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Polycationic photosensitizer conjugates: effects of chain length and Gram classification on the photodynamic inactivation of bacteria

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***Objectives*: We have shown previously that a polycationic conjugate between poly-L-lysine and the photosensitizer chlorin$_{e6}$ was effective in photodynamic inactivation (PDI) of both Gram-positive and Gram-negative bacteria. In this report we explore the relationship between the size of the polylysine chain and its effectiveness for mediating the killing of Gram-negative and Gram-positive bacteria.**

***Methods*: Conjugates were prepared by attaching precisely one chlorin$_{e6}$ molecule to the α-amino group of poly-(ε-benzyloxycarbonyl)lysines of average length eight and 37 lysine residues, followed by deprotection of the ε-amino groups, and were characterized by iso-electric focusing. The uptake of these conjugates and free chlorin$_{e6}$ by Gram-positive *Staphylococcus aureus* (ATCC 27659) and Gram-negative *Escherichia coli* (ATCC 29181) after washing was measured as a function of photosensitizer concentration (0–4 μM chlorin$_{e6}$ equivalent) and incubation time. After incubation the bacteria were exposed to low fluences (10–40 J/cm^2) of 660 nm light delivered from a diode laser, and viability was assessed after serial dilutions by a colony-forming assay.**

***Results*: *S. aureus* and *E. coli* took up comparable amounts of the two conjugates, but free chlorin$_{e6}$ was only taken up by *S. aureus.* After illumination *S. aureus* was killed in a fluence-dependent fashion when loaded with the 8-lysine conjugate and free chlorin$_{e6}$ but somewhat less so with the 37-lysine conjugate. In contrast, PDI of *E. coli* was only effective with the 37-lysine conjugate at concentrations up to 4 μM. PDI using the 8-lysine conjugate and free chlorin$_{e6}$ on *E. coli* was observed at a concentration of 100 μM. Transmission electron micrographs showed internal electron-lucent areas consistent with chromosomal damage.**

***Conclusion*: These results can be explained by the necessity of a large polycation to penetrate the impermeable outer membrane of Gram-negative *E. coli*, while Gram-positive *S. aureus* is more easily penetrated by small molecules. However, because *S. aureus* is more sensitive overall than *E. coli* the 37-lysine conjugate can effectively kill both bacteria.**

Keywords: photodynamic therapy, polylysine, *Escherichia coli*, *Staphylococcus aureus*, electron microscopy

Introduction

Photodynamic therapy (PDT) is based on the concept that a non-toxic dye, known as a photosensitizer (PS), can be localized preferentially in certain tissues or cells, and subsequently activated by low doses of visible light of the appropriate wavelength to generate singlet oxygen and free radicals that are cytotoxic to target cells.[1] Microorganisms such as bacteria,[2] fungi,[3] yeasts[4] and viruses[5,6] can also be killed by visible light after their treatment with an appropriate PS.[7] Several studies have demonstrated that Gram-positive bacteria are particularly susceptible to photodynamic inactivation (PDI)[7–9] but Gram-negative bacteria are significantly resistant to many PSs used commonly in PDT of tumours.[10] It was then shown

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that PDI of Gram-negative bacteria could be effective if the PS used had a cationic charge,[11–13] or if the bacterial membrane was permeabilized using polymyxin nonapeptide[14] or Tris/EDTA[15] together with a non-cationic PS. Cationic phenothiazines such as methylene blue and toluidine blue O have also been used to photoinactivate bacteria *in vitro*[16] and in *ex vivo* samples.[17,18]

Many naturally occurring antibacterial peptides have been discovered that all have in common *inter alia* a pronounced polycationic charge.[19] This is thought to be the initial factor that allows them to bind to negatively charged bacteria and subsequently disturb the outer-membrane permeability barrier.[20] Antibacterial polycations include polymyxins,[21] protamine,[22] insect cecropins,[23] reptilian magainins,[24] various cationic leucocyte peptides (defensins,[25] bactenecins,[26] bactericidal/permeability-increasing protein[27]), polymers of basic amino acids[28] and polyethyleneimine.[29] However, the cationic character is not the sole determinant required for the permeabilizing activity, and therefore some of the agents are much more effective permeabilizers than others.

We showed previously[30] that a polycationic covalent conjugate between the PS chlorin$_{e6}$ (c_{e6}) and poly-L-lysine (pL) (average molecular weight 2 kDa, 20 lysine residues) effectively delivered PS to both Gram-positive and Gram-negative oral bacteria after a short incubation, and enabled their photodestruction after illumination with red light. The activity of the cationic pL-c_{e6} was many times higher than the neutral acetylated pL-c_{e6}-ac and anionic succinylated pL-c_{e6}-succ conjugates against both Gram-positive and Gram-negative bacteria. With the relatively short incubation times used in this study, mammalian epithelial cells were essentially unharmed by the treatment. Other workers subsequently used conjugates between porphycenes and pL to carry out PDI of both Gram-positive and Gram-negative bacterial species. In order for bacterial PDI to have any clinical application it is necessary to demonstrate that a single conjugate can kill both classes of bacteria, so that the method can be used without prior identification of the infectious agent.

This report tests the hypothesis that a certain length of polycationic chain is necessary to allow the PS to gain entry through the outer membrane of Gram-negative bacteria, while this large polycationic carrier may be unnecessary or even detrimental with Gram-positive bacteria. In order to produce conjugates with a more defined structure, a synthetic strategy was devised that enabled precisely one c_{e6} molecule to be attached to the pL chain.

