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Journal of Photochemistry and Photobiology A: Chemistry 142 (2001) 145–150

Journal of Photochemistry and Photobiology A:Chemistry

www.elsevier.com/locate/jphotochem

The photosensitisation of *Escherichia coli* using disulphonated aluminium phthalocyanine

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Abstract

The lethal photosensitisation of the Gram-negative bacterium *Escherichia coli* using the anionic photosensitiser disulphonated aluminium phthalocyanine ($AlPcS_2$) and red laser or LED light is investigated. Disulphonated aluminium phthalocyanine can reduce the cell viability of *E. coli* but cannot achieve complete inactivation under the experimental conditions used. The greatest reduction in the viable count of *E. coli* is achieved by exposure to fractionated LED light (energy dose, 2.4 J; energy density, 4.0 mW cm^{-2}; fractionated, illumination time, 2 min; dark period, 2 min) in the presence of 35 μg ml^{-1} $AlPcS_2$ (86% reduction). High bactericidal activity is also observed when *E. coli* is exposed to red laser light (energy dose, 4.5 J; energy density, 30.0 mW cm^{-2}) in the presence of 27.5 μg ml^{-1} $AlPcS_2$ (72% reduction). No significant reductions in viability are obtained when bacterial suspensions are exposed to the same light doses in the absence of sensitiser.

Keywords: Gram-negative bacteria; Photodynamic therapy

1. Introduction

Photodynamic therapy has proved to be an effective way of killing tumour cells in the treatment of cancer. In recent years the light-induced inactivation of bacteria has been investigated. Phthalocyanines have been studied regarding their bactericidal effect [1,2]. Porphyrins have also been shown to be effective sensitisers for the inactivation of microbes [3–7]. Many studies have been completed using toluidine blue and $AlPcS_2$ as sensitisers to kill the microbes that are responsible for tooth decay and periodontal disease [8–11]. As bacteria are becoming increasingly resistant to antibiotics there is a need to develop new eradication techniques. The advantage of PDT as a method of killing bacteria is that because $^1O_2$ and free radicals are the species that mediate cell inactivation, it is highly unlikely that bacteria could develop a resistance to photodynamic action.

The overall electronic charge of a photosensitiser effects its PDT efficacy as this factor determines whether the dye is taken up by the cell and which cellular sites are available for the adsorption of the dye. Earlier studies have concluded that neutrally charged and anionic photosensitisers are not able to inactivate Gram-negative bacteria because these sensitisers are not taken up by the cells [5,6]. However, cationic photosensitisers have been shown to cause the successful inactivation of Gram-negative bacteria [2,12]. The main physiological difference between Gram-negative and Gram-positive bacteria is that the cell wall of a Gram-negative bacterium has an outer membrane outside of the peptidoglycan layer. The outer membrane provides a strong permeability barrier, for example many antibiotics that are effective against Gram-positive bacteria are ineffective against Gram-negative bacteria. In addition, Gram-negative bacteria have a negatively charged outer surface (due to the negative charges on the lipopolysaccharides of the outer membrane) which is thought to prevent adsorption and/or uptake of anionic dyes.

The photosensitisation of *Escherichia coli* using the sensitisers ZnPc (neutral), $ZnPcS_1$ and $ZnPcS_2$ (anionic) is unsuccessful unless the bacterial cells are pre-treated. *E. coli* can be inactivated using these sensitisers if the bacterial cells are pre-treated with a permeabilising agent such as EDTA (which induces the release of up to 50% of the lipopolysaccharide of the outer membrane), prior to photosensitisation [13]. Therefore, it is assumed that in order to inactivate Gram-negative bacteria using anionic or neutrally charged sensitisers, the outer membrane of the bacterial cells must first be compromised.

