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Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms

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***Objectives*: The purpose of this study was to evaluate the clinical plaque disclosing agent erythrosine as a photosensitizer in the photodynamic killing of the oral bacterium *Streptococcus mutans* grown as a biofilm.**

***Methods*: *S. mutans* biofilms of 200 μm thickness were grown in a constant-depth film fermenter. In addition to determining localization of the photosensitizer within biofilms using confocal laser scanning microscopy (CLSM), we compared the bacterial killing efficacy of erythrosine with that of two well-characterized photosensitizers, methylene blue (MB) and photofrin. Incubations were carried out with each photosensitizer (22 μM), and irradiation was for 15 min using a 400 W white light source.**

***Results*: The CLSM results showed that erythrosine is taken up into *S. mutans* biofilms, where it is associated with the biomass of the biofilm rather than the fluid-filled channels and voids. Comparison of the cell killing efficacy of erythrosine in *S. mutans* biofilms of different ages showed that erythrosine was 1–2 $\log_{10}$ more effective at killing biofilm bacteria than photofrin and 0.5–1 $\log_{10}$ more effective than MB. The results were statistically significant ($P < 0.01$). Photodynamic therapy (PDT) with all three photosensitizers was increasingly effective as biofilm age increased, suggesting that temporal changes in biofilm architecture and composition affect susceptibility to PDT.**

***Conclusions*: PDT using erythrosine as photosensitizer shows excellent potential as a treatment for oral plaque biofilms.**

Keywords: PDT, photofrin, methylene blue, *Streptococcus mutans*

Introduction

Photodynamic therapy (PDT) is an established treatment for localized tumours, involving the application and retention of an applied photosensitizing agent in malignant tissues. Upon irradiation with light of an appropriate wavelength the photosensitizer undergoes a transition from a low-energy 'ground state' to a higher-energy 'triplet state'. This triplet-state photosensitizer can react with biomolecules to produce free radicals and radical ions, or with molecular oxygen to produce singlet oxygen. These cytotoxic species can cause oxidation of cellular constituents such as plasma membranes and DNA, resulting in cell death.[1] A substantial body of work has shown that this photodynamic approach can also be used to kill bacteria.[2] PDT treatment of the ubiquitous species *Staphylococcus aureus* has been studied using photosensitizers such as haematoporphyrin,[3] phthalocyanine,[4] 5-aminolaevulinic acid and photofrin.[5] Bacteria that grow in biofilms, implicated in diseases such as cystic fibrosis (*Pseudomonas aeruginosa*) and periodontitis (*Porphyromonas gingivalis*), have been shown to be susceptible to PDT with photosensitizers such as methylene blue (MB),[6] toluidine blue O (TBO)[7] and phthalocyanine.[8] The photodynamic approach to killing bacteria is clearly a rapidly emerging alternative to current antimicrobial regimens.[9] Significantly, it is unlikely that bacteria could develop resistance to the photodynamic action of cytotoxic singlet oxygen or free radicals, as has been reported with conventional antimicrobials and antibiotics.[10]

Dental caries[11] and the periodontal diseases[12] are among the most common diseases in the Western world. Both result initially from a build-up of plaque biofilms on the teeth and soft tissues of the mouth. Mechanical removal of plaque and good oral hygiene are the most common treatments available. However, these can be the subject of poor patient compliance[13] and may be inappropriate, as in the case of the mechanoblistering disease epidermolysis bullosa, where brushing or scraping causes massive mechanical damage to the oral mucosa and is unbearable for the patient.

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Antibacterial agents are also widely used, but problems with general efficacy due to access of topical agents to plaque[14] and the possibility of development of bacterial resistance[15] mean alternative strategies are desirable to control plaque and treat caries, gingivitis and periodontal disease. Since they are localized infections, such plaque-related diseases would be well suited to PDT. In addition, local administration of both photosensitizer and light is relatively straightforward in the oral cavity.[16] Antibacterial photosensitizers currently under investigation for use in the mouth include TBO[7] and chlorin *e*6.[17] These agents show great promise, but will be subject to lengthy clinical and legislative assessment. More immediate benefit could be attained from photosensitizers already available for use in the mouth. One such photosensitizer is erythrosine.

Dental practitioners currently use erythrosine to stain and visualize dental plaque in the form of disclosing solution or tablets. Erythrosine has some reported antimicrobial activity against Gram-positive and Gram-negative oral bacteria.[18–20] However, erythrosine also belongs to a class of cyclic compounds called xanthenes, which absorb light in the visible region, and the ability of erythrosine to initiate photochemical reactions is well documented.[21,22] The efficacy of erythrosine in sensitizing non-oral microbes to killing by light is similarly well established.[23–25] However, despite the main medical application of erythrosine being its use in staining the aetiological agent of common oral diseases (dental plaque), to our knowledge there are no reports of the use of erythrosine as a photosensitizer in the mouth. Clearly, erythrosine has an advantage over other photosensitizers in development, as it already targets dental plaque and has full approval for use in the mouth.

