The effect of light-activated antimicrobial agents on bacterial virulence factors and key modulators of inflammation

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Declaration

I, Sarah Tubby, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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This runs the risk of becoming overly sentimental, so I shall just offer you all a very British pat on the back. Please know that you are all very much appreciated.

Abstract

Photodynamic therapy is a promising new strategy for the treatment of superficial skin infections and periodontitis. A limitation of antibiotic treatment for these diseases is that even after successful killing of the infecting organism, secreted virulence factors may still be present and cause significant damage to host tissues. If light-activated antimicrobial agents can inactivate microbial virulence factors in addition to killing the pathogenic microorganisms, this would represent an advantage of photodynamic therapy over conventional treatment options. The light-activated antimicrobial agents methylene blue and tin chlorin e6 in combination with laser light of 665 and 633 nm respectively, were assessed for their antibacterial activity and ability to reduce the activity of selected virulence factors of *Staphylococcus aureus* and *Porphyromonas gingivalis*. In addition to successfully reducing the microbial burden, it was demonstrated that photosensitisation was able to cause significant reductions in the activity of a number of secreted and cell wall-associated virulence factors produced by these species when irradiated with laser light of the appropriate wavelength. Photosensitisation was also shown to reduce the biological activities of the proinflammatory cytokines tumour necrosis factor-alpha and interleukin-6, which are produced in response to infecting bacteria and are associated with damage to host tissues. The results of these studies indicate that light-activated antimicrobial agents may be useful in reducing the pathology associated with bacterial virulence factors and host-mediated inflammation when used as part of an antimicrobial treatment regimen.

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List of Abbreviations

°C	Degrees Celsius
ANOVA	Analysis of variance
Arg-protease/gingipain	Arginine-specific protease/gingipain
BHI	Brain Heart Infusion
CA-MRSA	Community-acquired meticillin-resistant *Staphylococcus aureus*
CFU	Colony-forming units
cm	Centimetre
CO_2	Carbon dioxide
CSE	Control standard endotoxin
D_2O	Deuterium oxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPP IV	Dipeptidyl-peptidase IV
Eap	Extracellular adherence protein
ECM	Extracellular matrix
Efp	Extracelllular fibrinogen-binding protein
$FeSO_4$	Ferrous sulphate
H_2SO_4	Sulphuric acid
HeNe	Helium-Neon
IMDM	Iscove's Modified Dulbecco's Medium
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6

IL-8	Interleukin-8
J	Joules
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
Lys-gingipain	Lysine-specific gingipain
mA	milliampere
MB	Methylene blue
Mg	Magnesium
MgCl2	Magnesium chloride
µg	Microgram
µL	Microlitre
µM	Micromolar
mL	Millilitre
mm	Millimetre
mM	Millimolar
MRSA	Meticillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MSSA	Meticillin-sensitive *Staphylococcus aureus*
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
ng/mL	nanograms per mL
nm	nanometre
OD	Optical density
PBS	Phosphate buffered saline
PFW	Pyrogen free water
pg/mL	Picrograms per mL

PMSF	Phenylmethanesulfonylfluoride
PtpA	Prolyl tripeptidyl protease
PVL	Panton-Valentine leukocidin
Rpm	Revolutions per minute
SCV	Small colony variant
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SnCe6	Tin chlorin e6
TBO	Toluidine blue O
TEMED	Tetramethylethylenediamine
TNF-α	Tumour necrosis factor-alpha
TNPAL-Sphingomyelin	Trinitrophenylaminolauroyl-Sphingomyelin
Tris	Tris(hydroxymethyl)aminomethane
Tris-Hcl	Tris-hydrochloride
W	Watts
w/v	Weight per volume

1 Introduction

1.1 Management of infectious diseases

1.1.1 The "antibiotic era"

Fleming's famous discovery of penicillin in 1928 is generally hailed as the turning point in the war against infectious diseases; prior to the discovery of antibiotics, infectious diseases were the leading cause of death in the United States, and morbidity and mortality secondary to infection was a common occurrence (Demain & Sanchez, 2009; Yoshikawa, 2002). Although discovered over a decade earlier, it is the industrial manufacture of penicillin in the 1940s that truly heralded the beginning of the "antibiotic era" (Bentley, 2005). The discovery of penicillin was history-altering: commonly fatal infections were suddenly treatable; indeed, the availability of penicillin was considered a major turning point in the fortunes of Allied forces during World War II (Wainwright, 2004). Since the end of the pre-antibiotic era, the average life expectancy has risen by 30 years, almost entirely attributable to the decline in mortality due to infectious diseases (Lederberg, 2000).

During the golden age of the so-called antibiotic era, antibiotic discovery reached fever pitch; by 1957, the tetracycline, macrolide, aminoglycoside, glycopeptide, polyene, polymixin and semi-synthetic penicillin classes had all yielded budding new therapies (Owens, 2008). The number of new antibiotic classes peaked in the 1960s and 1970s (Ball, 2007); however, with the success of antibiotics also came a sense of complacency. By the mid-1960s, infectious diseases were believed to be mostly conquered and research funding was diverted to other areas (Lederberg, 2000). Consequently, the pace of antibiotic development slowed, and even at the beginning of the 1970s, concerns were raised over the decline in new antibacterial compound discovery (Bloom, 1971). The rate of antibiotic discovery has continued on its downward trajectory ever since; only five new antibacterial agents were

approved in the US between 2003 and 2007, a 50% reduction compared with applications approved between 1993 and 1997 (Spellberg *et al.*, 2008).

A number of factors have contributed to the decline in the development of new antibiotic agents; however, the lower return on investment for pharmaceutical companies compared with other disease areas is perhaps the highest contributing factor (Ball, 2007; Payne *et al.*, 2007; Spellberg *et al.*, 2008). There has been a clear switch in the pharmaceutical industry towards developing therapies for chronic diseases such as cancer and arthritis, which present more lucrative markets for pharmaceutical companies. Patients generally require a week of treatment for most antibiotics, compared with a year or more for cancer therapies and sometimes lifelong use in the case of antihypertensive agents (Ball, 2007; Katz *et al.*, 2006). From a financial standpoint, the pharmaceutical industry clearly has more to gain from turning its sights elsewhere.

Antimicrobial development has also become more complex, as increasing bacterial resistance to antibiotics limits exploitable target sites. Just two antibacterial agents with novel mechanisms of action, linezolid and daptomycin, have been developed in the last 20 years (Owens, 2008).

1.1.2 The rise of antimicrobial resistance

There is no doubt that the discovery and manufacture of antibiotics was crucial in the fight against infectious diseases; however, resistance to the newly-discovered antibacterial agents was swift in coming. The first case of resistance to penicillin was reported in 1940, three years before the drug was approved in the US (Bush, 2004). In fact, by just 1947, the rise in penicillin-resistant bacteria was described as "somewhat alarming" by one author (Barber, 1947).

The widespread use of penicillin led to the emergence of penicillinase-producing strains of *Staphylococcus aureus* in the mid 1940s, which had become pandemic by the 1950s (DeLeo & Chambers, 2009). By 1955, it was reported that almost 75% of *Staphylococcus* strains isolated from hospital patients were penicillin-resistant; the same year The New York Times sensationally declared that the "war on bacteria [was] seen as backfiring" (Creager, 2007). By 2004, it was estimated that over 70% of pathogenic bacteria were resistant to at least one antibiotic, and penicillin resistance as a result of β-lactamase production is now almost 100% amongst staphylococci (Katz *et al.*, 2006; Perera & Hay, 2005).

Resistance to antibiotics can be classified as intrinsic or acquired; intrinsic resistance is generally due to the absence or bypassing of the antibiotic target site, whereas acquired resistance depends on the acquisition of genetic material that may encode inactivating enzymes, altered target site, or antibiotic exclusion mechanisms (Perera & Hay, 2005). It is generally considered that resistance to naturally-occurring antibiotics evolved in bacterial species that encountered these products in their natural environment, which acted as a reservoir for resistance determinants. Resistance was then transferred to pathogenic bacteria via the transfer of genetic material following the introduction of antibiotics into clinical and agricultural use, which provided a selective pressure for their maintenance in the bacterial population. Resistance to synthetic antibiotics is generally thought to arise as a result of mutations and then initially spread by vertical transmission and clonal dissemination. The rapid spread of antibiotic resistance to taxonomically divergent bacteria has been associated with horizontal gene transfer, which is enhanced by the use of antibiotics at sub-inhibitory concentrations (Aminov & Mackie, 2007; Demain & Sanchez, 2009).

The rise of antimicrobial resistance is compounded by the inappropriate use of antibiotics (e.g. for viral infections), non-compliance of patients, and widespread

antibiotic use in animal husbandry (Dai *et al.*, 2009). The inappropriate use of antibiotics is considered a particularly important factor in the rise of antimicrobial resistance; reports have documented inappropriate prescription rates varying from 35 to 53% (Ball, 2007).

1.1.3 The search for novel therapeutic strategies

The global rise of antibiotic resistance has led to a major effort to develop new therapeutic strategies. Four new antibiotics have been approved in the last five years: tigecycline, daptomycin, linezolid and quinupristin-dalfopristin. However, these antimicrobial agents are costly, associated with adverse events, and of limited availability (Daum, 2007; Manfredi & Sabbatani, 2010). Other currently available antibiotics are associated with adverse effects such as gastrointestinal disturbance, rash, and photosensitivity, in addition to hepatic-, renal- and ototoxicity (Demain & Sanchez, 2009).

Due to the threat of antibiotic resistance and the toxicity of current antibiotic therapies, there has been a drive towards discovering novel treatment options that have a more favourable safety profile and do not select for bacterial resistance. A novel therapy would ideally act on multiple targets in order to avoid the development of resistance. Although this can be achieved by using combinations of antibiotics with differing mechanisms of action, this approach is associated with high cost, and drug interactions and formulation issues must also be considered (Wainwright, 2010).

The therapeutic avenues that are being explored in the search for new antimicrobials are many and varied. One novel antimicrobial strategy that has received much interest is the use of light in combination with a light-activated compound, termed photodynamic therapy (PDT), which will be discussed in more detail in later chapters.

In particular, alternative topical treatments for skin infections have been the subject of much investigation, in part due to the rise of meticillin-resistant *S. aureus* (MRSA). One such strategy is the use of antimicrobial peptides as topical agents in the treatment of skin infections (Schittek *et al.*, 2008). Antimicrobial peptides form part of the innate immune system, and have been investigated due to their broad spectrum of activity and lower propensity to select for resistance (Sang & Blecha, 2008). Bacteriophage therapy has also been suggested as a novel therapeutic option for bacterial infections, particularly for the treatment of chronic wound infections and for MRSA decolonization. The specificity of bacteriophages and their ability to rapidly kill bacteria regardless of antibiotic resistance phenotype has attracted interest in their therapeutic potential (Deresinski, 2009). Silver sulfadiazine is believed to be antibacterial due to the release of silver from the preparation and has also been investigated as a topical antibacterial agent; however it has been associated with burning sensations, rash and skin discolouration (Gelmetti, 2008).

