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Susceptibility of microcosm subgingival dental plaques to lethal photosensitization

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ABSTRACT

Photodynamic therapy (PDT) offers potential as a non-invasive treatment of periodontal disease. In this study, microcosm biofilms were grown *in vitro* under conditions designed to mimic subgingival plaques typically found in patients with periodontitis. To investigate potential PDT modalities, biofilms were exposed to light from a helium/neon laser in conjunction with a photosensitizer, toluidine blue O (TBO), at varying output and concentration, respectively. To determine cytotoxic effects, viability profiling was undertaken on whole biofilms using standard plating methods, and on horizontal cross-sections of biofilms using confocal laser-scanning microscopy (CLSM) in conjunction with a differential viability stain. A light energy dose of 94.5 J in combination with 81.7 μM TBO was found to be optimal, achieving significant kills of over 97%. CLSM enabled visualization of the effects of PDT in three dimensions. Viability profiling of the CLSM images revealed that lethal photosensitization was most effective in the upper layers of biofilm. PDT was found to reduce the viability of subgingivally modelled plaques *in vitro* by a magnitude similar to that of chlorhexidine digluconate, which is commonly used to treat periodontal disease. The findings of this study indicate that PDT may be an effective alternative to conventional modalities in the treatment of periodontal disease.

INTRODUCTION

Periodontal disease is caused by the accumulation of subgingival plaque biofilm near the gingival margin and is responsible for up to 60% of tooth loss in the UK. Chronic marginal gingivitis probably affects the whole dentate population at some stage and is characterized by inflammation of the gum margin. If the build-up of gum marginal plaque is left unchecked, gingivitis can develop into the more serious condition of chronic adult periodontitis (Pihlstrom *et al.*, 2005). This condition is characterized by the migration of the junctional epithelium tissue at the base of the gingival crevice down the tooth to form a periodontal pocket. The cause of this pocketing has been attributed to tissue destruction as a consequence of bacterial stimulation of host degradative enzyme production (e.g. matrix metalloproteinases) and invasion of host cells (Amano, 2003; Bodet *et al.*, 2007). The (probing) depth of the periodontal pocket is used as an indicator of the spread of the disease and the Adult Dental Health Survey 1998 (Kelly *et al.*, 2000) revealed that 54% of the UK adult dentate population had probing depths greater than 3.5 mm (moderate periodontal disease) whilst 8% of the population had a loss of attachment over 5.5 mm (severe destructive periodontitis). The annual cost of the UK National Health Service (NHS) periodontal therapy in 2001/2002 was £174 million on 15.5 million treatment episodes (Chapple, 2004). Conventional periodontal therapy routinely involves scaling and root planing, which may be complemented with adjunct antimicrobial chemotherapy, such as doxycycline (Tuter *et al.*, 2007).

Bacteria harboured within biofilms are notoriously recalcitrant to antimicrobial agents (Millward & Wilson, 1989; Anwar *et al.*, 1990; Costerton *et al.*, 2003) and their eradication represents a formidable challenge where they are associated with disease. The mechanisms by which biofilms resist conventional antimicrobial agents are generally considered to be multi-factorial, although current understanding is that the production of high numbers of essentially invulnerable persister cells, in response to shock proteins or "alarmones", is the most significant of these factors (Gilbert *et al.*, 2002b).

Treatment of bacterial infections by photodynamic therapy (PDT) offers an alternative to the use of antimicrobial agents (Malik *et al.*, 1990) and to invasive dental procedures, which are associated with patient trauma. The bactericidal effect associated with PDT (known as lethal photosensitization) is caused by the generation of cytotoxic free radicals, such as singlet oxygen, when a photoreactive agent (photosensitizer) is excited by light of an appropriate wavelength (Malik *et al.*,

1990). PDT has been used clinically to treat a number of systemic conditions including tumour (Choi *et al.*, 2006). Interest in its application to treat bacterial infections is gaining momentum – the treatment of periodontal disease being one of the most promising (Meisel & Kocher, 2005). A major benefit of PDT is that the bactericidal effect is localized to areas that are treated with both photosensitizer and light, preventing disruption of the indigenous microflora at sites distal to the treated area (Wilson, 2004). The non-specific mode of action of PDT, expressly the generation of singlet oxygen, means that the acquisition of bacterial resistance to these agents is unlikely.