Materials and methods

Preparation and characterization of polylysine-c_{e6} conjugates

All reactions were carried out in the dark at room temperature. Forty-seven milligrams (4.8 μmol) of poly-ε-(benzyloxycarbonyl)-DL-lysine [average molecular weight = 9700 (range = 5000–20 000), mean degree of polymerization = 37; Sigma Chemical Co., St Louis, MO, USA] were dissolved in 1 mL of dry dimethylsulphoxide (DMSO), to which 6.7 mg (11.2 μmol) of c_{e6} (Porphyrin Products, Logan, UT, USA) and 30 mg (157 μmol) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma) were added. Triethylamine (20 μL; Sigma) was then added and the mixture was stirred for 24 h. Likewise, 40 mg (20 μmol) of poly-ε-(benzyloxycarbonyl)-L-lysine [average molecular weight = 2000 (range = 1000–4000), mean degree of polymerization = 8] were reacted with 28 mg (47 μmol) of c_{e6} and 125 mg (654 μmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). 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. Trifluoroacetic acid (1 mL) was then added to the dry mixture and this solution was stirred for 2 h. Sodium acetate buffer (10 mM, pH = 5.5, 1 mL) and methanol (1 mL) were then added and the mixture was again evaporated to dryness under vacuum. The residue was dissolved in sodium acetate buffer (10 mM, pH = 5.5, 1 mL) and applied to a column of Sephadex G25 (60 × 1 cm) and eluted with sodium acetate buffer (10 mM, pH = 5.5) at a flow rate of 3.3 mL/h. Three millilitre fractions were collected and fractions 14–21 were combined and evaporated to give the product pL-c_{e6}. Concentrations of free c_{e6} and conjugates were measured routinely by absorption spectroscopy taking the extinction coefficient ε_{400nm} = 150 000. Isoelectric focusing (IEF) was carried out in agarose gels containing wide range ampholines (pI 3–12; Sigma) that were subjected to pre-focusing at 800 V for 30 min. Samples were then loaded followed by a desalting period of 150 V for 30 min and a focusing period at 1400 V for 60 min. c_{e6} was localized on the gel by fluorescence imaging using an excitation 400–440 nm bandpass filter, and an emission 580 nm longpass filter (ChemiImager 4000; Alpha Innotech Corp, San Leandro, CA, USA). pL was localized by Coomassie blue staining.

Bacteria

The bacteria used in this study were *Staphylococcus aureus* (ATCC 27659, penicillinase-positive, resistant to tetracycline, novobiocin, streptomycin and macrolide antibiotics) and *Escherichia coli* K12 (ATCC 29181, resistant to trimethoprim and streptomycin). The bacteria were grown in brain-heart infusion broth in an orbital shaker at 37°C for 18 h. An aliquot of this suspension was then added to nutrient broth and grown to mid-log phase (OD_{600} = 0.6, 10^8 cells/mL).

Uptake studies

Bacteria were used at a density of 10^9 cells/mL (prepared by concentrating a suspension of 10^8 cells/mL) in order to have sufficient material to measure the extracted c_{e6}. Bacteria were incubated in the dark at room temperature for 30 min with various concentrations of conjugates or free c_{e6} measured as μM c_{e6} equivalent (final concentration in nutrient broth). Experiments 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 of sterile phosphate-buffered saline (PBS) and centrifuged as above. Finally, the cell pellet was dissolved by digesting it in 1.5 mL of 0.1 M NaOH/1% SDS for at least 24 h to give a homogeneous solution. The fluorescence of the cell extract was measured on a spectrofluorimeter (model FluoroMax; SPEX Industries, Edison, NJ, USA). The excitation wavelength was 400 nm and the emission spectra of the cell suspensions were recorded from 580 to 700 nm. If necessary, the solution was diluted with 0.1 M NaOH/1% SDS to reach a concentration of c_{e6} where the fluorescence response was linear. Separate fluorescence calibration curves were constructed with known amounts of both conjugates and free c_{e6} dissolved in 0.1 M NaOH/1% SDS. The protein content of the entire cell extract was then determined by a modified Lowry method[31] using bovine serum albumin dissolved in 0.1 M NaOH/1% SDS to construct calibration curves. Results were expressed as nmol c_{e6}/mg cell protein.

PDI studies

Suspensions of bacteria (10^8/mL) were incubated in the dark at room temperature for 30 min with 0.5–4 μM c_{e6} equivalent of the two conjugates and free c_{e6} in nutrient broth as described above. Cell suspensions were centrifuged, cells were washed twice with sterile PBS and 1 mL of fresh nutrient broth was added. The bacterial suspensions (1 mL) were then placed in the wells of 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, USA) was coupled into a 1 mm optical fibre that delivered light into a lens that formed a uniform circular spot on the base of the 24-well plate 2 cm in diameter. Fluences ranged from 0 to 40 J/cm^2 at an irradiance of 50 mW/cm^2. 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, aliquots (100 μL) were taken from each well to determine colony-forming units (cfu). Care was taken to ensure that the contents of the wells were mixed thoroughly before sampling, as bacteria can settle at the bottom. The aliquots were serially diluted 10-fold in nutrient broth to give dilutions of 10^{-1}–10^{-6} times the original concentrations. Aliquots (10 μL) of each of the dilutions were streaked horizontally on square nutrient agar plates as described by Jett *et al*.[32] Plates were streaked in triplicate and incubated for 24 h at 37°C in the dark. In general, three dilutions could be counted on each plate. Controls were bacteria untreated with PS or light but kept in 24-well plates at room temperature covered with aluminium foil for the duration of the illumination, and bacteria exposed to light in the absence of PS.

Incubation time

A preliminary experiment was carried out in order to determine the effect of the incubation time on the efficiency of the PDI of bacteria. *S. aureus* and *E. coli* (10^8 cells/mL) were incubated with the 37-lysine conjugate (1 μM c_{e6} equivalent in nutrient broth) for times ranging from 1 to 30 min. Two aliquots were removed at each time point: one for the determination of PDT-mediated killing, and the other for the measurement of dark toxicity, both measured by colony-forming assay as described above.