Abbreviations: $AlPcS_2$, disulphonated aluminium phthalocyanine; TSB, tryptone soya broth; cfu, colony-forming units; PBS, phosphate buffered saline; LED, light emitting diode; PDT, photodynamic therapy; WC, Wilkins–Chalgren

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1010-6030/01/$ – see front matter
PII: S1010-6030(01)00508-1

A particularly interesting recent result shows that against expectation $AlPcS_2$ (an anionic sensitiser) is capable of causing the complete inactivation of the Gram-negative bacterium *Porphyromonas gingivalis* ([$AlPcS_2$], 25 μg ml^{-1}; laser light at 660 nm, 11 mW; energy dose, 3.3 J; 100% reduction in viability) [8,11]. Therefore, it is now important to study $AlPcS_2$ with other Gram-negative bacteria, such as *E. coli*.

A number of studies have suggested that the efficacy of the PDT of tumour cells may be enhanced by fractionation of the light source [14–16]. It is believed that the effect of fractionating the light source on the photosensitisation of bacterial cells has not been investigated previously.

2. Materials and methods

Disulphonated aluminium phthalocyanine was prepared according to the method of Ambroz et al. [17]. The $AlPcS_2$ synthesised by this procedure consists predominantly of the α,α-*cis*-isomer.

The laser light source used was provided by a frequency-doubled mode-locked Nd:YAG (Coherent, Antares 76-s) laser synchronously pumping a cavity dumped dye laser (Coherent /590-03/7220) with a gain medium of DCM. For the photoinactivation experiments, laser light at 670 nm was used (power, 15 or 30 mW at 3.8 MHz). The LED light source used emitted between 635 and 685 nm (power, 2 mW, continuous light source). The irradiating light was focused so that the whole experimental well of the micro-well plate was illuminated (one well has ca. 0.80 cm diameter, 0.50 cm^2 surface area). Therefore, the energy density of the LED was 4.0 mW cm^{-2} at the sample and the energy densities of the laser light were 30 or 60 mW cm^{-2} (at the sample) depending on the intensity used.

E. coli (strain AB1157) was maintained tri-weekly on WC blood agar plates. For experimental purposes, the bacteria were grown aerobically in TSB to the mid-log phase (2 h) at 37°C. The bacteria were then harvested by centrifugation (3000 × *g* for 5 min) and re-suspended in 0.85% (w/v) saline so that experimental aliquots (100 μl) contained ca. 1.25×10^6 cfu. The aliquots were transferred to individual wells of a 96 micro-well plate and an equal volume of a filter-sterilised solution of $AlPcS_2$ in PBS was added to give final concentrations of 14–55 μg ml^{-1}. All of the samples were incubated at 37°C in the dark for a pre-irradiation time of 20 min. A 4 mm magnetic stirrer bar was placed into each well, the plates were placed on a magnetic stirrer and individual wells irradiated for varying times (1–20 min) (L+S+).

Control wells containing saline in place of $AlPcS_2$ were treated in an identical manner to determine the effect of irradiation alone on bacterial viability (L+S−). In order to evaluate the anti-microbial activity of $AlPcS_2$ alone, a duplicate experimental sample was prepared but not irradiated (L−S+). Finally, control wells containing the bacterial suspension and saline were used to determine the number of cfu in the original samples (L−S−).

Following irradiation of the appropriate wells, serial 10-fold dilutions of the contents of each well were prepared in sterile TSB (to 10^{-6} of the original concentration), and 6×20 μl aliquots of each dilution were spread on to the surfaces of WC blood agar plates. After overnight incubation at 37°C, the number of colonies were counted and the mean determined.

The effects of varying both the laser or LED exposure time and the concentration of $AlPcS_2$ were evaluated. The effect of fractionating the LED light source was also investigated.

3. Results

No significant bactericidal activity is observed for any set of data when the *E. coli* cell suspensions are exposed to either the light source (L+S−) or the sensitiser (L−S+) alone.