Previous studies have examined the effects of PDT on single-species biofilms using streptococci to model supragingival plaque in conditions associated with onset of dental caries (Zanin *et al.*, 2005, 2006) and *Streptococcus pyogenes* to model a skin biofilm (Hope & Wilson, 2006). However, PDT also offers a promising, non-invasive treatment for periodontal disease. Previously, it was reported that toluidine blue O (TBO) and helium/neon (HeNe) laser light could kill significant numbers of bacteria associated with periodontal disease, using a simple system where bacteria were grown on nitrocellulose membranes (O'Neill *et al.*, 2002). In this investigation we describe the modelling of subgingival plaque using a sophisticated *in vitro* model more representative of *in vivo* conditions.

The aim of this study therefore was to investigate the susceptibility of microcosm subgingival periodontal plaques grown in a constant-depth film fermenter (CDFF) to PDT using a range of biofilm viability profiling techniques. We also compared the effectiveness of PDT with the commonly used oral antimicrobial chlorhexidine digluconate (CHX), representative of a conventional, non-invasive modality.

MATERIALS AND METHODS

Subgingival plaque inoculum

Subgingival plaque samples were obtained from ten patients attending the Eastman Dental Hospital, London, for treatment of moderate-to-advanced adult periodontitis. Each sample consisted of the contents of at least two periodontal pockets from each patient. The samples were pooled and mixed in pre-reduced BHI (brain–heart infusion) broth (Oxoid, Basingstoke, UK) containing 10% (v/v) glycerol and then aliquoted into 1 ml volumes and stored at −70 °C, to generate a bank of homogeneous innocula for use throughout this series of experiments. Two vials of homogenized plaques were used as the inoculum to produce the biofilms *in vitro*. Compositional studies (using culture and molecular methods) on biofilms produced from this pooled inoculum, using the method described below, demonstrated the presence of many of the genera found in subgingival plaque including *Veillonella, Streptococcus, Propionibacterium, Eubacterium, Micrococcus, Actinomyces*, *Fusobacterium* and *Porphyromonas* (O'Neill, 2006), the last three genera being especially associated with periodontal disease.

Production of microcosm subgingival dental plaques

Operation of a CDFF (University of Wales, Cardiff, UK) to produce multi-species oral biofilms modelling those associated with periodontal disease has been described (Allan *et al.*, 2002; Hope & Wilson, 2006). In this study, to model the microaerophilic conditions found in periodontal pockets (Mettraux *et al.*, 1984), the CDFF was operated by continuous flushing (60 cm^3/min) with a gas mixture consisting of 95% N_2, 3% CO_2, 2% O_2. The growth medium used to enable propagation of biofilms from the microcosm plaques consisted of RPMI medium (60%) (Sigma, Poole, UK) and horse serum (40%) (Oxoid, Basingstoke, UK) supplemented with 0.5 μg menadione/ml and 5.0 μg hemin/ml. The CDFF was housed within an incubator at 37 °C. The substratum for subgingival plaque is enamel/cementum, the mineral component of which is similar to hydroxyapatite (HA). HA discs were therefore used as the substratum for biofilm formation. The CDFF housed 15 sample pans on a rotating turntable, each pan containing five HA discs of 5 mm diameter. Discs were recessed to a depth of 100 μm.

The inoculum was added to 500 ml of growth medium and pumped onto the rotating HA substrata for 8 h, at a rate of 1 ml/min. The CDFF was then connected to fresh medium, which was supplied at 0.1 ml/min, and biofilms were then grown for 7 days before being removed for experimentation.

Photosenitization and laser application

TBO (Sigma) was used at concentrations of 32.7, 65.4, 81.7 or 163.4 μM in 0.85% (v/v) saline. The laser used was an HeNe gas laser (Spectra-Physics, Darmstadt-Kranichstein, Germany) with a measured power output of 35 mW. Light was emitted in a collimated beam of 3.5 mm diameter, with wavelength of 632.8 nm. To irradiate biofilms, laser light was passed through a lens (magnification 2×), allowing light coverage of the entire biofilm surface.