Transmission electron microscopy

Control and conjugate-treated bacteria (both dark- and light-treated) were suspended in PBS. PDI conditions were 4 μM c_{e6} equivalent and 20 J/cm^2. The bacteria were pelleted, PBS decanted and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4°C. The bacterial pellets were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 min at 12 000**g**. The glutaraldehyde was decanted, the pellets rinsed twice in 0.1 M cacodylate buffer and the bacteria spun down again. After decanting the buffer, the bacteria were resuspended in 2% molten Bacto agar (Difco) and centrifuged immediately. The agar pellets containing bacteria were cut into 3 mm^3 pieces and processed for routine electron microscopy. The bacterial pellets were co-fixed in 2% OsO_4, dehydrated in alcohols and embedded in Epon 812. Thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a transmission electron microscope.

Results

Preparation of conjugates

Because Sigma offered two lengths of poly-(ε-benzyloxycarbonyl)-L-lysine with average chain lengths of eight and 37 lysines, it was decided to compare the effect of chain length on the photosensitizing effects of pL-c_{e6}. We decided to use EDC as the coupling agent with free c_{e6} in DMSO in an attempt to avoid problems of aggregate formation. The results of the synthesis were favourable in that conjugates were obtained that could be purified easily by column chromatography and more importantly did not show any time-dependent aggregation behaviour.

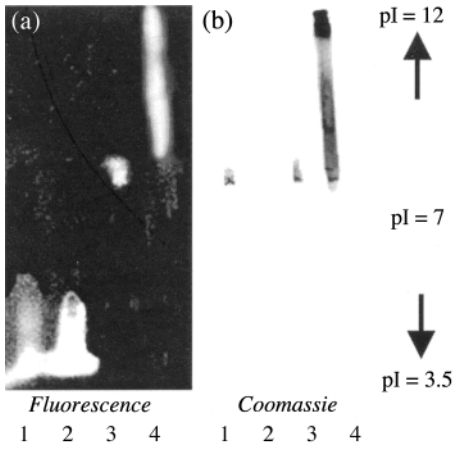

Figure 1. Agarose gel IEF (pI 3.5–12) visualized by (a) fluorescence imaging and (b) Coomassie blue staining. Lanes 1a and 1b: mixture of pL (8 Lys) and free c_{e6}; lanes 2a and 2b: free c_{e6}; lanes 3a and 3b: pL-c_{e6} (8 Lys); lanes 4a and 4b; pL-c_{e6} (37 Lys).

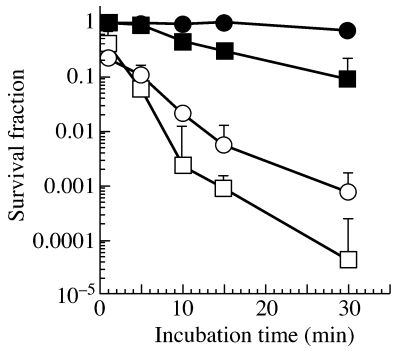

Figure 2. Effect of incubation time on phototoxicity and dark toxicity. Bacteria were incubated with pL-c_{e6} 37 Lys at 1 μM c_{e6} equivalent concentration for times ranging from 1 to 30 min in nutrient broth. They were then washed and resuspended in nutrient broth and either illuminated or not with 40 J/cm^2 660 nm light. Viability was determied by cfu assay on nutrient agar plates. All data points are expressed as survival fraction of untreated controls and are means of two separate experiments each containing three plates. Bars show S.D. Filled squares, *S. aureus* dark; empty squares, *S. aureus* light; filled circles, *E. coli* dark; empty circles, *E. coli* light.

The conjugates were characterized by IEF, taking advantage of their charged nature to effect separation, and the ability to localize c_{e6} independently by fluorescence imaging, and pL by Coomassie blue staining on the gel. Fluorescence and Coomassie images of the two conjugates and free c_{e6} are shown in Figure 1. The 37-lysine conjugate produced a streak that encompasses a higher range of pI values than the 8-lysine conjugate, while free c_{e6} had a low pI value of 3–4. The streaking of the 37-lysine conjugate is presumably due to the greater degree of polydispersity inherent in larger pL chains compared with small ones. In order to demonstrate that c_{e6} is attached covalently to the pL in the conjugates, we also loaded a mixture of pL (8 lysines) and free c_{e6} in lane 1. It can be seen that the c_{e6} fluorescence was well separated from the pL chain.

Effect of incubation time

In order to decide on an incubation time for subsequent experiments we investigated the effect of increasing the incubation time of the two bacteria with the 37-lysine conjugate at 2 μM c_{e6} equivalent in nutrient broth. On completion of incubations, the bacteria were either illuminated or kept in the dark, and the numbers of cfu were determined. Figure 2 shows that the survival fraction after illumination decreased fairly sharply with increasing incubation time for both species. *S. aureus* was significantly more sensitive than *E. coli* both in the light and the dark. Considering these data we decided to use an incubation time of 30 min for subsequent experiments rather than the time of 1 min used in our previous study.

Uptake studies

The uptake of c_{e6} from both the conjugates and unconjugated c_{e6} with increasing PS concentrations after 30 min incubation in nutrient broth is shown in Figure 3a (*S. aureus*) and Figure 3b (*E. coli*). The uptake of c_{e6} from the 8-lysine and 37-lysine conjugates was remarkably similar for both bacteria, and the uptake from the 8-lysine conjugate was significantly higher in both cases than the uptake from the 37-lysine conjugate. The most striking difference between the two species of bacteria was in the uptake of free c_{e6}, where the Gram-positive *S. aureus* took up more than 10 times as much as the Gram-negative *E. coli*. There appears to be a tendency for the uptake of all three species by *E. coli* to saturate with increasing concentration in the medium, as compared with the uptake by *S. aureus.*