The aim of this study was to carry out a preliminary assessment of erythrosine-mediated PDT on biofilms of *Streptococcus mutans* of different ages in terms of the localization of erythrosine within *in vitro*-generated biofilms and the cell killing efficacy of PDT with erythrosine compared with the well-characterized photosensitizers photofrin and MB.

Materials and methods

Photosensitizers and light source

Erythrosine (Sigma Ltd, Poole, UK), MB (Sigma) and photofrin (kindly supplied by Dr D. I. Vernon, Centre for Photodynamic Therapy, University of Leeds, UK) were stored as 1 mg/mL stock solutions in deionized water, foil-covered at 4°C after filter purification (0.2 μm). The light source used was a 400 W tungsten filament lamp. Samples were irradiated at 30 cm from the lamp, with a heat-dissipating water bath between the lamp and the biofilm samples. The average intensity of light in the presence of the water bath was 22.7 mW/cm^2 in the wavelength range 500–550 nm (region of maximal absorption by erythrosine) and 22.5 mW/cm^2 in the wavelength range 600–650 nm (region of maximal absorption by MB and photofrin).

Bacterial culture

S. mutans (NCTC 10449) was maintained by weekly subculture on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% horse blood.

S. mutans biofilms were grown in a constant-depth film fermenter (CDFF) at 37°C. This comprises a rotating stainless steel disc (2 rpm) inside a glass vessel containing 15 polytetrafluoroethylene (PTFE) pans, each of which has five PTFE plugs recessed to 200 μm, where the biofilms grow. A PTFE scraper bar distributes incoming inoculum and medium over the pans and maintains biofilms at a constant depth of 200 μm by removing material in excess of 200 μm.[26] Five 10 mL overnight cultures of *S. mutans* in brain heart infusion broth (BHI) (Oxoid) were added aseptically to 1 L of BHI, mixed and pumped into the pre-autoclaved CDFF over 24 h by a peristaltic pump. After inoculation, the CDFF was fed from a 10 L BHI medium reservoir, with the peristaltic pump delivering medium at a rate of ~0.7 L/day. Waste was withdrawn from the CDFF by another peristaltic pump via the outlet port. Pans were aseptically removed for examination or treatment of biofilms with sterilizable stainless steel instruments through the sampling port in the top plate of the CDFF.

Confocal laser scanning microscopy of biofilms

To examine the localization of erythrosine in the biofilms, confocal imaging of intact biofilms was performed using a Noran Odyssey confocal laser scanning microscope (CLSM) (Noran Instruments UK, Bicester, UK) equipped with an argon ion laser, mounted on a Nikon Optiphot microscope (Nikon UK, Kingston-upon-Thames, UK). The objective used was a water immersion 'dipping' lens (63×, Carl Zeiss Ltd, Welwyn Garden City, UK) with a working distance of 1.45 mm.

CDFF pans were aseptically removed after 168 h and immediately washed in 0.25 strength Ringers solution (RS) (Lab M, Bury, UK). The biofilm-containing pans were then placed in foil-covered vials containing 10 mL of 22 μM erythrosine in RS for 15 min incubation at room temperature. A cooling water bath placed between the light source and the sample prevented any temperature rise associated with the irradiation. The pans were then carefully washed in 10 mL of RS before being placed under the objective. Biofilm biomass was visualized with the CLSM in reflected-light mode. Areas of erythrosine localization were visualized by fluorescence imaging using 488 nm excitation (Ar laser) with detection >500 nm.

Photodynamic therapy of biofilms

Biofilm-containing pans were aseptically removed in triplicate at 48, 120, 168, 216 and 288 h after inoculation and immediately washed in 10 mL of RS to remove loosely adherent cells. Biofilm pans were then placed in foil-covered vials containing 10 mL of erythrosine, MB or photofrin in RS for 15 min at room temperature. To allow a direct comparison of the efficacy of the three photosensitizers, each was used at 22 μM, a concentration that is typical of those used by other groups in antibacterial PDT.[5,6,27] Dental practitioners use plaque disclosing solution at 0.72–2% w/v erythrosine,[20] which is equivalent to an erythrosine concentration of 9–25 mM. The 22 μM concentration of erythrosine used in the present study is 1000-fold lower than the currently acceptable clinically used concentration.