To test biofilm susceptibility to TBO/HeNe laser light, pans were removed and the biofilm-containing discs removed and placed in microtitre plate wells. TBO (10 μl) was added to each biofilm for sensitization. Laser light intensities of 63 J (163.8 J/cm^2) and 95.4 J (248 J/cm^2) were employed (L+S+). Controls were: TBO in the absence of laser light (L−S+); and addition of 10 μl sterile saline, with or without laser light (L+S− and L−S−, respectively). In addition to controlling for laser light and TBO exposure, L−S− also served to determine whether exposure to an aerobic atmosphere for the duration of the experiment had any effect on bacterial viability. A pre-exposure time of 5 min (in the absence of light) was applied to allow the solutions to penetrate into the biofilm. The L+S+ and L+S− samples were exposed to laser light for 15 min. Discs were then immersed in

1 ml pre-reduced BHI broth and biofilms resuspended by vortexing for 60 s. The total number of viable culturable bacteria (colony-forming units per millilitre (c.f.u./ml)) from resuspended biofilms before and after treatment was determined by viable counting on Fastidious Anaerobe Agar (FAA) (Bioconnections, Leeds, UK) containing 5% (v/v) defibrinated horse blood (E & O Laboratories, Bonnybridge, UK). Duplicate 25 µl aliquots were spread over the surface of FAA plates and the plates were incubated for 7 days at 37 °C in an anaerobic atmosphere. The resultant colonies were counted.

CLSM of biofilms subjected to PDT

Biofilms were subjected to either PDT using 63 J HeNe laser light and 81.7 µM TBO, or no treatment, as described above. Post PDT, biofilms were then placed into a miniature Petri dish, biofilm uppermost and stained using the *Bac*Light™ bacterial viability kit (Molecular Probes, Oregon, USA) with 5 min stain penetration time in darkness. Scans were taken of the biofilms using a Leica DMLFS fixed-stage microscope with a Leica TCS SP confocal scan-head. The objective lens was a 63× HCX water immersion dipping lens. Confocal image stacks were captured of both the viable (488 nm) and non-viable (568 nm) fluorescent emissions. Data are from two biofilms, one an untreated control (L−S−), and the other subjected to PDT (L+S+).

Analysis of CLSM data

Fluorescence intensity profiles (Hope *et al.*, 2002) through the biofilms were generated by measuring the brightness of each optical section in the confocal image stack, for both the viable (488 nm) and non-viable (568 nm) channels, using ImageJ computer software (ImageJ 1.36d, The National Institutes of Health, USA, website: <http://rsb.info.nih.gov/ij/>). To enable an effective comparison between the viable and non-viable channels, these data were normalized against the maximum image intensity for each individual channel (maximum value = 1) and plotted against depth into the biofilm. Zero depth was taken as the top of the confocal image stack.

Projection images were constructed by combining all confocal optical sections into one image.

Susceptibility of biofilms to chlorhexidine digluconate

Two pans, each containing five biofilms, were removed aseptically from the CDFF and placed in a sterile glass container. To this was added 5 ml 0.2% CHX (Sigma) or, in the case of the control pan, 5 ml phosphate-buffered saline (PBS: Oxoid, Basingstoke, UK) and incubated for 1 or 15 min. Biofilms were then prepared for viable counting as described above. In parallel, PDT was applied to biofilms of the same age using a regimen of 2.1 J HeNe laser light with 81.7 µM TBO for 1 min and then prepared for viable counting. In addition, the 15 min CHX exposure results were compared with the data generated using the PDT modalities described above (15 min treatment).

Statistical analyses

The statistical significance of the data was ascertained using the two-tail t-test assuming unequal variance. For viable counting following PDT, four individual biofilms were used to determine c.f.u./ml by plating in duplicate, $n = 8$. For CHX and TBO studies, three biofilms were plated in duplicate, $n = 6$. For ease of interpretation, viability is recorded as percentage kill as compared with untreated control.