3.1. Photosensitisation using an LED as the light source

The effect of varying the exposure time to the LED (635–685 nm) light on the viability of *E. coli* in the presence of $AlPcS_2$ at a concentration of 14 μg ml^{-1} is shown in Fig. 1. Irradiation for 5 min (energy dose, 0.6 J) causes a 0.47 $\log_{10}$ (66%) reduction in the viable count. Increasing the energy dose produces a slightly larger bactericidal activity; a 0.62 $\log_{10}$ (76%) reduction is achieved at an energy dose of 1.8 J.

Exposure of *E. coli* to light from the LED for 20 min (energy dose, 2.4 J) in the presence of $AlPcS_2$ at a concentration of 14 μg ml^{-1} results in a 0.56 $\log_{10}$ (73%) reduction in the viable count (see Fig. 2). Increasing the concentration of $AlPcS_2$ to 35 and 55.5 μg ml^{-1} does not cause an increase in the bactericidal activity in either case.

The effect of fractionating the LED light on cell viability is shown in Fig. 3. Irradiation of *E. coli* by light from the LED for 20 min (energy dose, 2.4 J) at a $AlPcS_2$ concentration of 35 μg ml^{-1} produces a 0.31 $\log_{10}$ (51%) reduction in cell viability. When the same energy dose of 2.4 J is given using fractionated light, a 0.86 $\log_{10}$ (86%) reduction in the viable count is obtained.

3.2. Photosensitisation using a laser light source

The effect of varying the exposure time to the laser light on the viability of *E. coli* in the presence of $AlPcS_2$ at a concentration of 27.5 μg ml^{-1} is shown in Fig. 4. Irradiation for 1.5 min (energy dose, 1.35 J; energy density, 30 mW cm^{-2}) causes a 0.32 $\log_{10}$ (53%) reduction in the viable count. Increasing the energy dose results in a greater bactericidal effect; reductions in the viable cell count of 0.45 $\log_{10}$ (65%) and 0.55 $\log_{10}$ (72%) are achieved at energy doses of 2.25 and 4.5 J, respectively.

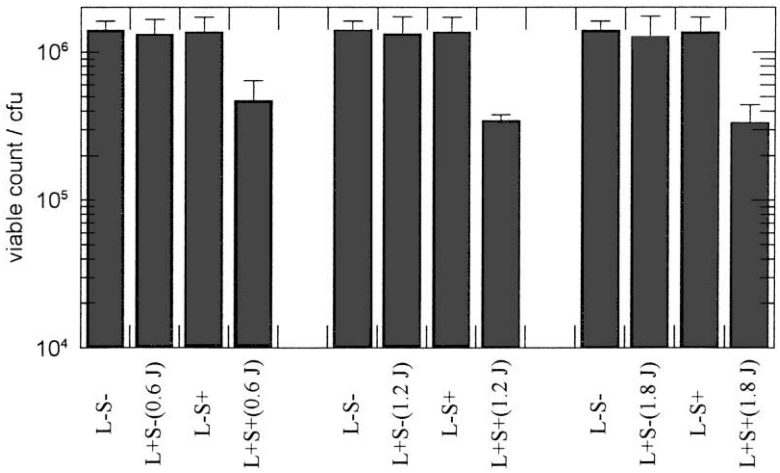

Fig. 1. The effect of varying the LED light exposure time on *E. coli* viability ([$AlPcS_2$] = 14 μg ml^{-1}, LED power = 4 mW cm^{-2}). (L+S+) denotes exposure to light in the presence of sensitiser; (L−S−) denotes no exposure to light or sensitiser (untreated control); (L+S−) denotes exposure to light in the absence of sensitiser; (L−S+) denotes exposure to sensitiser in the absence of light.