There has also been a renewed interest in "natural" topical antimicrobial approaches, such as honey and other bee-derived products such as propolis (Raghukumar *et al.*, 2010). Honey has attracted particular interest due to its potential for use in wound and surgical infections; however, there is currently a lack of robust clinical data to support its use (Al-Waili, 2004; Kwakman *et al.*, 2008; Moore *et al.*, 2001). Another area that has received a resurgence of interest is the medical use of maggots for the treatment of bacterial wound infections. It has been suggested that larvae may be useful in the removal of necrotic tissue, the facilitation of healing, and the eradication of MRSA (Bowling *et al.*, 2007). Maggot secretions/excretions have also been investigated for their ability to inhibit biofilm formation by various bacterial species, including *S. aureus* (Cazander *et al.*, 2010). Although antimicrobial action has been observed, it is not clear whether the

maggots and/or their secretions/excretions have any direct antibacterial action and conflicting reports exist in the literature (Cazander *et al.*, 2009; Jaklic *et al.*, 2008).

Although most current therapeutic strategies rely on direct bacteriocidal or bacteriostatic activity, the inactivation of virulence by targeting a microorganism's pathogenicity determinants has arisen as an alternative approach for the treatment of bacterial infections. According to Escaich, virulence is defined as "the relative capacity of a microbe to cause damage in a host". The definition of virulence therefore covers not only toxins, but also the mechanisms that contribute to colonisation of the host and allow a pathogenic organism to proliferate. As the targets are specific for mechanisms responsible for pathogenicity, a therapy aimed at inactivation of virulence should therefore not affect the normal microbiota of the host and would not select for resistance in other microorganisms (Escaich, 2008). By inhibiting the ability of the pathogen to damage the host, the immune system may then clear the infection (Clatworthy *et al.*, 2007).

Pathogenesis determinants may be divided into different groups, each of which represents a potential target for inhibitors of bacterial virulence: adhesion and colonisation, systems for nutrient acquisition and uptake, regulatory functions (e.g. quorum sensing), and resistance to the innate immune response (Escaich, 2010). Historically, the use of antibodies against diphtheria, tetanus and botulinum toxins, and the inhibition of *Clostridium difficle* toxin delivery by cholestyramine may be considered therapies targeted to virulence factors (Clatworthy *et al.*, 2007).

A number of strategies aimed at each of these groups are currently being investigated in the laboratory, and include peptidomimetic molecules to block pili synthesis, inhibition of iron uptake, synthetic ligands to block quorum sensing, and inhibition of antioxidant biosynthesis (Escaich, 2010). A major concern facing potential therapies targeted at specific bacterial virulence factors is one of economy.

An agent developed to inhibit a single virulence factor of a particular pathogen would have an extremely narrow range of activity, and would consequently offer little economic incentive for a pharmaceutical company (Clatworthy *et al.*, 2007). Therefore, it would be desirable for a therapy targeting pathogenicity to inactivate several virulence factors. In addition, as many such strategies do not have direct antimicrobial action, a therapy that combines antibacterial activity and also reduces a pathogen's virulence potential would be advantageous.

The antibiotic polymyxin B has been shown to affect virulence potential by inhibiting the release of pertussis toxin from *Bordetella pertussis* (Craig-Mylius & Weiss, 2000); it also shows lipopolysaccharide (LPS) -neutralising properties, although its toxicity limits its clinical use as an antibacterial (Bhor *et al.*, 2005). Sub-inhibitory concentrations of antibiotics have been shown to inhibit multiple virulence mechanisms, such as LPS and quorum sensing in *Pseudomonas aeruginosa* by the macrolides erythromycin and azithromycin, and inhibition of adherence to host cells by quinolones (Sonstein & Burnham, 1993; Tateda *et al.*, 2007). However, the use of antibiotics carries the associated risk of the development of bacterial resistance, and at therapeutic concentrations, macrolide antibiotics are neither bacteriostatic or bacteriocidal against *P. aeruginosa* (Tateda *et al.*, 2000). There is therefore a significant clinical need for a therapy that is both bacteriocidal and also inactivates bacterial virulence factors, without the concomitant risk of bacterial resistance.

1.2 Photodynamic therapy

In the search for novel antibacterial strategies, the use of light-activated antimicrobial agents has emerged as a promising candidate. Light has been used as a therapeutic agent since ancient times; its use can be traced back to ancient Egypt, India and China, where light was reportedly used to treat skin diseases, cancer and psychosis (Ackroyd *et al.*, 2001).

The use of a photosensitising agent in combination with light was first believed to be employed in India over 3000 years ago for the treatment of vitiligo (Ackroyd *et al.*, 2001). The photodynamic killing of microorganisms was first demonstrated in 1900 by Raab, who documented the antiprotozoal activity of acridine and visible light, and the term "photodynamic" was coined shortly afterwards in 1907 by Von Tappeiner and Jesionek (Dolmans *et al.*, 2003). PDT has also received much interest as a therapeutic option for the treatment of cancer; the first documented use for this indication was in 1903, in which topical eosin and white light was used to treat skin tumours (Ackroyd *et al.*, 2001).

1.2.1 Mechanism of action

Photodynamic inactivation relies upon the capacity of a dye, known as a photosensitiser, to be activated by light of a specific wavelength to generate reactive oxygen species that are toxic to microorganisms. Upon irradiation, the photosensitiser undergoes a transition from a low energy ground state to a higher energy triplet state, which can then react with biomolecules to produce free radicals (type I reaction), or with molecular oxygen to produce highly reactive singlet oxygen (type II reaction) (Maisch, 2007). These processes are outlined in Figure 1.1.

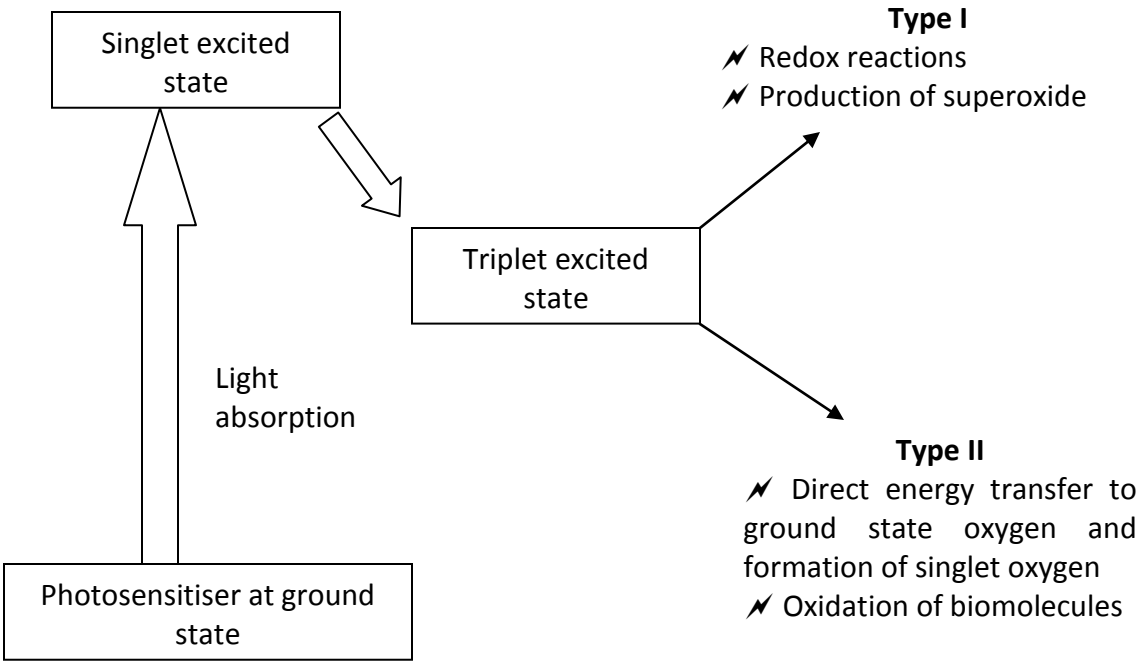

Figure 1.1 Mechanisms of photosensitiser action

(Modified from Wainwright, 2000)

Singlet oxygen produced by type II reactions can oxidise many biological structures such as proteins, nucleic acids and lipids. Although DNA damage has been shown to occur, it is not believed to be the principle cause of bacterial death; rather, death is thought to be due to damage to the cytoplasmic membrane and the subsequent leakage of cellular contents and inactivation of membrane transport systems and enzymes (Hamblin & Hasan, 2004). The diffusion of singlet oxygen is limited to a maximum of 30 nm, meaning that the localisation of the photosensitiser determines the site of action and reduces damage to host tissues at distant sites (Plaetzer *et al.*, 2009).

Type I reactions cause membrane damage by the formation of lipid hydroperoxides and hydroxyl radicals, which can react with biomolecules or combine to produce cytotoxic hydrogen peroxide *in situ*; however it is the singlet oxygen-producing type II reactions that are generally considered to be the major pathways in photodynamic

killing (Wainwright, 2000). As the mechanism of action of microbial killing is non-specific and multiple sites are affected, it is considered unlikely that resistance will evolve, thus representing a significant advantage over conventional antibiotic treatment where resistance is an ever-increasing problem (Wainwright, 2005).

A very desirable feature for PDT is the potential for inactivation of virulence factors, particularly secreted proteins, by reactive oxygen species (Hamblin & Hasan, 2004). Inactivation of membrane enzymes and receptors is also possible due to type I reactions at the cytoplasmic membrane (Wainwright, 1998).

The biological activity of certain virulence factors produced by some species of Gram-negative bacteria has been shown to be successfully reduced by photodynamic action. The inhibitory effect of red laser light and the photosensitiser toluidine blue O (TBO) on virulence factors from *Escherichia coli, P. aeruginosa* (Komerik *et al.*, 2000) and *Porphyromonas gingivalis* (Packer *et al.*, 2000) has previously been demonstrated; however the effect of photosensitisation on staphylococcal virulence factors has not yet been fully investigated. To date, photodynamic inactivation of biofilms is the only staphylococcal virulence determinant to have been reported in the literature (Sharma *et al.*, 2008). In addition, MRSA has been shown to be susceptible to photodynamic inactivation (Griffiths *et al.*, 1997), thus strengthening the position of photodynamic therapy as a promising new antimicrobial strategy for the treatment of staphylococcal infections.