Photodynamic inactivation

PDI of the two bacterial species was then carried out after 30 min incubation in nutrient broth with the three PSs at concentrations of 1, 2 and 4 μM. The illumination was also carried out in nutrient broth. The data are shown in Figure 4 (a–f). The survival fraction at 0 J/cm^2 represents the dark toxicity of the conjugate or PS. Under these conditions the limit of detection of the colony-forming assay was six logs of killing. *S. aureus* showed a clear PS dose- and light dose-dependent loss of cfu for all three PSs. The highest PS concentration of 4 μM in conjunction with 40 J/cm^2 of 660 nm light produced a near six log reduction of cfu in all cases. The lower concentrations of PS (1 and 2 μM) were less effective in the case of the 37-lysine conjugate compared with the killing observed with 1 and 2 μM concentrations of the 8-lysine conjugate and free c_{e6}. The effect of increasing the concentration of PS was less pronounced in the case of free c_{e6} (Figure 4c)

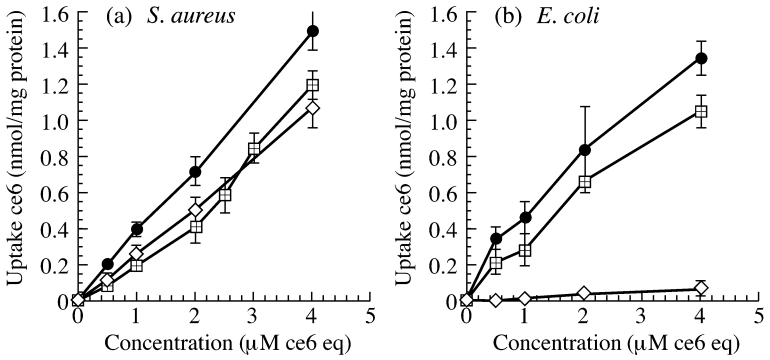

Figure 3. Uptake of c_{e6} by bacteria (a, *S. aureus*; b, *E. coli*) after incubation with conjugates or free PS. Bacteria were incubated with the indicated amount of conjugate or c_{e6} for 30 min in nutrient broth. They were then washed twice in PBS and the pellet was dissolved in 0.1 M NaOH/1% SDS, the fluorescence measured and c_{e6} content calculated from calibration curves. Cell protein was measured by a modified Lowry procedure. Values are means of triplicate determinations from two separate experiments. Bars show S.D. Squares, pL-c_{e6} 37; filled circles, pL-c_{e6} 8; diamonds, c_{e6}.

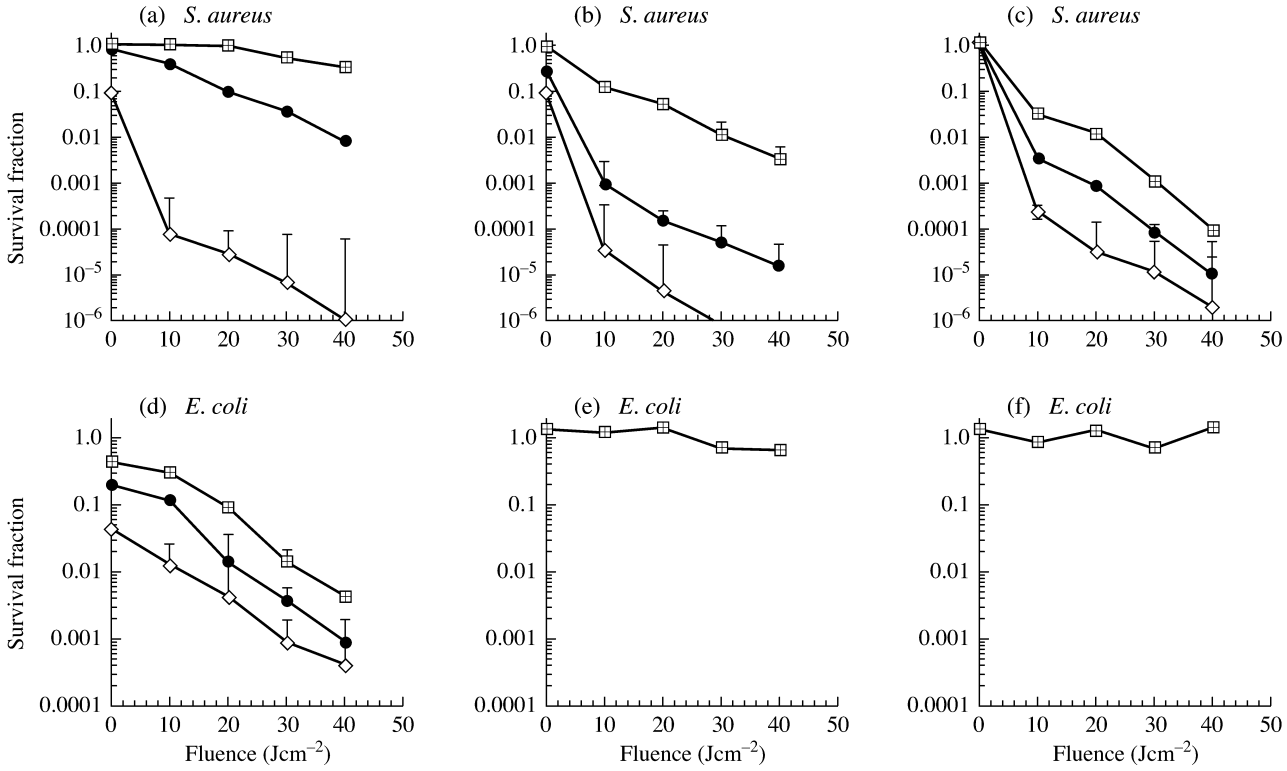

Figure 4. Bacteria were incubated with the indicated amount of pL-c_{e6} 37 Lys (a, *S. aureus*; d, *E. coli*), pL-c_{e6} 8 Lys (b, *S. aureus*; e, *E. coli*) or c_{e6} (c, *S. aureus*; f, *E. coli*) for 30 min in nutrient broth, washed twice with PBS, resuspended in nutrient broth and exposed to 660 nm light for indicated fluences at a fluence rate of 50 mW/cm^2. After serial dilutions they were plated on nutrient agar and colonies were counted 24 h later. Survival fractions were calculated from the number of cfu in the PDT-treated sample divided by the number of cfu in the sample treated with light but no PS. Data points at 0 J/cm^2 represent dark toxicity of the conjugate. Data points are means of counts from three plates for each fluence and from two independent experiments, and error bars show S.D. For parts (a) pL-c_{e6} 37, (b) pL-c_{e6} 8, (c) c_{e6} and (d) pL-c_{e6} 37: squares, 1 μM; circles, 2 μM; diamonds, 4 μM. For parts (e) pL-c_{e6} 8 and (f) c_{e6}: squares, 4 μM.