RESULTS

Effect of PDT on biofilm viability

Table 1 shows the effect of increasing light dose and photosensitizer concentration on the efficacy of lethal photosensitization in terms of the percentage of bacteria killed. The most effective combination of light and photosensitizer was 94.5 J and 81.7 µM TBO, which produced a significant kill of 97.4%. A reduced TBO concentration of 65.4 µM with the same light dosage still produced a significant kill of 95.0%. Increasing photosensitizer concentration to 163.4 µM but still using 94.5 J laser light reduced the efficacy of the procedure, producing a diminished, but significant, kill of 74.4%. A photosensitizer concentration of 32.7 µM in combination with a light dose of 94.5 J was sufficient to produce a significant bactericidal effect, with a kill of 82.7%.

Table 1: Effect of laser light intensity and sensitizer concentration on kill efficacy

Light intensity (J)	TBO concentration (μM)	% kill
63	32.7	41.9*
63	0	0*
0	32.7	72.6*
63	81.7	69.0
63	0	0*
0	81.7	36.3*
94.5	32.7	82.7
94.5	0	0*
0	32.7	0*
94.5	65.4	95.0
94.5	0	0*
0	65.4	0*
94.5	81.7	97.4
94.5	0	54.4
0	81.7	57.1
94.5	163.4	74.4
94.5	0	0*
0	163.4	0*

*Not significant.

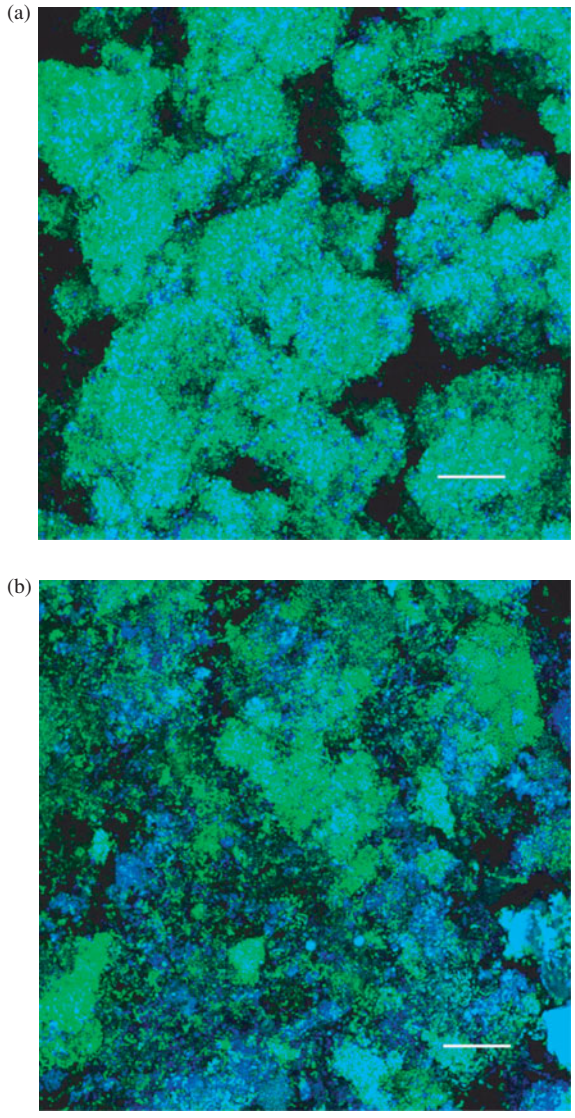

Fig. 1: (a) Projection of confocal image stack of a control CDFF-cultivated multi-species biofilm (L−S−). (b) Projection of confocal image stack of a CDFF-cultivated multi-species biofilm exposed to 63 J of HeNe laser light and 81.7 μM TBO (L+S+). (a) and (b) Scale bars represent 20 μm. Viable cells, green; non-viable cells, blue.

Reducing the light dosage from 94.5 to 63 J reduced the bactericidal efficacy of PDT. Laser light (63 J) with a photosensitizer concentration of 81.7 μM produced a significant kill of 69.0%; however, this kill was less than that observed at the higher light dosage using the same photosensitizer concentration, albeit the difference was not significant. Laser light (63 J) in conjunction with 32.7 μM TBO was insufficient to produce a significant lethal effect.