1.2.2 Light sources

The light source used for PDT generally depends on the depth of tissue penetration required, as the depth of light penetration increases in the visible and near-infrared regions, and the optimum wavelength at which maximum singlet oxygen production is achieved from a particular photosensitiser (Mitton & Ackroyd, 2008). The light source can either be coherent (i.e. laser light), or non-coherent.

1.2.2.1 Laser light

Laser is an acronym for Light Amplified by Stimulated Emission of Radiation, and is the most commonly used light source for PDT (Pervaiz & Olivo, 2006). Stimulated emission was first documented by Albert Einstein, and according to Silfvast & Robert, occurs "when a beam of light passes through a medium and stimulates atoms within the medium to radiate more light in the same direction, and of the same wavelength as the original beam." A laser device utilises mirrors to further amplify the beam, which is then emitted from the device via a partially transmitting mirror or a mirror with a small hole (Silfvast & Robert, 2001).

The light output from a laser device is collimated, coherent and highly monochromatic (Baxter, 1994). Monochromaticity and coherence are properties that make laser light an ideal light source for PDT: monochromaticity allows a laser to be matched to a photosensitising agent that has peak absorption at the wavelength of the laser light, and coherence allows the delivery of the laser light via fibre optics (Calin & Parasca, 2009). The high irradiance of laser light is also advantageous as shorter exposure times are required for a therapeutic effect. However, lasers are relatively expensive and high maintenance compared with non-coherent light sources (Choudhary *et al.*, 2009).

The most commonly used lasers for PDT are pumped dye lasers, such as argon dye and potassium-titanyl phosphate dye lasers. Argon dye lasers have been much utilised, as the wavelength of the laser light may be altered to match the peak absorbance of a photosensitiser (Ackroyd *et al.*, 2001). Argon dye lasers were used in early anticancer PDT investigations and were previously the standard in clinical PDT; however the cost, immobility, and size of the units limits their practicality, as does the requirement for an external cooling system and power source (Mang, 2004; Mitton & Ackroyd, 2008).

Another commonly used laser is the helium-neon (HeNe) laser. The HeNe laser system was the first gas laser to be developed (in 1961), and emits red laser light with a wavelength of 633 nm. This laser has many applications in addition to PDT, including scanners, printers and surveying, and can be manufactured in a variety of sizes (Silfvast & Robert, 2001). The use of HeNe laser light has been well documented in antimicrobial PDT, in particular for the photodynamic inactivation of *S. aureus* and *P. aeruginosa* (Calin & Parasca, 2009). This wavelength is ideal for use in PDT as haemoglobin does not absorb in this region (Vladimirov *et al.*, 2004). In addition, HeNe laser light does not penetrate far below the skin surface (approximately 0.5 mm), and therefore does not affect deeper tissues; it can also have beneficial biostimulatory effects on the epidermis and upper dermis (Hawkins & Abrahamse, 2005).

Semiconductor diode lasers have been hailed as the "new generation of laser systems", as this type of laser system is user-friendly, compact, lightweight, and easily portable (Mang, 2004). Diode lasers are now commonly used for PDT, and have the additional advantages of being relatively low cost and use normal mains voltage as a power source (Choudhary *et al.*, 2009; Meisel & Kocher, 2005). Diode lasers have been commonly utilised in periodontal PDT investigations, but have also been used for dermatological indications (Fimple *et al.*, 2008; Garcez *et al.*, 2010; George & Kishen, 2008; Kim *et al.*, 2007; Salah *et al.*, 2009; Teichert *et al.*, 2002). Diode lasers are approved for clinical use in certain therapeutic indications throughout Europe. As the wavelength cannot be altered, the laser system must be matched with a particular photosensitiser (Mitton & Ackroyd, 2008).

1.2.2.2 Incoherent light sources

Natural light is incoherent, polychromatic and divergent (Wilson, 1993). One of the first documented uses of sunlight as a therapeutic agent is that of Herodotus in the 2nd century BC, who believed exposure to sunlight was important for the restoration of health (Daniell & Hill, 1991). Photodynamic inactivation of microorganisms has been demonstrated using incoherent light sources such light-emitting diodes and tungsten, halogen and xenon lamps (Calin & Parasca, 2009). Incoherent light sources are easy to use and comparatively cheap (Ackroyd *et al.*, 2001). Incoherent light has several advantages as a light source for PDT: the polychromatic nature of the light means that different photosensitisers with different absorption maxima may be used, and large illumination fields may be achieved for use over large areas, which is particularly relevant to dermatological indications. Incoherent light sources are also relatively cheap, easy to operate and are readily available (Choudhary *et al.*, 2009). However, the disadvantages of incoherent light are low intensity, difficulties in management of light dose, and significant thermal effects (Pervaiz & Olivo, 2006). The low intensity of incoherent light means that long exposure times are generallly required for a therapeutic effect to be achieved (Wilson, 1993).

1.2.3 Photosensitisers

The use of an exogenous photosensitising agent can be traced back to India in 1400 BC and the use of psoralens for the treatment of vitiligo (Daniell & Hill, 1991). Haematoporphyrin is believed to be the first modern photosensitiser to be discovered in the laboratory: it was first isolated in 1841; however, its properties as a photosensitiser were not discovered until 1911 (Mitton & Ackroyd, 2008). Most photosensitisers consist of a heterocyclic ring structure, with structural similarities to chlorophyll and haemoglobin (Ackroyd *et al.*, 2001).

There are several photosensitisers currently approved for clinical use for certain cancer and dermatological indications, including: Photofrin (porfimer sodium), Levulan, Benzvix, Hexvix and Metvix, (5-aminolevulinic acid and various esters), Lutex (lutetium texaphyrin), Visudyne (verteporfin) and Foscan (5, 10, 15, 20-tetra(3-hydroxyphenyl)-2,3-dihydroporphyrin) (Ackroyd *et al.*, 2001; Detty *et al.*, 2004). Methylene blue has been widely utilised in medicine for indications other than PDT, and is also commercially available for clinical use in periodontal and nasal decolonisation PDT applications (Jose *et al.*, 2010; Street *et al.*, 2009; Wainwright, 2010).

Favourable attributes for a photosensitiser to be used in an antimicrobial PDT regimen are: a broad spectrum of activity, activity independent of antibiotic resistance, selectivity for microorganisms over host tissues, low probability of selecting for resistance to PDT, low mutagenicity potential, availability of formulations for delivery, and the availability of a suitable light source (Jori *et al.*, 2006).

1.2.3.1 Methylene blue

Methylene blue is a phenothiazinium dye, and one of the photosensitisers described in this thesis. Methylene blue strongly absorbs light in the 600-700 nm region, giving the dye its characteristic colour, with maximum absorption occurring at 656 nm (Wainwright, 2000). Methylene blue was initially synthesised in 1876 for use in the textile industry, and was first employed in medicine in 1891 as a treatment for malaria (Wainwright, 2005). The structure of methylene blue is shown in Figure 1.2.

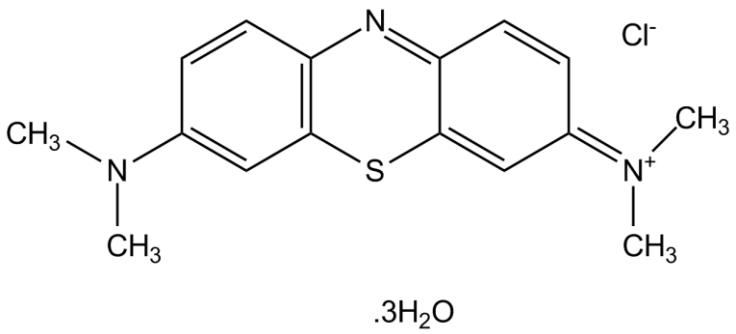

Figure 1.2 The chemical structure of methylene blue

Phenothiazinium-based photosensitisers have a core structure consisting of a planar tricyclic heteroaromatic ring system and at physiological pH are generally cationic, allowing them to target the negatively-charged bacterial membrane (Phoenix & Harris, 2003). Cationic photosensitisers are useful for antimicrobial PDT as they are effective for the photodynamic killing of both Gram-positive and Gram-negative microorganisms. In addition, they are more selective for bacterial cells compared with mammalian cells as cationic molecules are taken up comparatively slowly by mammalian cells, and thus damage to host cells may be limited by carrying out PDT within a short time of applying the photosensitiser (Dai *et al.*, 2009).

The use of methylene blue is well established in medicine, where it is used for the routine staining of vital organs and the treatment of septic shock (Wainwright, 2000). The concentrations used for staining are usually 1% w/v, which equates to 27 mM. As the concentrations of photosensitiser required for the photodynamic inactivation of bacteria are in the micromolar range, this is favourable when considering toxicity concerns (Wainwright, 2000). The minimum lethal concentration of methylene blue for the photosensitisation of *S. aureus* has been reported to be 1 µM, and therefore well below the concentrations used for medical staining procedures (Wainwright *et al.*, 1997).

Methylene blue has been shown to cause photodamage to the outer membrane, cell wall, ribosomes and nucleic acids of bacteria (Wainwright, 2010). At increasing concentrations, methylene blue may aggregate and form dimers, which may cause a shift in the absorption maximum of the photosensitiser. It has been proposed that dimerisation is further induced at the bacterial cell surface due to electrostatic interactions between methylene blue and negatively charged polymers on the bacterial cell surface, and that these dimers are also involved in cell photodamage, as well as monomeric species (Usacheva *et al.*, 2003).

1.2.3.2 Tin chlorin e6

The second photosensitiser used in the work described in this thesis was the anionic metalloporphyrin tin chlorin e6, the structure of which is shown in

Figure 1.3. Chlorin e6 is a derivative of chlorophyll A and demonstrates maximum absorption at 654 nm (Detty *et al.*, 2004). Tin chlorin e6 shows a prominent absorption peak at 634 nm and has been shown to have a 2-fold greater quantum efficiency of singlet oxygen generation compared with metal-free chlorin e6 (Gil-Tomas *et al.*, 2011; Rakestraw *et al.*, 1990). The addition of tin to the chlorin structure increases the stability of the compound against photodecomposition, therefore increasing the lifetime of the photosensitiser upon illumination (Strong *et al.*, 1994). Additionally, free chlorin is associated with long-term skin photosensitisation and high doses are required for therapeutic activity (Detty *et al.*, 2004).