than the two conjugates (Figure 4a and b). There was a small amount of dark toxicity associated with the higher concentrations of the conjugates but none with free c_{e6}. The results were completely different for *E. coli*. The only phototoxicity observed was with the 37-lysine conjugate (Figure 4d), which showed a PS dose- and light dose-dependent loss of cfu up to three logs. A dose-dependent dark toxicity was observed with survival fractions of 0.43, 0.2 and 0.04 for 1, 2 and 4 μM concentrations. Only the 4 μM concentrations of the 8-lysine conjugate and free c_{e6} are shown in Figure 4 (e and f) as the lower concentrations had no effect on bacterial survival in either the light or dark.

Increasing concentrations of pL-c_{e6} 8 and c_{e6} with E. coli

As mentioned earlier, there was no apparent phototoxicity of either pL-c_{e6} or free c_{e6} with *E. coli*, despite the fact that *E. coli* took up more pL-c_{e6} 8 than pL-c_{e6} 37. The absence of phototoxicity by free c_{e6} may be explained by the low uptake (less than one-tenth of the value from both the pL-c_{e6} conjugates). It was, therefore, of interest to increase the concentrations of pL-c_{e6} 8 and free c_{e6} substantially with *E. coli* to try to resolve these anomalies. Figure 5a shows the uptake of c_{e6} by the cells after incubation with concentrations up to 100 μM of both pL-c_{e6} 8 and free c_{e6} and Figure 5 (b and c) shows the resultant phototoxicity. Both pL-c_{e6} 8 and free c_{e6} were taken up in a linear fashion with increased concentration, and the ratio of c_{e6} in cells incubated with both species was similar at 100 μM to that pertaining at 4 μM. When the phototoxicity was studied, it could be seen that, although 100 μM pL-c_{e6} 8 killed up to two logs of bacteria, the increase was much more pronounced in the case of free c_{e6}, with 20 μM killing two logs and 100 μM killing four logs.

Relative phototoxicity

By defining the phototoxicity as the inverse of the survival fraction and then dividing this by the uptake of c_{e6} in nmol/mg cell protein and plotting against light fluence, it is possible to depict graphically and compare the efficiency of the different species for PDI of the bacteria in terms of how much killing is accomplished by each molecule of c_{e6} taken up by the cells. Figure 6b shows that the relative phototoxicity of the 37-lysine conjugate and free c_{e6} both incubated at 4 μM was identical for *S. aureus* while that of the 8-lysine conjugate at 4 μM was *c.* 10-fold higher. For *E. coli*, since only the 37-lysine conjugate showed phototoxicity at 4 μM, we also plotted the values obtained from free c_{e6} and pL-c_{e6} 8 at 100 μM. Figure 6a shows that pL-c_{e6} 8 had very low values at both concentrations, while the values for free c_{e6} showed a remarkable (≤100 times) rise from 4 to 100 μM. Indeed, the values from free c_{e6} at 100 μM were somewhat higher than pL-c_{e6} 37 at 4 μM.

Electron micrographs

Figure 7 shows the morphology of *S. aureus* (Figure 7a–e) and *E. coli* (Figure 7f–j) under control conditions, and after treatment with the two conjugates in the dark and exposed to 20 J/cm^2 light. *S. aureus* showed similar internal areas that were electron lucent in cells treated with pL-c_{e6} 37 Lys and light (Figure 7c) and pL-c_{e6} 8 Lys and light (Figure 7e), which were not seen in control cells or cells treated with conjugates in the dark (Figure 7b and d). In contrast, *E. coli* only showed these internal cavities after treatment with pL-c_{e6} 37 Lys and light (Figure 7h). These electron-lucent areas may be explained by membrane damage leading to chromosomal alteration and DNA condensation. They bear a remarkable resemblance to those transmission electron micrographs presented by Nitzan *et al.*,[14] who used a mixture of polymyxin nonapeptide and deuteroporphyrin to photoinactivate *E. coli* and *Pseudomonas aeruginosa*.

Discussion

It has been proposed that effective PDI of Gram-negative bacteria requires either a PS with a pronounced cationic charge[13] or the use of a method of increasing the permeability of the outer membrane (such as a combination of a lipophilic or anionic PS with a polycationic peptide).[14] Our approach to carrying out effective PDI of bacteria involves covalently linking the PS to a polycationic molecule to produce a conjugate that will bind to and photoinactivate both Gram-positive and Gram-negative species. We showed previously[30] that c_{e6} attached to a pL chain of 20 amino acids to give a conjugate with an approximate 1:1 substitution ratio was effective in