CLSM studies on biofilms subjected to PDT

Microcolony formations interspersed with water channels can be seen in the control biofilm shown in Fig 1a. Viable cells (green) appeared to be far more numerous than the non-viable cells (blue). (It should be noted that the red, non-viable stain used in the procedure was digitally altered to appear blue using ImageJ for ease of interpretation.) Fig. 1b shows a multi-species biofilm that was subjected to lethal photosensitization with 63 J of HeNe laser light in the presence of 10 μl of 81.7 μM TBO. Visually, there was a discernible difference between this biofilm and the control (Fig. 1a). The structure of the biofilm seems to have been disrupted by exposure to the light in the presence of TBO. In addition, there appeared to be a larger proportion of non-viable to viable cells as compared with the control biofilm.

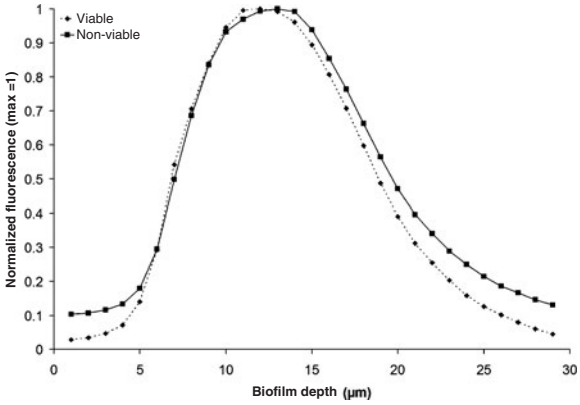

Fig. 2: Normalized fluorescence intensity profile of viable and non-viable stain as a function of depth in a control biofilm (L−S−), as determined by CLSM.

The normalized fluorescence image intensity profile through the control biofilm (Fig. 2) conformed to those observed elsewhere (Hope *et al.*, 2002; Hope & Wilson, 2006) in similar supragingival and subgingival plaque biofilm models. Biofilm that was exposed to 63 J of HeNe laser light in the presence of TBO (Fig. 3) showed a broader distribution of non-viable fluorescence, particularly in the upper layers closest to the illumination source.

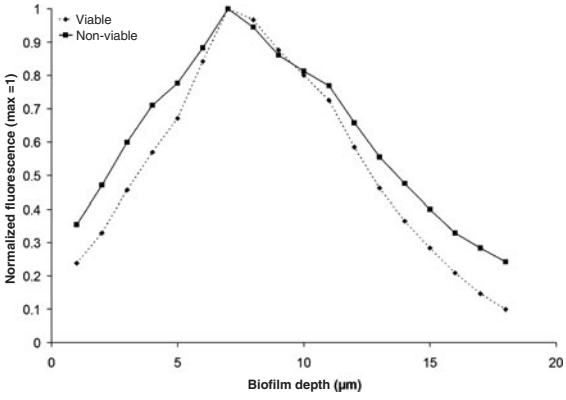

Fig. 3: Normalized fluorescence intensity profile of viable and non-viable stain in biofilms exposed to 63 J of HeNe laser light and 81.7 μM TBO (L+S+), as determined by CLSM.

Effect of chlorhexidine digluconate on biofilm viability

Exposure of biofilms to 0.2% CHX for 1 min did not result in a statistically significant reduction in the number of recovered viable organisms post-exposure when compared with the control, whereas exposure for 15 min with this treatment resulted in a significant reduction of 99.7%. Exposure to PDT for 1 min using a combination of 2.1 J of laser light and 81.7 μM TBO did not produce a significant kill, whereas (as described above) combinations of 63 J (163.8 J/cm^2) with 81.7 μM TBO and 94.5 J (248 J/cm^2) with 32.7, 65.4, 81.7 and 163.4 μM TBO produced significant reductions in biofilm viability.

DISCUSSION

PDT was performed using two light doses, 63 J (163.8 J/cm^2) and 95.4 J (248 J/cm^2), with a range of photosensitizer concentrations, the latter light dosage proving the most effective.

Inconsistencies in effect of laser and photosensitizer activity alone on biofilm viability were observed. In all but one instance, these were not significant. However, 94.5 J laser light, and 81.7 μM TBO alone, produced significant kills (54.4% and 57.1%, respectively), although these kills were much lower than those achieved in combination (97.4%). It is possible that the variability encountered in these experiments was due to the heterogeneous nature of these multi-species biofilms, where perhaps unculturable bacteria accounted for varying proportions of biofilm biomass. Alternatively, the treatments alone may have contributed a small antimicrobial effect. Further experiments, with larger sample sizes are required to build on this preliminary study.