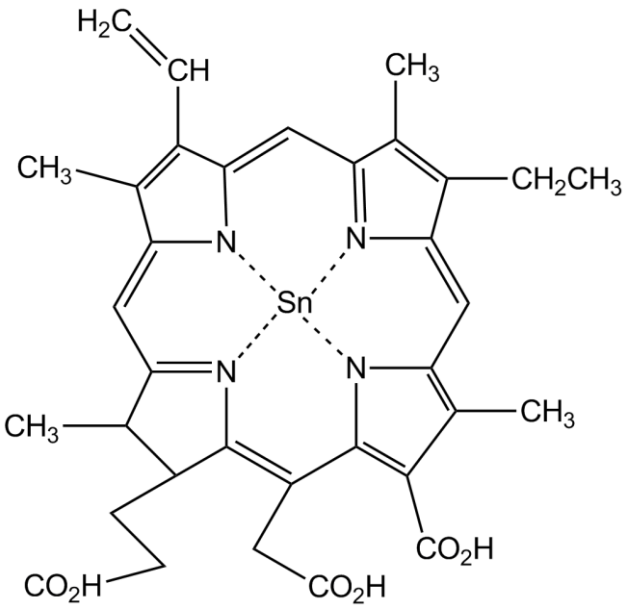

Figure 1.3 The chemical structure of tin chlorin e6

Tin chlorin e6 has been shown to be an effective photosensitiser for the photodynamic killing of meticillin-sensitive and meticillin-resistant *S. aureus*, (Embleton *et al.*, 2002). It has also demonstrated activity against Gram-negative microorganisms, as antibody-conjugated tin chlorin e6 has been shown to be successful in the selective killing of *P. aeruginosa* (Friedberg *et al.*, 1991; Lu *et al.*, 1992). Disruption and reduction in viability of biofilms formed by the Gram-negative periodontal pathogen *Aggregatibacter actinomycetemcomitans* has also been reported (Suci *et al.*, 2010). The photosensitiser also has applications outside the field of antimicrobial PDT. A tin chlorin e6-monoclonal antibody-dextran conjugate has been used for the *in vitro* photolysis of malignant melanoma cells, suggesting it may also be of use in anticancer PDT (Rakestraw *et al.*, 1990).

1.2.4 Clinical applications of PDT

PDT has most extensively been studied for use as a treatment modality for various cancers and has been approved for clinical use in this setting. PDT using Photofrin (hematoporphyrin derivative) was first approved in Canada for the prophylaxis of bladder cancer in 1993; since then, PDT has been approved by regulatory bodies for various cancer indications in Europe, the US and Japan. PDT is also employed in the treatment of age-related macular degeneration and is being investigated for a number of other applications, including arthritis, cardiovascular diseases and dermatology (Dolmans *et al.*, 2003).

1.2.5 Antimicrobial photodynamic therapy

Although the concept of antimicrobial photodynamic killing had been known since the time of Raab's discovery, the advent of the antibiotic era prevented the potential of photodynamic therapy for the treatment of microbial infections from being realised (Jori, 2006). Currently, no antimicrobial PDT regimen has been approved by the US Food and Drug Administration for clinical use (Cassidy *et al.*, 2009). However, the Periowave™ and MRSAid™ systems have been approved in Canada and the European Union, which utilise laser light of 660-675 nm in combination with a formulation of methylene blue. The Periowave™ system is indicated for the treatment of chronic periodontitis in adults as part of a periodontal health maintenance program (Ondine Biomedical, 2010c), and the MRSAid™ system is approved for the nasal decolonisation of MRSA carriers (Street *et al.*, 2009), (Ondine Biomedical, 2010b). The HELBO® photodynamic system is also commercially available for periodontal PDT, which uses phenothiazine chloride as a photosensitising agent and diode laser light of 670 nm (Jose *et al.*, 2010).

The main issues that must be addressed in order for PDT to be successfully used in the treatment of localised bacterial infections are the selectivity of the treatment for

bacterial cells in order to avoid unacceptable damage to host cells, the efficacy of the treatment to sufficiently kill the pathogen, and the prevention of regrowth of the pathogen if eradication is not 100% (Dai *et al.*, 2009). Repeat applications of PDT may be useful in preventing bacterial regrowth, and have not been associated with resistance (Lauro *et al.*, 2002). The use of selectivity-enhancing agents to produce more targeted photosensitisers may help to overcome the issue of selectivity. The selectivity of a photosensitiser may be increased by conjugating an antibody or peptide to the molecule, or through the use of bacteriophages (Cassidy *et al.*, 2009; Embleton *et al.*, 2002; Embleton *et al.*, 2005). However, it has been postulated that the light doses required for microbial killing have a negligible effect on neighbouring host tissues, and host tissues can also undergo repair if necessary following treatment (Wilson, 2004).

The photodynamic killing of microorganisms has other applications beyond the direct treatment of infections. There has been interest in the incorporation of photosensitisers into antimicrobial coatings in order to reduce the incidence of nosocomial infections acquired as a result of contaminated surfaces in hospitals (Decraene *et al.*, 2008b). Coatings containing the photosensitisers TBO and rose bengal have been shown to achieve significant killing of *S. aureus* when illuminated with a domestic light source, raising the possibility of self-disinfecting surfaces, which would be of great benefit in frequently contaminated environments such as hospital clinics (Decraene *et al.*, 2008a).

1.2.5.1 Skin and soft tissue infections

Skin and soft tissue infections represent an area where new therapies are urgently required. Surgical wound infections are commonly associated with antibiotic-resistant strains of bacteria, and account for a quarter of all nosocomial infections (Dai *et al.*, 2009). The mainstays of treatment for infected wounds are antibiotics and surgical debridement; however, with the increasing problem of antimicrobial

resistance to antibiotics and the invasive nature of surgery, PDT could present an attractive alternative (Lipovsky *et al.*, 2008).

S. aureus is the most commonly isolated pathogen from skin and skin structure infections, being isolated in 46% of cases, and therefore represents a relevant target for the studies described in this thesis (Lee *et al.*, 2005). Uncomplicated skin and skin structure infections include impetigo, cellulitis, folliculitis and abscesses (Lee *et al.*, 2005). Photodynamic therapy is particularly appropriate for the treatment of superficial staphylococcal infections, as the site of infection is likely to be accessible for topical application of a photosensitiser and its subsequent irradiation (Wainwright, 1998). The susceptibility of *S. aureus* to lethal photosensitisation has been well documented, and there is much scope for the investigation of the effect of photosensitisation on the activities of the numerous virulence factors produced by this pathogen.

Complicated skin and skin structure infections are rapidly-spreading, associated with high morbidity and mortality rates, and usually require hospitalisation (Nichols, 1999; Raghavan & Linden, 2004). Due to the serious nature of the infections, it is unlikely that PDT would be the only treatment modality employed; however, it could be useful for reducing the bacterial burden and consequently reducing the level of surgical debridement required (Dai *et al.*, 2009). Broad-spectrum antibiotic therapy is often initiated empirically (Lee *et al.*, 2005); as broad-spectrum antimicrobials can be associated with side effects, a more specific treatment would be advantageous to the patient (Demain & Sanchez, 2009).

Topical mupirocin is generally the drug of choice for the treatment of skin infections and recommended for the decolonisation of the nares in patients colonised with MRSA (Coia *et al.*, 2006; Jacobs *et al.*, 2007). Traditional topical antimicrobial therapy also has associated problems. The use of topical antibacterial agents is

associated with several disadvantages, including the development of bacterial resistance, contact dermatitis, low penetration and the potential for disruption of the normal skin microbiota. High-level resistance to mupirocin has been well documented in both meticillin-sensitive and meticillin-resistant strains of *S. aureus* (Hogue *et al.*, 2010; Liu *et al.*, 2010; Perez-Roth *et al.*, 2006; Rasmussen *et al.*, 2010). Although less likely to select for bacterial resistance, some topical antiseptics (e.g. iodine and hydrogen peroxide) may be associated with host toxicity and/or hypersensitivity (Englander & Friedman, 2010; Gelmetti, 2008). Topical antibiotics are also used for the treatment of impetigo, and are the treatment of choice for external ear infections; these treatment areas therefore also represent circumstances in which novel antimicrobial strategies such as PDT may be employed (Gelmetti, 2008).

The application of PDT to the treatment of superficial infections has several advantages over standard antibiotic therapy. PDT is fast-acting and selective; laser light may be applied only to the area of interest via a fibre optic, resulting in a beam size of only mm in diameter. The specificity of PDT may be further increased by the conjugation of antibodies to the photosensitising agent, which would allow more specific targeting of the causative organism and consequently less damage to host cells. The conjugation of targeting molecules to the photosensitiser has been successfully demonstrated for tin chlorin e6 (Embleton *et al.*, 2002; Embleton *et al.*, 2005). However, this approach would be costly and would also require the infecting organism to be correctly identified prior to treatment. Zeina *et al*. found that treatment of keratinocytes with methylene blue and visible light did not result in DNA damage to the cells, indicating that the treatment is not associated with high levels of genotoxicity (Zeina *et al.*, 2003). If PDT does not cause appreciable damage to human cells, then such targeting methods may not be necessary.

Photodynamic treatment is also associated with lower systemic toxicity compared with oral antibiotic agents. The photosensitiser may be applied topically, thus avoiding damage to cells at sites distant to the treatment area. In the case of antimicrobial PDT, the treatment does not rely on actively-growing cells to be effective, as the mechanism of killing is structural rather than related to the growth of the organism. Perhaps most crucially, the potential for resistance is very low compared with antibiotic therapy. As PDT is non-specific and multiple sites are affected, there is a low possibility that bacterial resistance would evolve (Wainwright, 2010).

Laser light has been shown to induce epithelialisation and consequent wound closure; HeNe laser light in particular has been shown to cause tissue regeneration and high levels of mitosis in keratinocytes and fibroblasts, which may be beneficial in the healing of wounds following treatment (Hawkins & Abrahamse, 2005; Lipovsky *et al.*, 2008) Irradiation with laser light has also been shown to induce activation of a number of cell types involved in bactericidal activity, such as leukocytes. Irradiation with low power laser light can cause photoreactivation of superoxide dismutase and stimulate the release of nitric oxide, thus enhancing the antimicrobial effect of photodynamic treatment (Vladimirov *et al.*, 2004). HeNe laser light also does not penetrate tissue as deep as laser light of longer wavelengths, and therefore causes less damage to the skin and skin structures.