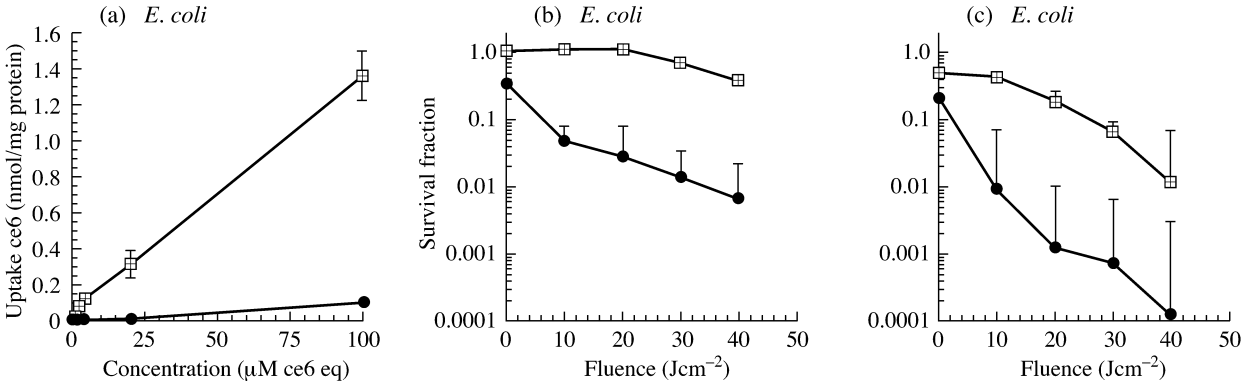

Figure 5. Uptake and phototoxicity of pL-c_{e6} 8 and free c_{e6} after incubation at concentrations up to 100 μM by *E. coli.* (a) Uptake was measured as described in Figure 3 after incubation for 30 min in nutrient broth. Bacteria were then illuminated and the survival fractions determined, as described for Figure 4, are shown for pL-c_{e6} (b) and for free c_{e6} (c). (a) Squares, pL-c_{e6} 8; circles, c_{e6}. (b) Squares, pL-c_{e6} 8 at 20 μM; circles, pL-c_{e6} 8 at 100 μM. (c) Squares, c_{e6} 20 μM; circles, c_{e6} 100 μM.

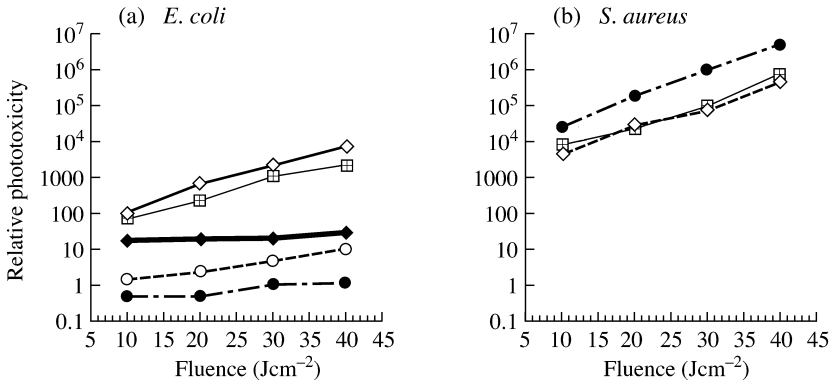

Figure 6. Relative phototoxicity, i.e. reciprocal of survival fraction divided by cellular uptake in nmol c_{e6}/mg protein plotted as a function of light fluence. (a) *E. coli* incubated with pL-c_{e6} 37 Lys at 4 μM (squares), pL-c_{e6} 8 Lys at 4 (full circles) and 100 μM (empty circles), and free c_{e6} at 4 (full diamonds) and 100 μM (empty diamonds) concentrations. (b) *S. aureus* incubated with pL-c_{e6} 37 Lys (squares), pL-c_{e6} 8 Lys (circles) and free c_{e6} (diamonds) all at 4 μM.

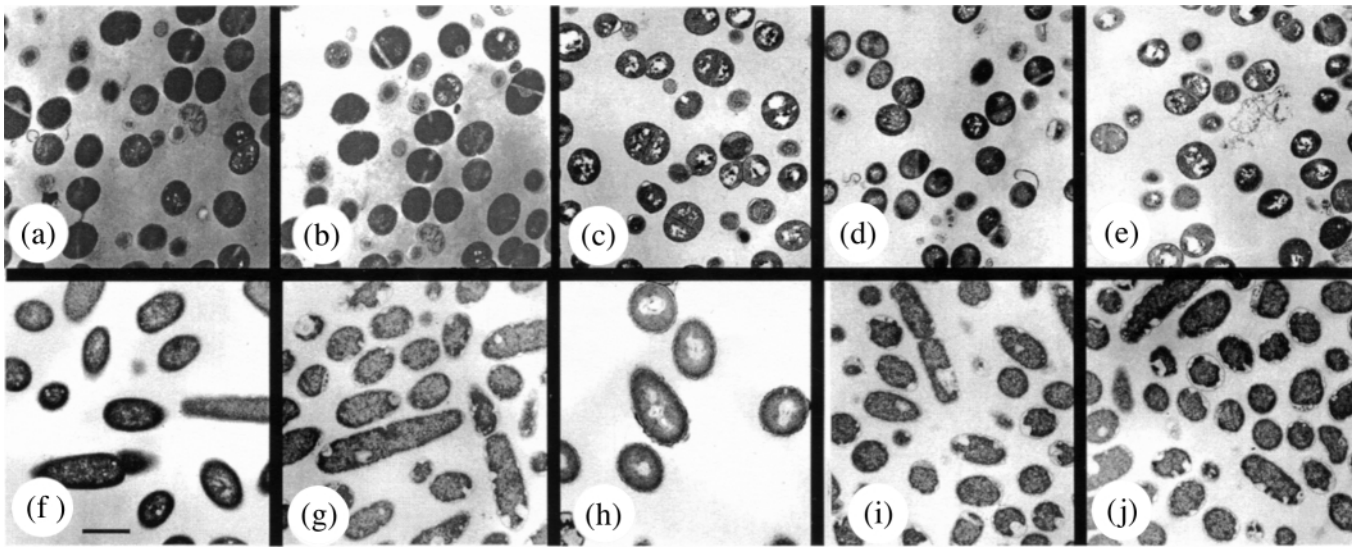

Figure 7. Transmission electron micrographs. Bacteria were incubated with the conjugates or free c_{e6} at 4 μM concentration and illuminated with 20 J/cm^2 light and then processed as described. *S. aureus* control cells (a), treated with pL-c_{e6} 37 Lys in the dark (b), and in the light (c), and with pL-c_{e6} 8 Lys in the dark (d) and light (e). *E. coli* control cells (f, bar = 1 μm), treated with pL-c_{e6} 37 Lys in the dark (g), and in the light (h), and with pL-c_{e6} 8 Lys in the dark (i) and light (j).