A combination of 63 J laser light and 81.7 μM TBO produced the greatest bactericidal effect, as determined by viability studies using plate culture. An increase in the photosensitizer concentration (to 163 μM) did not effect an increase in bactericidal activity of PDT. This observation seems counter-intuitive; one would have envisaged that increasing the concentration of photosensitizer applied to the biofilm would result in an increase in kill. However, if the TBO solution was too concentrated, penetration of the solution by the laser light would be impeded. Thus a shielding effect (Herzog *et al.*, 1994) could occur, preventing the excitation of TBO proximal to the biofilm bacteria, with a consequent reduction in efficacy of killing. Alternatively, TBO may also aggregate at higher concentrations, localizing its presence and preventing adequate pervasion of the target material. This may also account for the lack of increased efficacy at higher concentrations (Sternberg & Dolphin, 1996). If the concentration of TBO used is sub-optimal for the light dose administered, photobleaching will occur (Rigaut & Vassy, 1991) resulting in degradation of photosensitizer and diminished bactericidal activity. Thus there appears likely to be a concentration of TBO optimal for photosensitization – sufficient to prevent bleaching but below a threshold where its presence becomes detrimental to the procedure.

CLSM studies utilized the *Bac*Light™ bacterial viability kit (LIVE/DEAD) to determine the viability profile of bacteria within the biofilms and revealed biofilms of typical architecture, similar to those observed in other CLSM studies (e.g. Costerton *et al.*, 1994; Hope *et al.*, 2002). Microcolonies interspersed with water channels were visible (Fig. 1a).

Analyses of the control biofilms (L−S−) (Fig. 2) revealed that in general there was little difference in the spatial distribution of live cells relative to dead cells, with the exception that at the margins of the upper and, in particular, lower biofilm interfaces there seemed to be a slight increase in dead cells. In the case of the upper surface it is possible that obligately anaerobic bacteria were killed during the sampling process (Hope & Wilson, 2006). At the biofilm/substratum interface there may have been nutrient limitation or a build up of toxic products (especially in areas relatively distant from water channels) that may have resulted in the death of some cells.

Biofilms exposed to laser light and photosensitizer showed evidence of lethal photosensitization, as indicated by an increasing proportion of dead cells, particularly in the upper portion of the biofilm (0–10 μm), suggesting that PDT was most effective in this area (Fig. 3). In addition to the possible limitations associated with photosensitizer efficacy discussed above, it is also possible that, with increasing depth, biofilms became increasingly more resistant to PDT. This may be due to reduced growth rate of cells with increasing depth in the biofilm, a known contributor to antimicrobial resistance in biofilm populations (Evans *et al.*, 1990; Roberts & Stewart, 2004).

CHX is used in the treatment of a wide range of oral infections, including periodontitis (Pietruska *et al.*, 2006). Its efficacy against subgingivally modelled *in vitro* biofilms was compared with that of PDT to provide an indication of the suitability of PDT as a putative therapeutic regimen in treating periodontal disease. CHX (at 0.2%) did not produce a significant kill when applied to the biofilms for 1 min, suggesting that this may be an ineffective treatment when directed against periodontal plaques of similar dimensions *in situ*. CHX did produce a statistically significant kill after 15 min, although this would be an impractical exposure period clinically. However, it is possible that a similar kill could have been achieved with an exposure period somewhere between 1 and 15 min or at increased concentration (not tested). When PDT was employed with a low light dosage (2.1 J) for 1 min, no significant reduction in viability of the biofilms was observed. However, as discussed above, comprehensive, significant kills were achieved with 15 min exposure at increased light intensities, and optimally with a light dose and photosensitizer concentration of 94.5 J and 81.7 μM, respectively. Hence, from these preliminary *in vitro* data, one can conclude that PDT (using TBO and HeNe laser light) can be as effective as CHX when directed against biofilms similar to those found in periodontal disease.