1.2.5.2 Oral infections

The treatment of oral infections represents the fastest-growing area of antimicrobial PDT. Three companies have marketed photosensitiser/laser systems for the treatment of periodontitis and endodontic infections, including the Periowave™ and HELBO® systems, marketed by Ondine Biopharma and HELBO Photodynamic Systems, respectively (Dai *et al.*, 2009; Jose *et al.*, 2010). The periodontal pocket is easily accessible and therefore well-suited to treatment by PDT, and the rapid killing

of microorganisms associated with PDT overcomes the problem of maintaining high concentrations of the therapeutic agent in the disease lesion seen with antibiotic treatment (Wilson, 2004).

Wilson *et al.* have shown that significant eradication of periodontopathogens from subgingival plaque can be achieved using the photosensitiser TBO and HeNe laser light, regardless of the presence of blood (Wilson *et al.*, 1993c). Methylene blue is well-established as an effective photosensitiser for the photodynamic killing of *P. gingivalis*, an important periodontopathogen (Wilson *et al.*, 1993a). Methylene blue has also successfully been shown to be effective in the killing of periodontal pathogens in biofilms (Dobson & Wilson, 1992; Fimple *et al.*, 2008) . In addition, methylene blue-based PDT has been proposed for the treatment of oral candidiasis (Teichert *et al.*, 2002). PDT has several applications in the field of periodontics, including the treatment of aggressive, refractory or recurrent periodontitis, as an adjunct to scaling and root planing, and disinfection of roots and furcation areas during surgical procedures (Jose *et al.*, 2010).

The use of PDT for endodontic disinfection (disinfection of root canals) has also been proposed as an adjunct to conventional endodontic treatment, and the combination of PDT and mechanical debridement plus antiseptic irrigation has been shown to significantly reduce bacterial load and bacterial regrowth in infected root canals compared with either treatment alone (Garcez *et al.*, 2007). Methylene blue in combination with laser light of 665 nm has demonstrated efficacy against several endodontic pathogens in both planktonic phase and multispecies root canal biofilms (Fimple *et al.*, 2008; Soukos *et al.*, 2006).

The issue of photosensitiser delivery for the photodynamic treatment of oral infections has been raised, as photosensitisers such as methylene blue would stain the buccal mucosa and teeth and be cosmetically undesirable (Cassidy *et al.*, 2009).

However, PDT also has advantages over other treatments for periodontal disease such as scaling, mouth washes and surgery in that it is fast, relatively painless, does not require anaesthesia and does not interfere with taste (Jose *et al.*, 2010; Soukos *et al.*, 2005).

PDT using a chlorin e6 conjugate in combination with endodontic treatment has recently been shown to significantly reduce the numbers of antimicrobial-resistant bacteria (predominantly *Enterococcus, Prevotella* and *Porphyromonas* species) in necrotic periapical lesions compared with endodontic treatment alone; in fact, complete eradication of bacteria was achieved following the combination treatment (Garcez *et al.*, 2010). This study demonstrates that PDT may be of use in antibiotic-resistant oral infections.

1.3 Microbial species studied in this project

The bacterial species studied in this project are *S. aureus* and *P. gingivalis*. Skin infections and periodontal diseases represent two diseases that are well-suited to treatment with light-activated antimicrobial agents; as previously discussed, *S. aureus* and *P. gingivalis* are considered important aetiological agents in skin infections and periodontitis, respectively. Therefore, these two species were the focus of this project.

1.3.1 *Staphylococcus aureus*

S. aureus is a facultatively aerobic, Gram-positive coccus belonging to the *Staphylococcaceae* family, so named due to its microscopic appearance as grape-like clusters (see Figure 1.4) (from the Greek *staphule*, meaning “bunch of grapes”) and the golden colour of colonies formed on agar (from the Latin *aureus*) as a result of carotinoid production (Bloch, 2001). Staphylococci are catalase-positive and differentiated from the Gram-positive, catalase-positive genus *Micrococcus* by a

lower G+C content (33-40 mol% compared with approximately 70 mol% for micrococci) (Götz *et al.*, 2006). The main characteristics that distinguish *S. aureus* from other staphylococci are the production of coagulase and the ability to ferment mannitol; *S. aureus* is also β-haemolytic due to the production of haemolysins, and can grow in high salt conditions of 7.5 to 10% sodium chloride (Somerville & Proctor, 2009).

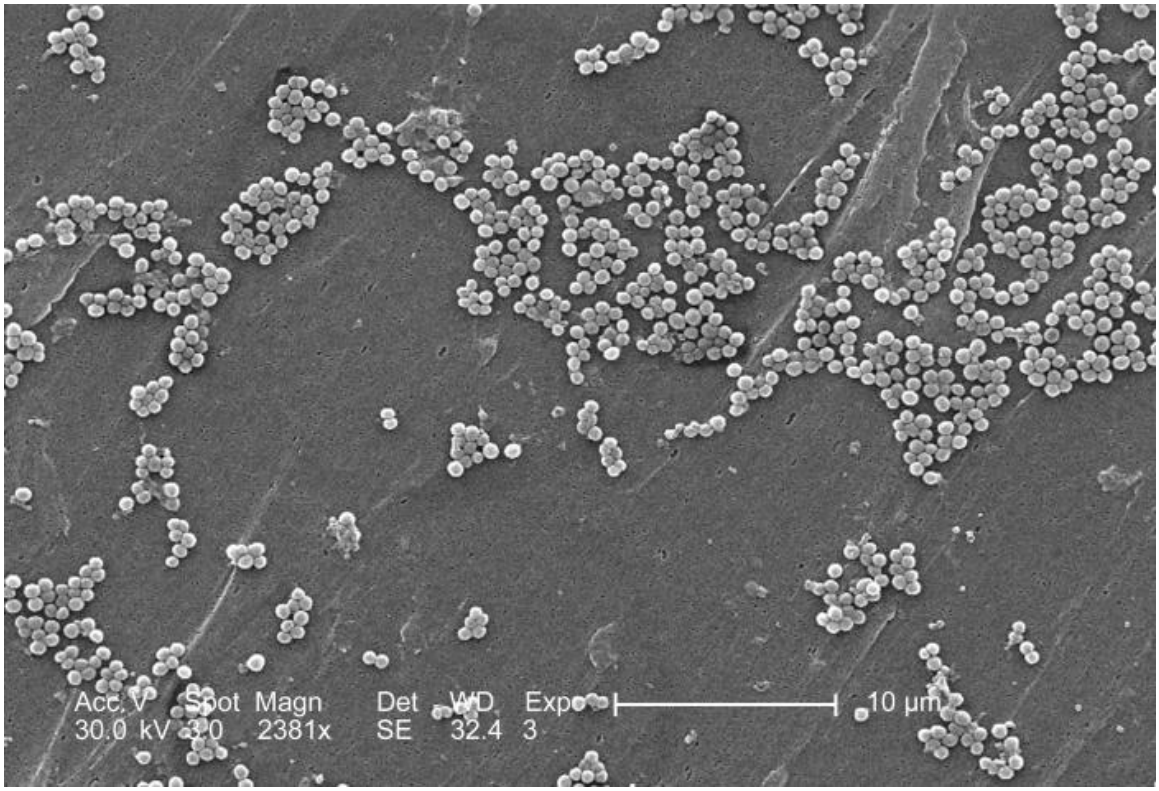

Figure 1.4 Scanning electron micrograph of *S. aureus*

Photograph courtesy of Janice Haney Carr, Public Health Image Library, Centers for Disease Control and Prevention (http://phil.cdc.gov/phil/).

1.3.1.1 Interactions with humans

S. aureus is an amazingly versatile opportunistic pathogen. Infections caused by *S. aureus* can range from superficial skin infections to severe, invasive diseases such as bacteraemia and necrotising pneumonia (Alekshun & Levy, 2006). There is also a

huge diversity in the organs affected by staphylococcal infections. Almost no organ or tissue is safe: *S. aureus* is responsible for disorders of the eye, skin, connective tissue, bone and joints, heart, lungs and gastro-intestinal and urinary tracts (Wertheim *et al.*, 2005).

Over 120 years after staphylococcal disease was described by Ogston in 1880, *S. aureus* remains an important human pathogen. Its success is highlighted by the fact that despite the development of antibiotic therapy, the frequency of staphylococcal infections has increased steadily with little change in the mortality rate (Lowy, 1998). Developments in medicine can at least be partly attributed to this rise: *S. aureus* is the most common cause of surgical site infections in England and an increasing number of infections are related to the use of joint prostheses, immunosuppressants and catheter use (Casey *et al.*, 2007). Indeed, *S. aureus* is the most frequent cause of hospital-acquired infections overall (Jones, 2003). A recent analysis of the burden of skin and skin structure infections caused by *S. aureus* in the US found that in addition to the increasing incidence of such infections, these infections were also associated with substantial treatment costs, on average costing approximately $4500 ± $11,000 per patient per episode (Marton *et al.*, 2008). The SENTRY Antimicrobial Surveillance Program found *S. aureus* to be the most common causative agent of skin and soft tissue infections across all geographical regions surveyed in North and Latin America and Europe (Moet *et al.*, 2007). *S. aureus* was found to be the most common causative agent of surgical site infections in the UK, causing 31% of such infections during the period 2009 to September 2010 (Health Protection Agency, 2010).

What makes this pathogen even more remarkable is its ability to colonise the human body and, for the most part, remain a harmless commensal. In the general population mean carriage rates of almost 40% have been found, and it is estimated that 20% of the population are persistent carriers of *S. aureus*, with 60% being

intermittently colonised (Kluytmans *et al.*, 1997). Carriage of *S. aureus* is principally localised to the anterior nares where it can either adhere to the epithelium directly or via mucus or serum constituents; it may also colonise the pharynx, axillae and perineum (Peacock *et al.*, 2001).

Despite this commensalism, it is thought that nasal carriage of *S. aureus* is a major risk factor for staphylococcal infection, as infection rates are higher in carriers and individuals are often infected with their own carriage isolate (Peacock *et al.*, 2001). Autoinfection rates are estimated at 76-80% (Coates *et al.*, 2009). In addition, nasal carriage also carries a risk for inter-patient transmission (Nashev *et al.*, 2004). Colonisation with *S. aureus* is not generally a problem in healthy individuals; however infection may occur when there is a breach in the skin or mucous membranes, or if the immune system is compromised (Lindsay & Holden, 2006).

1.3.1.2 Virulence factors and their role in disease

The success of *S. aureus* as a human pathogen is facilitated by its vast arsenal of virulence factors, which are involved in almost all processes from colonisation of the host to nutrition and dissemination. Over 40 different virulence factors have been identified in *S. aureus* (Arvidson & Tegmark, 2001), a summary of which are shown in Figure 1.5. The genes encoding virulence determinants are carried on both the core genome, such as proteases and adhesin proteins, and mobile genetic elements including bacteriophages, pathogenicity islands and transposons, which encode genes for antibiotic resistance, superantigens, enterotoxins and leukocidins (Lindsay & Holden, 2004).