Biofilms were then irradiated under the white light system (as described above) for 15 min. Three biofilms per time point were disrupted from the plugs by vortexing in 1 mL of RS for 1 min, and serially diluted in RS. Bacterial survival was enumerated by serially diluting samples and plating in triplicate on Columbia agar supplemented with 5% horse blood. Plates were incubated aerobically at 37°C for 48 h and the colony count was determined. Controls consisted of biofilms subjected to (i) 22 μM photosensitizer alone; (ii) light alone after incubation with RS only; and (iii) incubation with RS only and no irradiation. For each treatment regime, $n \geq 9$, and each experiment was repeated on a different occasion.

Results and discussion

A 2 μm section of an *S. mutans* biofilm, imaged on the CLSM in reflected-light mode, is shown in Figure 1(a). Highly reflective regions correspond to regions of cellular biomass, while poorly reflecting areas are the channels and voids typical of biofilm

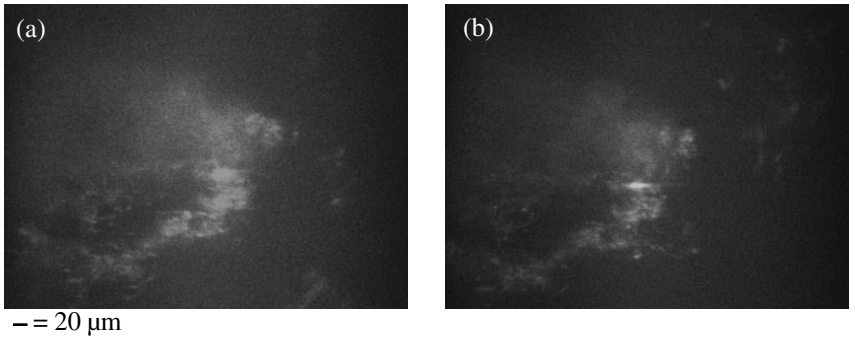

Figure 1. CSLM imaging showing uptake of erythrosine into the biomass of *S. mutans* biofilms. Reflective cellular material was located using CSLM reflection-mode imaging (a) and erythrosine fluorescence was detected in fluorescence mode (b); λ_{ex} = 488 nm; λ_{em} > 500 nm. A colour version of this figure is available as Supplementary data at *JAC* Online.

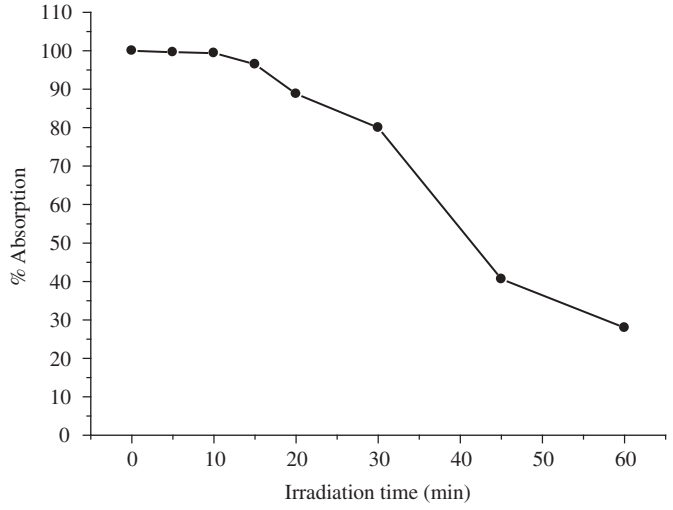

Figure 2. Photobleaching of a 22 μM solution of erythrosine as a function of irradiation time.

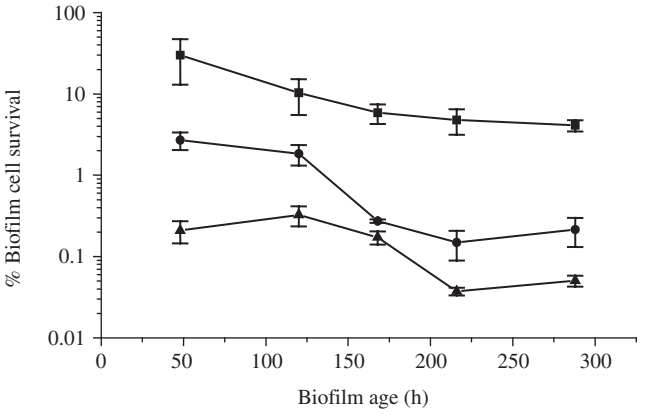

Figure 3. A direct comparison of the efficacy of erythrosine with that of methylene blue and photofrin in the PDT of *S. mutans* biofilm cells. Data have been normalized and cell survival expressed as a percentage of the untreated controls. Biofilms were incubated with 22 μM erythrosine (filled triangles), methylene blue (filled circles) or photofrin (filled squares) for 15 min then irradiated for 15 min. Error bars indicate the SD of 9–18 determinations. All treatments are statistically significantly different from one another ($P < 0.01$).

architecture.[28] By viewing the same image in fluorescence mode, the areas of erythrosine localization are revealed (Figure 1b). The two images are superimposable, indicating that erythrosine localizes primarily in the biomass of the biofilm, although whether the photosensitizer is associated with the bacterial cells, the extracellular matrix or a combination of the two is not known at present.