PDI of both the Gram-positive *Actinomyces viscosus* and the Gram-negative *Porphyromonas gingivalis*. The conjugate was more effective than an equivalent mixture of pL and c_{e6}, and the efficacy was lost if the conjugate was rendered neutral by polyacetylation, or rendered anionic by polysuccinylation. The present study has examined conjugates with different degrees of polycationic character, and asked whether they would achieve PDI of Gram-negative species, and compared their effectiveness in killing Gram-positive species.

In our previous publications [30,33] we described a synthetic scheme to give pL-c_{e6} conjugates using dicyclohexylcarbodiimide to prepare an *N*-hydroxysuccinimide ester from c_{e6} 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) that made purification and storage difficult. In an attempt to avoid this aggregation problem we formed the hypothesis that it might be caused by the structure of the pL-c_{e6} having c_{e6} substitution at random points throughout the length of the pL chain. To test this hypothesis we devised a synthetic scheme using a fully ε-amino-protected pL chain that could therefore only react on the single α-amino group. Not only would this allow substitution of c_{e6} on the end of the chain instead of throughout its length, but it would also give chains with only one c_{e6} molecule per pL molecule. This would eliminate the uncertainty caused by random substitution, whereby although one could have an average of one c_{e6} molecule per chain it is only an average value and some chains could have two while others have none. This method allowed the conjugates to be readily purified using Sephadex

chromatography, and they could be characterized using IEF on agarose gels. The 37-lysine conjugate appeared to have a higher pI range than the 8-lysine conjugate, thus providing evidence of its greater polycationic character.

The results of the present study imply that, in the case of polycationic PS conjugates, it is necessary for the PS to gain access through the outer membrane permeability barrier to more sensitive parts of the cell. The efficiency with which this occurs depends on the size of the polycationic chain. All three species (conjugates with eight and 37 lysines and free c_{e6}) efficiently inactivated *S. aureus* while only the 37-lysine conjugate killed *E. coli*. The binding of the 8-lysine conjugate to *E. coli*, however, was higher than that of the 37-lysine conjugate, implying that the 8-lysine conjugate was located at a non-sensitive site. The most obvious explanation of our findings is that the 37-lysine conjugate was able to interact with the outer membrane structure of *E. coli*, perhaps causing loss of some lipopolysaccharide (LPS) and rendering the remaining LPS more permeable, thus allowing the conjugate to penetrate through the outer membrane to the periplasmic space and to the cytoplasmic membrane beyond. The 8-lysine conjugate, presumably, did not have sufficient polycationic character to accomplish this and, although its overall uptake was higher, the reactive species produced upon illumination were unable to cause lethal damage. In the case of *S. aureus* it is generally accepted that the peptidoglycan outer layer has much higher permeability[34] than the Gram-negative outer envelope, and, although it can carry out 'molecular sieving' for relatively large molecules (up to 50 kDa),[35] it presents little barrier to the diffusion of smaller molecules into the periplasmic space. It is possible that the higher uptake and phototoxicity of the 8-lysine conjugate compared with the 37-lysine conjugate could be explained by a molecular-sieving effect that restricts uptake of the larger molecule.

Poly-L-lysines are among the polycations that bind to the anionic sites of LPS. This binding may weaken the intermolecular interactions of the LPS constituents, disorganize the structure and render it permeable to drugs[20] by enabling them to cross the outer membrane. Hancock *et al.*[36] coined the term 'self-promoted uptake' to describe the uptake of cationic peptides across outer membranes of Gram-negative bacteria. The first step is the interaction of polycations with divalent cation-binding sites on the cell surface, and since these peptides have much higher affinities for LPS compared with the native divalent cations Ca^{2+} and Mg^{2+}, they competitively displace these ions and, being so bulky, disrupt the normal barrier property of the outer membrane. The affected membrane is thought to develop transient 'cracks' that permit the passage of a variety of molecules, including hydrophobic compounds and small proteins and/or antimicrobial compounds, and, more importantly, promote the uptake of the perturbing peptide.[37]

Vaara[38] studied the permeabilizing effect on *P. aeruginosa* of pL chains 3, 4 and 5 lysines in length. Only the 5-lysine chain permeabilized *P. aeruginosa* but not other Gram-negative species (*E. coli* or *Salmonella typhimurium*), and only in low salt and low Mg^{2+} ion buffers. This was explained by a very weak binding of the 5-lysine chain to the LPS, which could be competitively reversed by Na^+ ions as well as Mg^{2+} ions. The effect of a pL with 20 lysine residues on *S. typhimurium* with smooth LPS after a short treatment (10 min) was a rapid release of 20–30% of the LPS from the outer membrane and the subsequent sensitization of the bacteria to the anionic detergent sodium dodecyl sulphate.[39] The same authors also showed that 20-lysine sensitized smooth *E. coli* and *S. typhimurium* strains to hydrophobic antibiotics by a factor of ≥100.[40] In both studies the polymer was not found to be bactericidal.