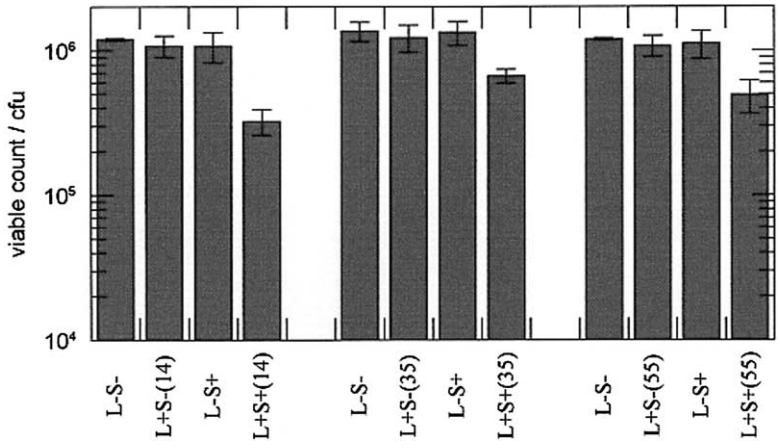

Fig. 2. The effect of varying the concentration of $AlPcS_2$ on *E. coli* viability. $AlPcS_2$ concentrations given in μg ml^{-1}. (LED energy dosage = 2.4 J).

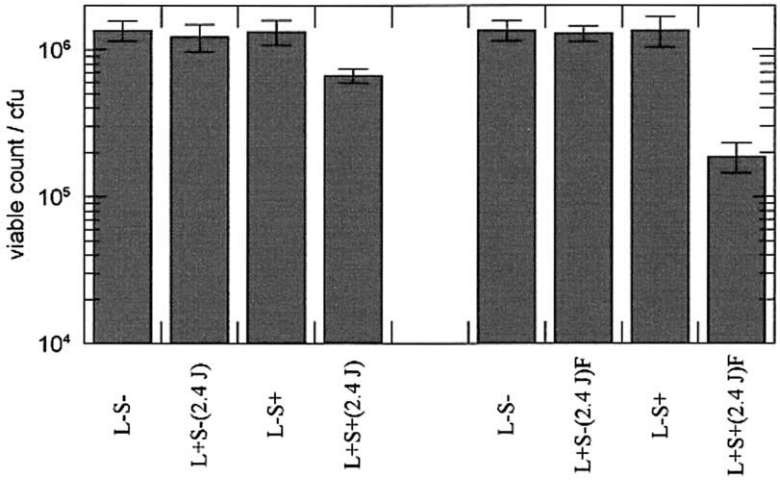

Fig. 3. The effect of fractionating the LED light on *E. coli* viability ([$AlPcS_2$] = 35 μg ml^{-1}, LED power = 4 mW cm^{-2}). 'F' denotes the fractionated light sample (LED 'on' for 2 min and then 'off' for 2 min cyclically).

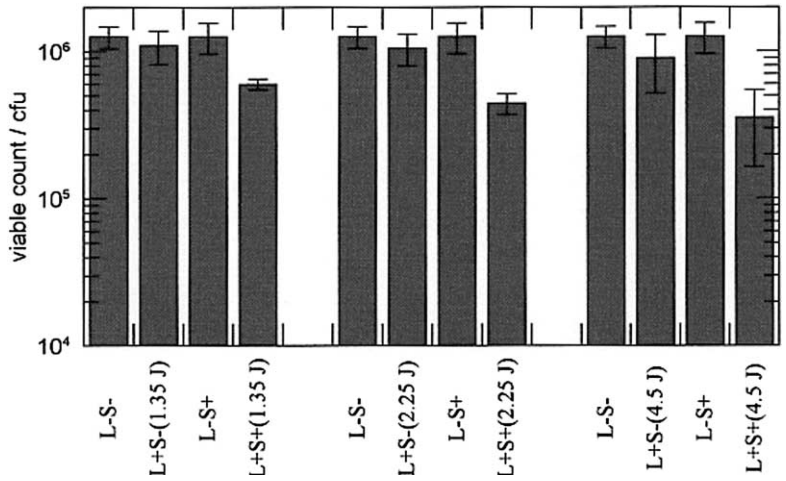

Fig. 4. The effect of varying the laser light exposure time on *E. coli* viability ([$AlPcS_2$] = 27.5 μg ml^{-1}, laser power = 30 mW cm^{-2}).