It has been proposed that the environment which *S. aureus* colonises (i.e. the anterior nares) maintains the selective pressure to retain virulence factors by constantly challenging it with both the innate and acquired immune response. Increased virulence is also thought to enhance the transmission of the pathogen via

the production of infectious materials by the host, thus favouring the maintenance of such genes (Massey *et al.*, 2006).

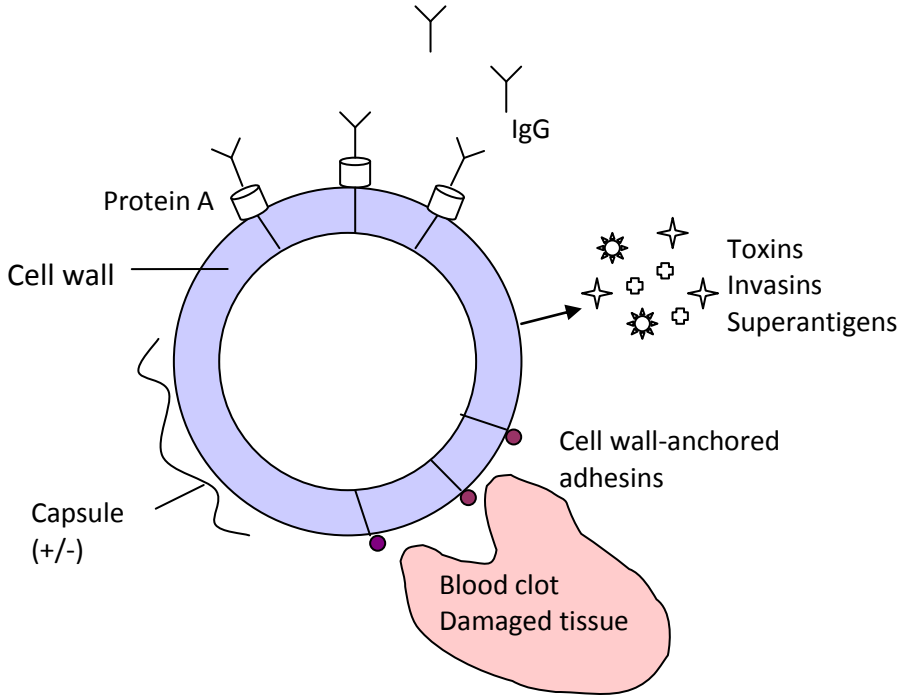

Figure 1.5 A summary of some classes of virulence factors produced by *S. aureus*

(Modified from Foster, 1996)

Virulence factors have a huge role to play in every step of the infection process. The starting point for colonisation and infection is binding to host tissues, and *S. aureus* produces a wide range of proteins that facilitate binding to host extracellular matrix components such as fibronectin and collagen (Götz, 2004). Adherence of *S. aureus* to components of the extracellular matrix is mediated predominantly by proteins belonging to the MSCRAMMs (Microbial Surface Components Recognising Adhesive Matrix Molecules) family, which are cell wall-anchored surface proteins (Foster & Höök, 1998). It is thought that exposure of extracellular matrix molecules may be involved in colonisation of the nasal epithelium, and may be particularly relevant in

lesions caused by trauma to the nasal mucosa and dermis, such as those caused by nose-picking (Wertheim *et al.*, 2006). Wall teichoic acids are also believed to play a major role in nasal colonisation by *S. aureus* and may interact with nasal epithelial cells (Burian *et al.*, 2010; Weidenmaier *et al.*, 2004).

The binding of *S. aureus* to fibronectin has a suggested role in not only the colonisation of the anterior nares, but also adhesion to the airway epithelium, intravascular catheters and biomaterials (Menzies, 2003). Fibronectin-binding proteins are also believed to play a role in invasive staphylococcal disease, as the percentage of isolates positive for the *fnbA* gene, which encodes a fibronectin-binding protein, has been found to be higher in invasive isolates compared with carriage isolates (Peacock *et al.*, 2002). Fibronectin-binding proteins are believed to facilitate the invasion of osteoblasts, epithelial cells and endothelial cells (Ahmed *et al.*, 2001; Lammers *et al.*, 1999; Peacock *et al.*, 1999).

Once colonisation/infection has been initiated, the next challenge facing the bacterium is for nutrients. *S. aureus* produces a wide range of enzymes and toxins that are thought to be involved in the conversion of host tissues into nutrients for bacterial growth, such as haemolysins, proteases, lipases and hyaluronidase (Dinges *et al.*, 2000). As well as degrading host proteins directly, proteases also have the ability to dysregulate the kallikrein-kinin pathway, resulting in increased vascular permeability and hence ensuring the supply of nutrients to the site of infection, in addition to having numerous modulatory effects on the host immune response (Travis *et al.*, 1995). Proteases produced by *S. aureus* have been implicated in the pathogenesis of skin disorders such as atopic dermatitis (Miedzobrodzki *et al.*, 2002).

In order to persist in the human host, not only does *S. aureus* have to acquire nutrients, it also faces the double challenge of the innate and acquired immune

systems. What makes *S. aureus* such a successful pathogen is its extensive array of virulence factors that facilitate avoidance of the host immune response. *S. aureus* produces a variety of toxins and proteins that kill leukocytes, inhibit neutrophil chemotaxis, resist phagocytosis, inactivate complement and neutralise host antimicrobial peptides (Foster, 2005). Many of these virulence factors are multifunctional; for example protein A, a cell wall-associated protein, not only elicits the release of proinflammatory cytokines from monocytes and fibroblasts (Fournier & Philpott, 2005), but also has the ability to bind the Fc portion of human IgG and consequently disrupt opsonisation (Forsgren & Sjoquist, 1966).

1.3.1.3 The rise of meticillin-resistant *Staphylococcus aureus*

Further compounding the problem of staphylococcal infection is the increasing resistance of *S. aureus* to antibiotics; and of particular concern is the rise of MRSA. MRSA was first reported in the United Kingdom just two years after the introduction of meticillin in 1959 (Elston, 2007). Horizontal transfer of the *mecA* gene, which encodes a penicillin-binding protein, results in resistance not only to meticillin, but also to broad spectrum β-lactams such as the third-generation cephalosporins, cefamycins and carbapenems (Foster, 2004).

The proportion of meticillin-resistant *S. aureus* isolates from blood cultures taken from cases of bacteraemia in England has risen dramatically from less than 5% in 1990 to around 40% by the end of the 1990s (Health Protection Agency, 2007). In the US, it has been reported that 52% of *S. aureus* isolates from intensive care units and 42% of isolates from non-intensive care units are resistant to meticillin, with MRSA causing approximately 20% of all nosocomial skin and skin structure infections (Lee *et al.*, 2005). According to the SENTRY Antimicrobial Surveillance Program, the overall rate of MRSA in North America during the period 1998 to 2004 was 35.9%, compared with 22.8% in Europe (Moet *et al.*, 2007). During the 2009/2010 reporting

period, 32% of *S. aureus* strains isolated from surgical site infections in the UK were resistant to meticillin (Health Protection Agency, 2010).

In 1984, it was discovered that the prevalence of MRSA in London hospitals was largely due to the spread of a single strain, subsequently designated epidemic MRSA-1 (EMRSA) (Johnson *et al.*, 2005). Following this discovery, a number of distinct EMRSA strains have been identified, most notably EMRSA-15 and EMRSA-16, which have become the dominant strains in UK hospitals. EMRSA-15 and 16 are not only resistant to β-lactam antibiotics, but frequently also erythromycin and ciprofloxacin (Johnson *et al.*, 2001). EMRSA-16 has also been detected in the community, as well as in nursing and residential homes (Cox *et al.*, 1995).

Previously thought to be limited to the hospital setting, MRSA infections are also on the rise outside of the hospital (Kluytmans-Vandenbergh & Kluytmans, 2006). Although once primarily associated with hospital-acquired infections and infections in specific populations such as prison inmates, intravenous drug users and military recruits, MRSA infections are now seen in the wider community, affecting otherwise healthy people (Schraga, 2008). Community-acquired MRSA (CA-MRSA) first emerged as a significant public health threat in the late 1990s (Klevens *et al.*, 2008). Although there is no universally accepted definition of CA-MRSA, classification is usually based on isolation of MRSA less than 24 to 72 hours following hospital admission. CA-MRSA is primarily associated with skin and soft tissue infections and the production of exotoxin production, such as the Panton-Valentine leukocidin (PVL). CA-MRSA isolates generally have a distinct pattern of antimicrobial resistance, with community strains typically showing resistance only to β-lactams, compared with the multi-drug resistance profile usually seen in nosocomial isolates (Kowalski *et al.*, 2005). The distinction between hospital- and community-acquired MRSA has started to become less clear, with CA-MRSA strains entering the hospital environment and acquiring new resistance patterns; indeed, CA-MRSA is now

endemic in many hospitals in the US. This is of particular concern as CA-MRSA tends to be more virulent and generally affects younger, otherwise healthy people (Deurenberg & Stobberingh, 2008; Klevens *et al.*, 2008).

As well as mortality rates of almost double those associated with meticillin-sensitive *S. aureus* infections, MRSA has put a considerable financial burden on both hospitals and society in general (Gould, 2006). Lodise and McKinnon found that patients with MRSA infections were at increased risk of delayed treatment with an appropriate antimicrobial, 1.5-fold longer length of hospital stay and a 2-fold increased cost of hospitalisation (Lodise & McKinnon, 2005).

1.3.1.4 Management of infections

Uncomplicated skin infections caused by *S. aureus* include impetigo, folliculitis, furunculosis and superficial cellulitis (Merlino & Malangoni, 2007). Impetigo is the most common bacterial skin infection, accounting for 50 to 60% of infections, and is the most common infectious skin infection in children, with incidence peaking between two and six years of age. A range of topical antimicrobials are available for the treatment of impetigo, including mupirocin, retapamulin, fusidic acid and bacitracin; however, these treatments are associated with adverse events such as irritation and contact dermatitis at the application site (Feaster & Singer, 2010). No recommended therapeutic strategy exists for the treatment of chronic furunculosis, which is often difficult to treat and may rely on long-term treatment with topical and systemic antibiotics. Eradication of staphylococcal carriage by decolonisation of the anterior nares is commonly used in an effort to prevent recurrence (Bernard, 2008).