Our results on the effect of conjugate structure on the PDI efficiency are in accordance with many other reports concerning the use of PDI to kill Gram-negative bacteria. It has been found that the efficacy of a PS in sensitizing Gram-negative bacteria to PDI is related to the charge on the PS itself.[11,13] Meso-substituted cationic porphyrins were efficient PSs of Gram-negative bacteria such as *Vibrio anguillarum* and *E. coli* after incubation for 5 min.[11] In another study, the authors suggested that it was the positive charge that promoted the binding of the porphyrin to the outer membrane, inducing a limited damage that favoured the penetration of the PS.[12] They also showed that the photosensitizing activity of cationic porphyrins toward Gram-negative bacteria was inhibited by their incorporation into liposomes.[12] Gram-negative bacteria *E. coli* and *P. aeruginosa* could be photoinactivated when illuminated in the presence of a cationic water-soluble zinc pyridinium phthalocyanine (PPC) for 30 min but not by illumination in the presence of a neutral tetra-diethanolamine phthalocyanine or a negatively charged tetra-sulphonated phthalocyanine.[13] These workers showed recently[41] that incubation of *E. coli* cells with a cationic phthalocyanine in the dark caused alterations in the outer membrane permeability barrier and increased the uptake of hydrophobic compounds, with little effect seen with hydrophilic compounds. Addition of Mg^{2+} to the medium before incubation of the cells with the PS prevented these alterations in the outer membrane permeability barrier and also prevented the PDI of *E. coli*. PDI of Gram-negative species has been achieved using non-cationic PS in the presence of membrane-disorganizing polycations such as polymyxin B nonapeptide[42] or Tris-EDTA, which is also known to disorganize the outer membrane permeability barrier.[15]

However, there are other reports of PDI of Gram-negative bacteria in which it is clear that the PS does not have to penetrate the bacterium to be effective, or indeed even come into contact with the cells.[43–46] According to these reports, if singlet oxygen can be generated in sufficient quantities near

to the bacterial outer membrane it will be able to diffuse into the cell to inflict damage on vital structures.[43–46] In one set of studies the bacteria were separated from the PS 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. 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.[44] In another study, the PS rose bengal was bound covalently to small polystyrene beads thus preventing the dye from penetrating the bacteria.[45] Other workers have bound PS covalently to monoclonal antibodies that recognize and bind to cell surface antigens expressed on *P. aeruginosa*, and demonstrated specific killing after illumination.[46] It is very unlikely that covalent antibody-bound PS could penetrate the outer membrane.

How can these two conflicting sets of findings on the necessity for PS penetration into Gram-negative bacteria be reconciled? One possibility is that singlet oxygen can indeed diffuse into bacteria, producing fatal damage if it is produced at the outer surface or in solution in close proximity. The diffusion distance of singlet oxygen in solution has been estimated to be *c.* 50 nm,[47] and that would indeed allow the molecule to diffuse across the outer membrane to the cytoplasmic membrane. The failure of the 8-lysine conjugate (which had the highest binding to *E. coli*) to produce any killing must mean that the reactive species produced on illumination were unable to diffuse inwardly to sensitive sites on the cytoplasmic membrane. The fact that this conjugate was the best at killing *S. aureus* shows that the molecule has not undergone any conformational change that has rendered the PS no longer photoactive. This is presumptive evidence that the reactive species responsible for cell death in the case of Gram-negative species is not singlet oxygen produced by the Type II photoprocess, but rather free radicals and electron transfer processes typical of the Type I photoprocess. In agreement with this hypothesis is a recent report[48] in which the PDI of a range of oral pathogenic bacteria was studied using c_{e6} conjugated to a pL chain with five lysines. It was found that this conjugate efficiently killed a range of Gram-positive species and several Gram-negative anaerobes in the presence of 2.5 mM EDTA and 662 nm light. However, there may be considerable differences between anaerobic and aerobic species in this respect,[49] with anaerobes such as *P. gingivalis* showing sensitivity to both atmospheric oxygen and exogenous reactive oxygen species.[50] After completion of the experiments described in the present work, a report by Polo *et al.*[51] appeared reporting similar results. These workers used pLs with two sizes (either 1–4 kDa or 15–30 kDa mean molecular weights) conjugated to porphycene PS and tested their PDI efficacy against *E. coli* and *S. aureus.* They found that 1 μM PS equivalent of both conjugates and 27 J/cm^2 white light killed *S. aureus* efficiently, but only the large pL conjugate killed *E. coli.* The concentration of the small pL conjugate needed to be raised to 10 μM PS equivalent to kill *E. coli.* They did not examine the uptake of the conjugates by the bacteria and left the conjugates in the bacterial suspension during illumination.

Most reports that discuss the PDI of bacteria propose that the lethal event is damage to the cytoplasmic membrane.[52] This damage has been shown to allow vital constituents to leak out into the medium.[53] Although damage to DNA has been shown to occur after PDI this is not thought to be the main cause of cell death.[54] *Deinococcus radiodurans,* which has highly efficient DNA repair mechanisms, was still found to be susceptible to PDI.[52,55] In agreement with these reports, our data from transmission electron microscopy show that bacterial killing coincides with the appearance of cavities within the cytoplasm, presumably caused by a combination of damage to the membrane and chromosomal changes including DNA condensation.

We have shown previously that both epithelial and endothelial cells accumulate sufficient c_{e6} from charged pL conjugates to enable efficient photoinactivation to take place.[33] However, in mammalian cells this process of uptake is time dependent due to the necessity of these macromolecules being actively internalized via endocytosis. Furthermore, the sparing of HCPC-1 cells in that study under conditions that killed both Gram-positive and Gram-negative bacteria was probably due to the short incubation time.

We have carried out preliminary studies using cationic pL-c_{e6} conjugates and red light to treat animal models of infected wounds.[56] Although it was necessary to use higher concentrations of PS and higher light doses than were required *in vitro*, it was possible to obtain several logs of bacterial killing *in vivo*.[56] Experiments are underway to define further the utility of this method of PDI of pathogenic bacteria as a means of treating wound and burn infections.

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