Biofilms of *S. mutans* were subjected to PDT using erythrosine, MB and photofrin as photosensitizers. Fifteen minutes white light irradiation of intensity 22.5–22.7 mW/cm^2 (in the wavelength range 500–650 nm) was used in this study as irreversible photobleaching of erythrosine, due to reactive oxygen species,[29,30] occurs at irradiation times >15 min (Figure 2).

Incubation of the *S. mutans* biofilms with 22 μM erythrosine alone and no irradiation had a negligible effect on the number of cfu compared with completely untreated controls, except for a slight reduction of 0.3 ± 0.1 $\log_{10}$ cfu in the earliest (48 h) biofilms tested. Clearly this is of clinical value, where it is desirable to target the PDT to regions of interest via careful administration of photosensitizer and light. Upon irradiation with white light for 15 min, the cfu count in the erythrosine-incubated biofilms was reduced by between 2.2 ± 0.2 $\log_{10}$ (for 48 h biofilms) and 3.0 ± 0.3 $\log_{10}$ cfu (for 288 h biofilms). PDT using 22 μM MB and 15 min irradiation resulted in $\log_{10}$ reductions in cfu of between 1.5 ± 0.1 (for 48 h biofilms) and 2.6 ± 0.2 (for 288 h biofilms). Finally, photofrin-mediated PDT had less effect on *S. mutans* biofilm cell viability than the other photosensitizers, with 0.5 ± 0.2 to 1.1 ± 0.1 $\log_{10}$ cfu reductions observed, again with the older biofilms being more susceptible to photoinactivation.

Direct comparisons of the efficacies of the three photosensitizers tested was achieved by normalizing the data from each experiment against the untreated controls, and expressing cell killing as percentage cell survival (Figure 3). Erythrosine was a significantly more effective ($P < 0.01$) photosensitizer than either photofrin or MB at all time points studied. Erythrosine is some 1.5 $\log_{10}$ to 2 $\log_{10}$ more efficient a photosensitizer of *S. mutans* biofilm bacteria than photofrin. The mixture of porphyrin oligomers that makes up photofrin is an established tumour photosensitizer[31] and has been used experimentally to treat bacteria, but its efficacy has often been reported to be inferior to more recently developed photosensitizers.[5] Figure 3

also shows that erythrosine-mediated PDT is 5–10 times more effective than MB-mediated PDT at killing *S. mutans* biofilm bacteria. This is extremely encouraging, as MB is an established and effective tumour[32,33] and antimicrobial photosensitizer.[27,34–36] MB-mediated antibacterial PDT has been demonstrated to damage bacterial cell DNA, and to a lesser extent the outer cell membrane.[37] *S. mutans* is reported to be photoinactivated mainly by membrane damage due to lipid peroxidation,[38] which is the likely mechanism of action of erythrosine-mediated PDT.

These results also highlight the trend in cell killing after PDT with all three photosensitizers, where 'young' biofilms are less susceptible than 'older' biofilms. This may be due to the temporal development of the putative circulatory systems typical of oral biofilms,[28] allowing greater access of photosensitizer to cells as the biofilm architecture develops. CLSM has been used to study the structure of microcosm dental plaques cultured in a CDFF.[39] The authors reported biofilms containing voids and channels through the extracellular matrices, which developed temporally over a 9 day period. Another possibility is that young biofilms are more metabolically active and may have more effective repair systems. Further work is needed to study this, particularly as a recent study using TBO as photosensitizer has reported that younger biofilms of *S. mutans* are more susceptible to PDT.[40] The reasons for this difference are unclear, but may be the properties of the photosensitizer used or differences in extracellular matrix composition.

In the present study we report the use of the dental plaque-disclosing agent erythrosine in the PDT of oral biofilm bacteria. We have demonstrated erythrosine to be a more effective photosensitizer than two established photosensitizers in terms of the killing of the cariogenic bacterium *S. mutans*, which highlights the excellent clinical potential of erythrosine-mediated PDT in the control and treatment of dental plaque biofilm bacteria. Further work is now required to evaluate more clinically acceptable treatment times (<15 min), perhaps by increasing the light intensity or by the use of more efficient light delivery systems such as light guides. Future studies will also investigate the possibility of using erythrosine-mediated PDT for the treatment of oral malignancies.

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Transparency declarations

None to declare.

Supplementary data

A colour version of Figure 1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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