Incision and drainage remain the mainstays of treatment for superficial cutaneous abscesses. In cases where MRSA is prevalent and cellulitis of the surrounding tissue is observed, treatment options include trimethoprim-sulfamethoxazole, clindamycin

and doxycycline (Schraga, 2008). In cases of cellulitis caused by meticillin-sensitive strains of *S. aureus*, semisynthetic penicillins or cephalosporins remain the treatment of choice (Merlino & Malangoni, 2007). Antibiotic treatment is also indicated in abscesses over 5 cm in length, in particular body sites such as the head and neck, or when systemic signs of infection such as fever are observed. In cases of impetigo, topical antimicrobial therapy with bacitracin, mupirocin or retapamulin is indicated (Daum, 2007). In cases of surgical site infections, removal of sutures and reopening of the incision is generally required, with antibiotic therapy indicated in cases where there are signs of systemic toxicity, necrosis or immunocompromise (Merlino & Malangoni, 2007).

The optimal treatment regimen for staphylococcal skin and soft tissue infections has not been determined. Due to the prevalence of resistant strains of *S. aureus*, β-lactams can no longer be considered as an empirical treatment choice for skin and soft tissue infections. Although clindamycin has been shown to be effective, its use has been associated with rising rates of resistance and *Clostridium difficile*-associated diarrhoea. Linezolid, whilst active against the majority of community-acquired MRSA isolates, is costly and associated with haematological side effects. Rifampin has also been shown to be effective; however, the high frequency of mutations conferring resistance contraindicates use of this antibiotic alone. Doxycycline and minocycline are associated with side effects such as photosensitivity, and cannot be used in children under nine years of age due to the propensity for deposition in teeth and bones; in addition, there is a lack of available data to support their efficacy against community-acquired MRSA. The fluoroquinolones are not indicated for the treatment of skin and soft tissue infections caused by *S. aureus* due to the widespread prevalence of resistance (Daum, 2007).

In the hospital setting, vancomycin remains the antibiotic of choice for invasive staphylococcal infection, although intermediate resistance to vancomycin has been observed, as well as a shift towards decreased susceptibility, known as “MIC creep”. Tigecycline and daptomycin may also be efficacious in more severe *S. aureus* skin and soft tissue infections (Daum, 2007); however, tigecycline is expensive and both drugs can only be administered intravenously (Merlino & Malangoni, 2007).

Several new antibacterial agents active against MRSA have been developed; however, their use is primarily reserved for severe, complicated skin and skin structure infections (Raghavan & Linden, 2004). There is therefore an urgent unmet need for novel therapeutic strategies against MRSA

MRSA decolonisation has been proposed in order to prevent outbreaks and disease recurrence. In the UK, nasal decolonisation with mupirocin is recommended in patients colonised with MRSA (Coia *et al.*, 2006); however the use of mupirocin is not without its problems. MRSA decolonisation is complicated by factors such as concomitant exposure to antibiotics that select for MRSA, and pre-existing conditions in colonised patients, for example skin lesions and catheterisation. Successful decolonisation also depends on the intensity of the decolonisation regimen and patient compliance (Kluytmans & Harbarth, 2009). Mupirocin resistance has also been noted in some strains of MRSA, most notably in USA300 isolates, which raises concern over the use of mupirocin for nasal decolonisation (Daum, 2007). The MRSAid™ antimicrobial PDT system is being investigated in clinical trials for its ability to eradicate MRSA from the anterior nares. The PDT system is being evaluated as part of an extensive decolonisation regimen including antibacterial body wash and shampoo in an attempt to achieve sustained decolonisation (Street *et al.*, 2009).

Relapse of nasal colonisation is common, adding to the problem of microbial resistance to mupirocin (Coates *et al.*, 2009). In one study, hospitalised patients who had previously been colonised with MRSA had an approximately 5-fold greater risk of being colonised, compared with patients who had not previously been colonised (Robicsek *et al.*, 2009a). Recolonisation is largely believed to be due to persistence of MRSA at extranasal sites, as recolonising strains are often identical to the initial strain; however, other risk factors such as residence in a long-term care facility also exist (Robicsek *et al.*, 2009b). The recurrence rate of MRSA infection is thought to be at least 10%, although it is not clear whether this is due to reinfection by a new strain or autoinfection (Daum, 2007).

Adding to the controversy regarding the efficacy of decolonisation, nasal decolonisation using mupirocin has been shown to delay the time to infection, but not reduce the overall infection rate (Robicsek *et al.*, 2009b). In addition, the efficacy of decolonisation regimens in outpatients colonized with CA-MRSA has not been investigated (Kowalski *et al.*, 2005).

1.3.2 *Porphyromonas gingivalis*

Porphyromonas gingivalis is a Gram-negative, non-motile, non-spore-forming, obligately anaerobic bacillus belonging to the *Bacteroideaceae* family, which can be found in the gingival sulcus, tongue, buccal mucosa and tonsillar area. Unlike most other members of the genus *Porphyromonas*, *P. gingivalis* possesses significant proteolytic activity (Cutler *et al.*, 1995; Gibson & Genco, 2006). The bacterium also produces large amounts of cell-associated protoheme that gives colonies their characteristic black colour, which can be observed in Figure 1.6 (Holt *et al.*, 1999).

Figure 1.6 The colony morphology of *P. gingivalis*

Photograph courtesy of Dr Derren Ready, UCL Eastman Dental Institute

1.3.2.1 Interactions with humans

Although primarily associated with periodontal disease, *P. gingivalis* may also be found in healthy people (Cutler *et al.*, 1995). During periodontitis, the proportion of *P. gingivalis* in the oral microbiota can rise significantly, and generally declines on return to oral health (Holt *et al.*, 1999). It is thought that *P. gingivalis* primarily colonises new hosts via the transmission of saliva (Greenstein & Lamster, 1997). It has been observed that *P. gingivalis* genotypes cluster within families and thus hypothesised that the bacteria may be transmitted between family members; saliva and direct mucosal contact were considered the most likely primary routes of transmission (Van Winkelhoff & Boutaga, 2005). *P. gingivalis* is predominantly a late coloniser of the oral cavity, requiring other microorganisms to generate suitable conditions for colonisation via the creation of adherence sites, supply of growth substrates, and the reduction of oxygen (Lamont & Jenkinson, 1998).

Periodontal diseases are a collection of pathologies with similar symptoms and can be divided into a number of subcategories, of which one is periodontitis. Periodontitis is a polymicrobial inflammatory disease of the oral cavity, characterised by destruction of the periodontal tissues that support the teeth (Hajishengallis, 2009). The disease occurs as a result of microbial colonisation of the tooth at or below the gingival margin, and follows a population shift in the subgingival plaque towards proteolytic Gram-negative anaerobes, which are associated with disease initiation and progression (Socransky & Haffajee, 2002; Soukos *et al.*, 2005). Plaque formation is the first step in the development of periodontitis and consists of a diverse microbial biofilm comprising of several hundred different bacterial species. In health, plaque is primarily composed of Gram-positive microorganisms such as *Streptococcus* and *Actinomyces* species. As gingivitis develops, an increase in anaerobic and Gram-negative species is observed, including *Fusobacterium nucleatum* and *Capnocytophaga* species. The progression to periodontitis is associated with an increase in *P. gingivalis, Prevotella intermedia, Tanerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans* (Dumitrescu, 2010).

Periodontitis may be classified as aggressive periodontitis (previously named early-onset periodontitis) or chronic periodontitis (previously named adult periodontitis); aggressive and chronic periodontitis may then be further subdivided into localised or generalised forms (Armitage, 2000). Chronic periodontitis is regarded as localised if $\leq$ 30 sites are affected, and generalised if > 30 sites are affected (Lindhe, 1999).

Chronic periodontitis is the most common form of periodontitis, and is characterised by a significant increase in gingival crevicular exudate, bleeding, destruction of connective tissue and consequent bone resorption and tooth loss. *P. gingivalis* has been associated with the majority of patients with chronic periodontitis, and therefore is believed to be one of the primary aetiological agents (Dumitrescu, 2010;

Travis & Potempa, 2000). Although bacterial plaque is considered essential to the initiation of disease, the host immune response is critical in the pathogenesis of the chronic periodontitis (Lindhe, 1999). The host immune response is stimulated by an inflammatory cascade initiated by oral pathogens, and is believed to exacerbate tissue destruction in periodontal disease via the production of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) (Graves & Cochran, 2003).

Cytokines play an essential role in the regulation of the immune response and are produced by a number of cell types, including lymphocytes, macrophages, epithelial cells and fibroblasts; increased levels of the proinflammatory cytokines TNF-α, IL-1β and interleukin 6 (IL-6) have been detected in lesions associated with periodontitis (Takashiba *et al.*, 2003). A summary of some of the mechanisms involved in host-mediated tissue destruction is shown in Figure 1.7. In particular, the local production of proinflammatory cytokines has been strongly implicated in periodontal bone destruction via the induction and activation of osteoclasts; it is believed that IL-1 and TNF-α are co-produced and act synergistically to induce bone resorption (Assuma *et al.*, 1998). Therefore, these proinflammatory cytokines also represent a target for PDT, as inactivation or reduction of their biological activity may reduce damage to host tissues and facilitate healing.