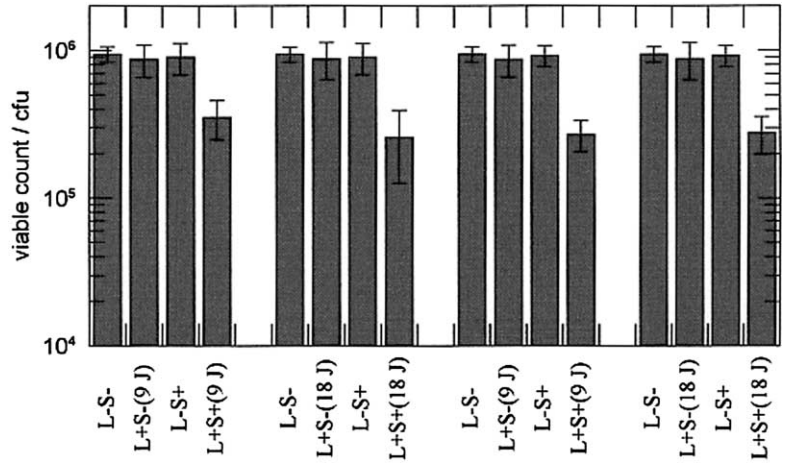

Fig. 5. The effect of varying the laser light exposure time on *E. coli* viability. First two data series (left hand side), [$AlPcS_2$] = 35 μg ml^{-1}, and the last two data series, [$AlPcS_2$] = 27.5 μg ml^{-1}, laser power = 60 mW cm^{-2}.

The effect of varying the exposure time to the laser (energy density, 60 mW cm^{-2}) on cell viability is shown in Fig. 5. Irradiation for 5 min (energy dose, 9 J) produces 0.54 $\log_{10}$ (71%) and 0.43 $\log_{10}$ (62%) reductions at $AlPcS_2$ concentrations of 27.5 and 35 μg ml^{-1}, respectively. On increasing the exposure time to 10 min (energy dose, 18 J) no increase in the bactericidal activity is observed for either of the $AlPcS_2$ concentrations used.

4. Discussion

In all of the photosensitisation experiments carried out in the present study, large reductions in the viability of *E. coli* are obtained although none of the experimental conditions used achieves complete inactivation of the bacterium (i.e. 100% reduction in cell viability). The greatest reduction in the viable count of *E. coli* (86%) is caused by exposure to fractionated LED light in the presence of 35 μg ml^{-1} $AlPcS_2$. High bactericidal activity is also observed when *E. coli* is exposed to laser light (energy density, 30 mW cm^{-2}) using 27.5 μg ml^{-1} $AlPcS_2$ (a 72% reduction in the viable cell count is achieved). The lower intensity light sources employed in the present work are more effective than the high power laser light source used.

Recently, the photobleaching of $AlPcS_2$ in the presence of *E. coli* has been investigated (paper in preparation). Spectral data shows that $AlPcS_2$ molecules are highly aggregated in the presence of *E. coli* cells (data not shown). The photobleaching of $AlPcS_2$ in this cellular environment results in a loss of the photoactive $AlPcS_2$ monomer species. If this decrease in the concentration of the $AlPcS_2$ monomer is prevented, higher levels of cell inactivation will be obtained.

In the experiments carried out to determine the effect of varying exposure times on the bacterial activity of $AlPcS_2$ with *E. coli*, it is found that at the lower energy density of

4.0 mW cm^{-2} (LED light), the increase in bactericidal activity (with increasing the exposure time) reaches a plateau, that is no significant increase in the reduction in the viable count is observed for the energy doses 1.2, 1.8 and 2.4 J compared with that of 0.6 J (see Figs. 1 and 2). This indicates that once the concentration of $AlPcS_2$ monomer has been reduced to a sufficiently low value (due to $AlPcS_2$ photobleaching) the dye becomes virtually ineffective.