The site-specific clinical application of PDT would enable avoidance of the use of antibiotics (which are

often used following periodontal surgery) and the associated problem of perturbation of the indigenous systemic microflora, for example gastrointestinal upset. The increasing prevalence of bacterial antibiotic resistance necessitates the development of alternative antibacterial therapies. Sublethal concentrations of biocides may also select for mutants expressing multidrug efflux pumps, which can also render them antibiotic resistant (Gilbert *et al.*, 2002a). Thus routine use of antimicrobial oral health-care products could conceivably select for a population of bacteria with an intrinsic ability to resist antibiotic therapy, and consequently limit the effectiveness of antibiotic treatment following periodontal surgery. Development of resistance to chlorhexidine has been demonstrated in some Gram-negative bacteria including *Pseudomonas aeruginosa* (Thomas *et al.*, 2000) and *Klebsiella pneumoniae* (Fang *et al.*, 2002). With PDT, lethal photosensitization mediates bacterial killing via singlet oxygen and free radicals. Resistance development would be unlikely owing to the multiplicity of target sites, which include the outer and plasma membranes of Gram-negative bacteria, DNA and photolabile surface-associated proteins (Bhatti *et al.*, 1998, 2001), against which the generated singlet oxygen and free radicals are active.

REFERENCES

Allan, I., Newman, H. & Wilson, M. (2002) Particulate bioglass reduces the viability of bacterial biofilms formed on its surface in an *in vitro* model. *Clinical Oral Implants Research* **13**, 53–58

Amano, A. (2003) Molecular interaction of *Porphyromonas gingivalis* with host cells: implication for the microbial pathogenesis of periodontal disease. *Journal of Periodontology* **74**, 90–96

Anwar, H., Dasgupta, M. K. & Costerton, J. W. (1990) Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrobial Agents and Chemotherapy* **34**, 2043–2046

Bhatti, M., MacRobert, A., Meghji, S., Henderson, B. & Wilson, M. (1998) A study of the uptake of toluidine blue O by *Porphyromonas gingivalis* and the mechanism of lethal photosensitization. *Photochemistry and Photobiology* **68**, 370–376

Bhatti, M., Nair, S. P., Macrobert, A. J., Henderson, B., Shepherd, P., Cridland, J. & Wilson, M. (2001) Identification of photolabile outer membrane proteins of *Porphyromonas gingivalis. Current Microbiology* **43**, 96–99

Bodet, C., Andrian, E., Tanabe, S. & Grenier, D. (2007) *Actinobacillus actinomycetemcomitans* lipopolysaccharide regulates matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase, and plasminogen activator production by human gingival fibroblasts: a potential role in connective tissue destruction. *Journal of Cell Physiology* **212**, 189–194

Chapple, I. L. (2004) Management of chronic periodontal diseases within the NHS. *British Dental Journal* **196**, 505

Choi, Y., Wiessleder, R. & Tung, C.H. (2006) Selective antitumor effect of novel protease-mediated photodynamic agent. *Cancer Research* **15**, 7225–7229

Costerton, J. W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D. & James, G. (1994) Biofilms, the customized microniche. *Journal of Bacteriology* **176**, 2137–2142

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C. & Ehrlich, G. (2003) The application of biofilm science to the study and control of chronic bacterial infections. *Journal of Clinical Investigation* **112**, 1466–1477

Evans, D. J., Brown, M. R., Allison, D. G. & Gilbert, P. (1990) Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. *Journal of Antimicrobial Chemotherapy* **25**, 585–591

Fang, C. T., Chen, H. C., Chuang, Y. P., Chang, S. C. & Wang, J. T. (2002) Cloning of a cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella pneumoniae. Antimicrobial Agents and Chemotherapy* **46**, 2024–2028

Gilbert, P., Allison, D. G. & McBain, A. J. (2002a) Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *Journal of Applied Microbiology* **92** Suppl, 98S–110S

Gilbert, P., Maria-Litran, T., McBain, A.J., Rickard, A.H., & Whyte, F.W. (2002b) The physiology and collective recalcitrance of microbial biofilm communities. *Advances in Microbial Physiology* **46**, 202–256

Herzog, M., Moser, J., Wagner, B. & Broecker, J. (1994) Shielding effects and hypoxia in photodynamic therapy. *International Journal of Oral Maxillofacial Surgery* **23**, 406–408

