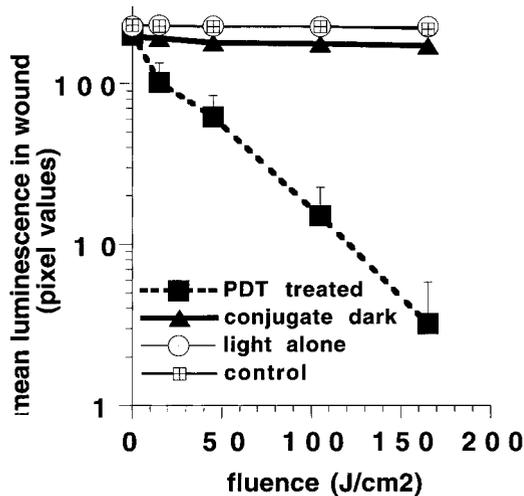


**Figure 2.** Successive overlaid luminescence false-color images and monochrome LED images of a mouse with four excisional wounds infected with equal numbers of *E. coli* (A). Wounds 1 (nearest tail) and 4 (nearest head) received topical application of conjugate (B). Wounds 1 and 2 (two nearest tail) were then illuminated with successive fluences (40–160 J cm<sup>-2</sup>) of 665 nm light (C–F). The same mouse 24 h (G) and 48 h (H) later.

at several timepoints after inoculation and during the period of action of antibiotics.

The present model of an infected wound was rather artificial in that it used a clean excision, a noninvasive bacterial strain and a large inoculum ( $5 \times 10^6$  bacteria). Nevertheless, it served to establish a proof-of-principle that PDT could destroy infection *in vivo*. There is only one other report of this process in the literature by Berthiaume *et al.* (18). These

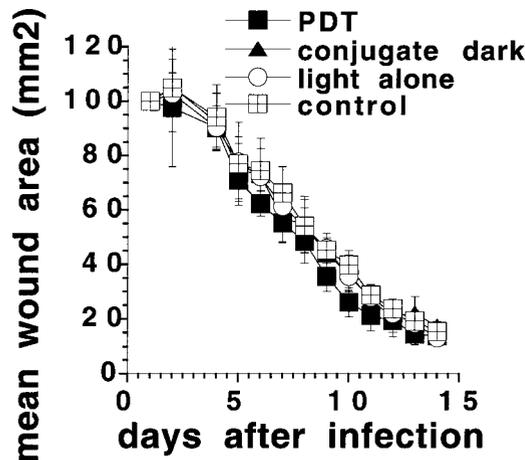
workers used a monoclonal antibody that recognizes *Pseudomonas aeruginosa* covalently conjugated to a *c<sub>66</sub>* derivative. The bacteria were injected subcutaneously in the mouse to form a bubble, into which was subsequently injected the conjugate, followed by illumination. They showed a 75% loss of cfu from bacteria extracted from skin removed from sacrificed animals, not seen with conjugates formed from a nonspecific antibody.



**Figure 3.** Mean pixel values of luminescence signals from defined areas of wounds, measuring 1200 pixels, determined by image analysis. Data points are the means of values from the corresponding wound on six mice per group, and bars are the SD.

### Wound healing

The assumption that considerable amounts of conjugate bound to the tissue in the wound suggested that illumination might have caused damage to the host cells, blood vessels or extracellular matrix in the wound. However, we observed that PDT of infected wounds did not lead to any inhibition of wound healing as seen in Fig. 4. There was an indication that the PDT-treated wounds actually healed somewhat faster relative to the other control wounds, but this was not statistically significant. The lack of host tissue phototoxicity may have been because of the necessity for a macromolecular species such as pL-*c<sub>66</sub>* (molecular weight approximately 18500) to be taken up into mammalian cells by the time-dependent process of endocytosis. There have been some literature reports of PDT mediated by systemically administered PS causing inhibition of wound healing (28,29), whereas other reports showed no effect (30) or an acceleration (31). In the present experiments the absence of wound healing inhibition may be explained by a combination of the topical delivery method together with the large conjugate size and the relatively short incubation time. The fact that



**Figure 4.** Mean areas of wounds from six mice per group treated as described earlier. Wounds were measured daily in two dimensions, and areas were calculated. Bars are the SD.

treated wounds healed as well as control wounds suggests that PDT may have advantages over topical antimicrobial products that have been reported to cause tissue damage or have other undesirable side effects. Although silver nitrate and silver sulfadiazene are effective in destroying bacteria in wounds and burns (32), they have limited penetration into tissue (33), bacteria can develop resistance (34), there have been reports of local toxicity manifested by delayed wound healing (35) and silver may undergo systemic absorption (36) leading to argyria (37). Likewise, alternative topical products such as cerium nitrate, mafenide acetate, mupirocin and polymyxin B may have problems with resistant bacteria (38) or toxicity (39).

## CONCLUSIONS

The present study demonstrated for the first time, rapid eradication of bacteria infecting a wound in living mice, using targeted PDT, and showed the strength of an *in vivo* assay for infection in living animals. The fact that until now PDT of wound infections has not been explored may be attributed to inefficient PS, lack of selectivity for prokaryotic cells compared to eukaryotic cells or to difficulties inherent in monitoring the response of localized infections in small rodents. Further studies concerning our bacterial PDT approach *in vivo* are underway. These will include comparing loss of luminescence to reduction in cfu extracted from tissue, use of invasive strains of bacteria and lower initial bacterial inocula, use of more realistic animal infection models including full-thickness burns and studies on whether this treatment is capable of inactivating bacteria that have penetrated the tissue.

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