During the photobleaching of $AlPcS_2$, it is possible that the position of the $AlPcS_2$ monomer:dimer equilibrium may shift to compensate for the reduction in the concentration of monomer. If the disaggregation of the $AlPcS_2$ dimers is relatively slow within a bacterial suspension then fractionating the light source may result in more photoactive $AlPcS_2$ monomer molecules being ultimately available than were present in the initial concentration. It has been found that a fractionated light source does not produce any changes in the overall rate of photobleaching of $AlPcS_2$ in the presence of *E. coli* (paper in preparation). Thus, a further explanation for the increase in photoinactivation achieved by fractionating the irradiating light source observed in the present study is required.

Some of the processes that ultimately lead to cell inactivation are only initiated by the primary species generated from the $AlPcS_2$ triplet excited state. For example, lipid peroxidation continues after the initiating radical event via many propagating steps that do not involve species directly generated from the $AlPcS_2$ triplet state. Thus, when the light source is fractionated, the $AlPcS_2$ triplet states formed during illumination generate the species necessary to start the cytotoxic mechanisms and these processes will continue in the absence of irradiation. In this way, the number of photoactive $AlPcS_2$ monomer molecules present in the initial $AlPcS_2$ concentration are used more efficiently; it is possible that the same reduction in viable count will be achieved when a sample of *E. coli* is illuminated either for 10 min at 2 mW, or for 5 min at 2 mW if using a fractionated light source (1 min illumination, 1 min dark interval).

Previous studies conclude that Gram-negative bacteria cannot be inactivated by anionic photosensitisers such as $AlPcS_2$ because the sensitiser molecules are localised extracellularly. However, $AlPcS_2$ has recently been shown to be effective in the lethal photosensitisation of *P. gingivalis* [11] and *E. coli* (the present study). In order for a sensitiser to inactivate a bacterial cell, the cytoplasmic membrane of the cells must be damaged; thus sensitisers that localise within bacterial cells (e.g. cationic sensitisers localise within Gram-negative bacterial cells) are generally more effective. When a sensitiser is localised extracellularly, the initial photo-induced mechanisms need to damage the outer membrane of the Gram-negative bacterial cell which will then allow the sensitiser molecules to relocalise at internal cellular sites where more effective PDT damage is achieved (i.e. to the cytoplasmic membrane).

The $AlPcS_2$ isomer used in this work is an amphiphilic molecule; therefore it can enter the lipid environment of cellular membranes more readily than other non-amphiphilic disulphonated isomers [18]. Even though, $AlPcS_2$ is an anionic sensitiser (and thus close association with the surface of Gram-negative bacteria is inhibited), the amphiphilic nature of the sensitiser molecule enhances the possibility of interaction with Gram-negative bacterial cell outer membranes (the negative charges on the sulphonate groups of the $AlPcS_2$ molecule are on the hydrophilic side of the planar molecule). It is proposed that $AlPcS_2$ molecules are able to cause photo-induced damage to the outer membranes of both *E. coli* and *P. gingivalis* due to the partial interaction between the dye molecules and the surface of the bacterial cells. The relocalisation of the $AlPcS_2$ molecules to internal cellular sites subsequent to this initial photodamage is also favoured as a result of the ability of the amphiphilic $AlPcS_2$ molecule to enter lipid environments.

It is possible to predict the most effective conditions to achieve maximum photo-inactivation of bacterial cells if parameters such as sensitiser photobleaching and sensitiser localisation are understood when the sensitiser is in in vivo environments and thus this is the direction of our future work.

Acknowledgements

We are grateful to Dr. Mike Wilson and Manny Bhatti (The Eastman Dental Hospital, University College London) for providing the bacteria, Dr. Paula Booth (Imperial College, London) for laboratory equipment and the EPSRC for funding J.A.L. (PGRA).

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