Hope, C. K. & Wilson, M. (2006) Biofilm structure and cell vitality in a laboratory model of subgingival plaque.*Journal of Microbiological Methods* **66**, 390–398

Hope, C. K., Clements, D. & Wilson, M. (2002) Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling. *Journal of Applied Microbiology* **93**, 448–455

Kelly, M., Steele, J., Nuttall, N., Bradnock, G., Morris, J., Nunn, J., *et al.* (2000) *Adult Dental Health Survey: Oral Health in the United Kingdom 1998.* Edited by A. Walker & I. Cooper. London: The Stationary Office

Malik, Z., Hanania, J. & Nitzan, Y. (1990) Bactericidal effects of photoactivated porphyrins – an alternative approach to antimicrobial drugs. *Journal of Photochemistry and Photobiology* B **5**, 281–293

Meisel, P. & Kocher, T. (2005) Photodynamic therapy for periodontal diseases: state of the art. *Journal of Photochemistry and Photobiology* B **79**, 159–170

Mettraux, G. R., Gusberti, F. A. & Graf, H. (1984) Oxygen tension (pO_2) in untreated human periodontal pockets. *Journal of Periodontology* **55**, 516–521

Millward, T. A. & Wilson, M. (1989) The effect of chlorhexidine on *Streptococcus sanguis* biofilms. *Microbios* **58**, 155–164

O'Neill, J. F. (2006) Characterisation and susceptibility to lethal photosensitisation of *in vitro* grown subgingival biofilms. Ph.D. thesis, University College London, University of London

O'Neill, J. F., Hope, C. K. & Wilson, M. (2002) Oral bacteria in multi-species biofilms can be killed by red light in the presence of toluidine blue. *Lasers in Surgery and Medicine* **31**, 86–90

Pietruska, M., Paniczko, A., Waszkiel, D., Pietruski, J. & Bernaczyk, A. (2006) Efficacy of local treatment with chlorhexidine gluconate drugs on the clinical status of periodontium in chronic periodontitis patients. *Advances in Medical Sciences* **51**, 162–165

Pihlstrom, B. L., Michalowicz, B. S. & Johnson, N. W. (2005) Periodontal diseases. *Lancet* **366**, 1809–1820

Rigaut, J. P. & Vassy, J. (1991) High-resolution three-dimensional images from confocal scanning laser microscopy. Quantitative study and mathematical correction of the effects from bleaching and fluorescence attenuation in depth. *Analytical and Quantitative Cytology and Histology* **13**, 223–232

Roberts, M. E. & Stewart, P. S. (2004) Modelling antibiotic tolerance in biofilms by accounting for nutrient limitation. *Antimicrobial Agents and Chemotherapy* **48**, 48–52

Sternberg, E. & Dolphin, D. (1996) Pyrrolic photosensitizers. *Current Medicinal Chemistry* **3**, 239–272

Thomas, L., Maillard, J. Y., Lambert, R. J. & Russell, A. D. (2000) Development of resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a "residual" concentration. *Journal of Hospital Infections* **46**, 297–303

Tuter, G., Kurtis, B., Serdar, M., Aykan, T., Okyay, K., Yucel, A., *et al.* (2007) Effects of scaling and root planing and sub-antimicrobial dose doxycycline on oral and systemic biomarkers of disease in patients with both chronic periodontitis and coronary artery disease. *Journal of Clinical Periodontology* **34**, 673–681

Wilson, M. (2004) Lethal photosensitisation of oral bacteria and its potential application in the photodynamic therapy of oral infections. *Photochemistry and Photobiology Sciences* **3**, 412–418

Zanin, I. C., Goncalves, R. B., Junior, A. B., Hope, C. K. & Pratten, J. (2005) Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: an *in vitro* study. *Journal of Antimicrobial Chemotherapy* **56**, 324–330

Zanin, I. C., Lobo, M. M., Rodrigues, L. K., Pimenta, L. A., Hofling, J. F. & Goncalves, R. B. (2006) Photosensitization of *in vitro* biofilms by toluidine blue O combined with a light-emitting diode. *European Journal of Oral Sciences* **114**, 64–69