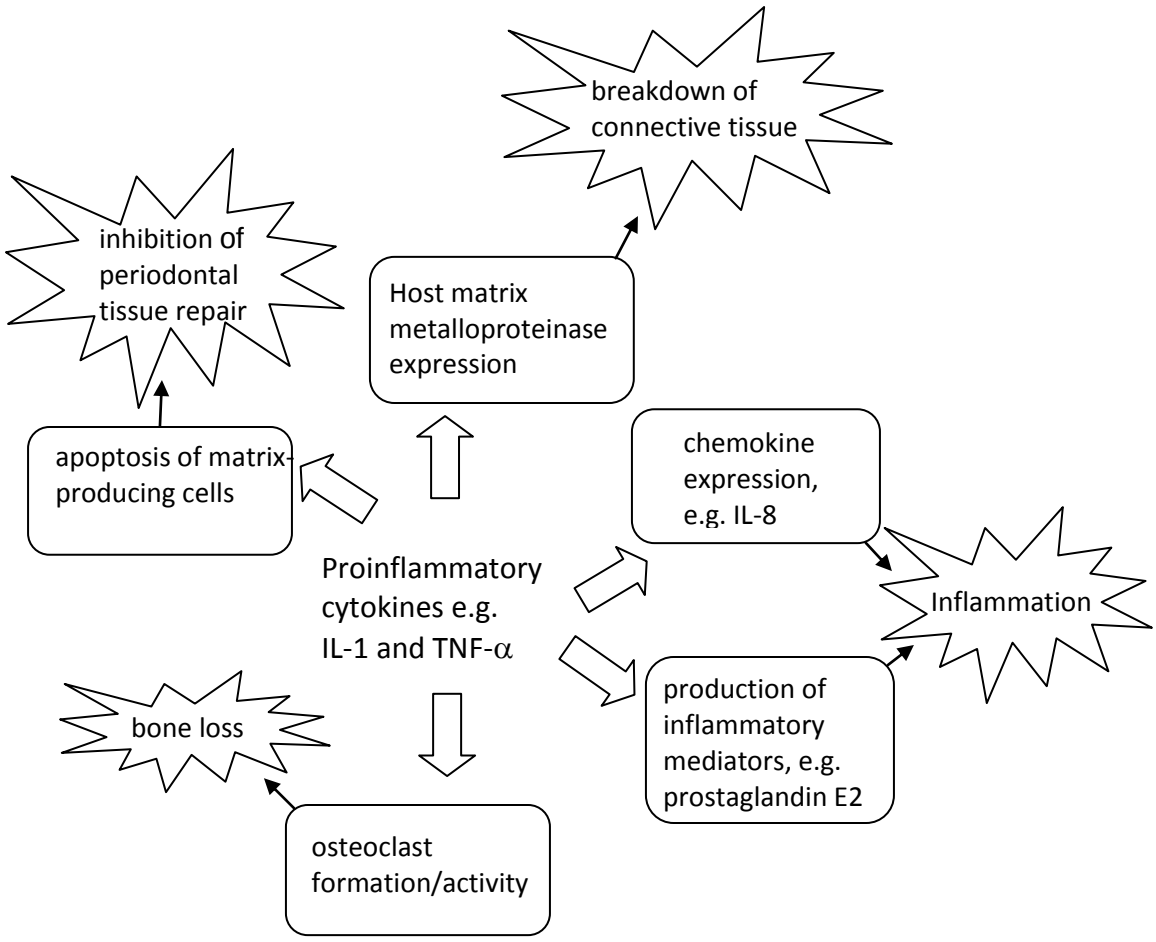

Figure 1.7 A summary of some of the mechanisms of host-mediated periodontal tissue destruction.

(Adapted from Graves & Cochran, 2003)

P. gingivalis has also been implicated in a number of conditions outside of the oral cavity, including pulmonary infections, appendicitis and otitis media (Van Winkelhoff & Slots, 1999). The invasion of epithelial cells and connective tissue by *P. gingivalis* and the resultant bleeding allows oral microorganisms entry to the bloodstream,

which may result in endocarditis. Although not directly implicated as a causative agent of endocarditis, *P. gingivalis* therefore plays a role in the aetiology of the disease (Meyer & Fives-Taylor, 1998). The bacterium has been identified in atherosclerotic plaques, although it is unclear whether *P. gingivalis* is the causative agent or simply invades damaged arteries (Seymour *et al.*, 2007). In addition, periodontal disease has been associated with pre-term delivery and low birth weight (Lamont & Jenkinson, 1998).

1.3.2.2 Virulence factors and their role in disease

P. gingivalis produces an array of virulence factors, which are summarised in Figure 1.8. These virulence factors include extracellular proteases, lipopolysaccharide (LPS), fimbriae, haemagglutinins and an invasin, which are all thought to be involved in the invasion of host tissues (Yilmaz, 2008). It has been demonstrated that virulence is not limited to one particular clonal type, supporting the hypothesis that *P. gingivalis* is an opportunistic pathogen, rather than certain strains being classed as pathogenic and others as commensal (Lamont & Jenkinson, 1998).

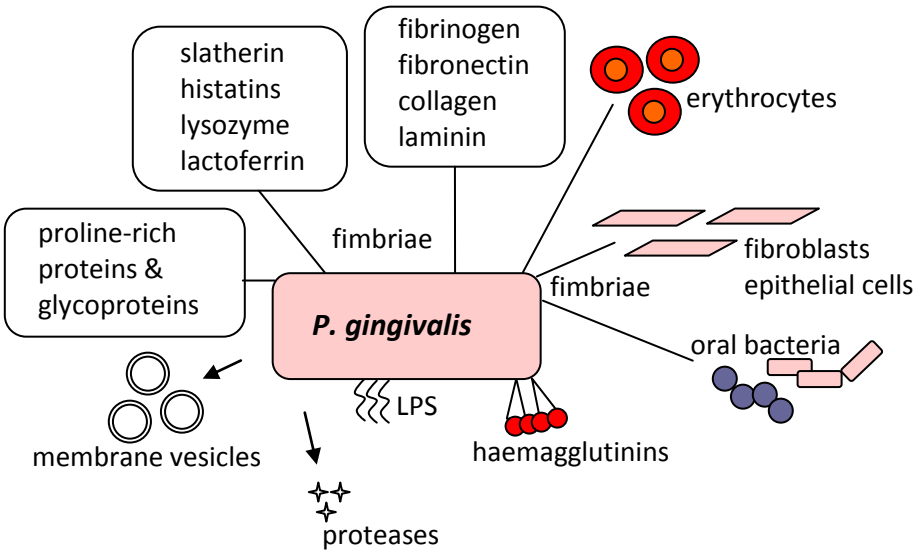

Figure 1.8 A summary of virulence factors produced by *P. gingivalis*.

(Modified from Lamont & Jenkinson, 1998)

P. gingivalis demonstrates multimodal adherence to many substrates, associated with both fimbriae and outer membrane proteins (Lamont & Jenkinson, 1998). The fimbriae of *P. gingivalis* are involved in adherence of the organism to host and microbial cells, bacterial internalisation, and the induction of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α (Ezzo & Cutler, 2003). As summarised in Figure 1.7, these proinflammatory cytokines play an important role in the initiation of host-mediated tissue destruction, which exacerbates the damage directly caused by bacterial virulence factors.

The proteolytic enzymes of *P. gingivalis* are multifunctional enzymes and have been proposed to play a role in almost every aspect of the bacterium's interaction with the human host, from colonisation through to nutrition and the disease state. The proteolytic enzymes of *P. gingivalis* have been suggested as potential targets for

novel antibiotics due to their multifunctionality and importance in periodontal disease (Travis & Potempa, 2000).

P. gingivalis produces a number of enzymes with proposed proteolytic functions, including the arginine-specific gingipains (arg-gingipains / gingipain R), lysine-specific gingipain (lys-gingipain / gingipain K), PrtT proteinase, tpr proteinase, collagenase, prolyl tripeptidyl protease (PtpA), dipeptidyl-peptidase IV (DPP IV), dipeptidyl peptidase VI, aminopeptidase P, oligopeptidase O, collagenase, and gelatinase (Potempa *et al.*, 2000).

The arg-gingipains are thought to play a role in colonisation of the host by cleavage of host proteins, resulting in exposed arginine residues that *P. gingivalis* can then use for attachment via its fimbriae (Curtis *et al.*, 2001). The haemagglutinin/adhesion properties of the gingipains are also thought to be essential for the colonisation of host tissues. It is believed that the gingipains are involved in the colonisation of periodontal pockets and/or the gingival sulcus through the binding of gingipain complexes to extracellular matrix molecules such as fibronectin and fibrinogen (Potempa *et al.*, 2000). In addition, proteases are involved in the processing of other cell surface-associated virulence factors required for colonisation, such as the fimbriae (Curtis et al., 2001).

The proteases are believed to be involved in nutrition, either via direct proteolytic activity or by the activation of host proteolytic enzymes. Lys-gingipain has been shown to be crucial for the acquisition of carbon, nitrogen and hemin, and is involved in the lysis of erythrocytes (Curtis *et al.*, 2001). The bacterium has an essential requirement for hemin, and it is believed that the ability of its proteolytic enzymes to cleave transferrin and haemoglobin may be an important iron-acquisition mechanism (Holt *et al.*, 1999). Proteolytic enzymes released by *P. gingivalis* also have a number of effects on the host immune response, including

activation of the kallikrein/kinin pathway, complement and coagulation cascades, dysregulation of the fibrinogen cascade, and polymorphonucleocyte migration (Travis & Potempa, 2000). They can also activate host proteinases, which further degrade extracellular matrix components and thus further exacerbate host tissue damage (Potempa *et al.*, 2000).

The gingipains are reported to be responsible for 85% of *P. gingivalis*'s general proteolytic activity and have additional haemagglutinating, adhesin and haemoglobin-binding properties (Potempa *et al.*, 2000). Lys-gingipain can hydrolyze many substrates in the human body, including IgG, IgA, type I collagen and haemoglobin (Curtis *et al.*, 2001). Laminin, fibronectin, collagen types III, IV and V have also been identified as substrates for gingipains *in vitro*, and are believed to contribute to damage to basement membranes, extracellular matrix and host cells (Potempa *et al.*, 2000).

Although it is unknown precisely how many haemagglutinins are produced by *P. gingivalis*, it is thought that the bacterium produces at least eight haemagglutinating molecues, including those complexed with LPS and lipid on the cell surface and an extracellular exohaemagglutinin. The majority of non-protease haemagglutinating activity is believed to be associated with the haemagglutinins encoded by *hagA, hagB, hagC, hagD* and *hagE* (Lamont & Jenkinson, 2000). The haemagglutinins are thought to mediate binding of the bacterium to erythrocytes as well as to host epithelial cells. The haemagglutinins may also play a role in aiding colonisation of host tissues by facilitating the acquisition of iron or hemin (Holt *et al.*, 1999). It is thought that *P. gingivalis* haemagglutinins cause lysis of erythrocytes via the formation of small pores (Shah *et al.*, 1992). In addition, haemagglutinin B has been shown to induce production of the proinflammatory cytokines interleukin-12, interferon-γ and TNF-α, suggesting an immunomodulatory role for the haemagglutinins (Zhang *et al.*, 2005).

LPS is commonly believed to be the one of the most important virulence factors of periodontopathogens (Wilson, 2004). The LPS of *P. gingivalis* is generally considered to be distinct from the classic LPS of the *Enterobacteriaceae* and is believed to be less endotoxic, although conflicting reports have been published in both regards (Holt *et al.*, 1999). Septic shock-like symptoms and lethality have been observed in a murine model, suggesting stimulation of the systemic immune response (Huang *et al.*, 2006). In other reports, *P. gingivalis* LPS has been shown to strongly stimulate the local inflammatory response, but only minimally stimulate the systemic inflammatory response (Liu *et al.*, 2008). *P. gingivalis* LPS has been shown to induce the production of IL-1, IL-1β, IL-6, IL-8, TNF-α, nitric oxide and prostaglandin E_2, although once again, conflicting reports exist in the literature, and it may be concluded that LPS from different strains of the bacterium may have differing biological properties (Holt *et al.*, 1999).

In addition to the presence of surface-associated LPS, *P. gingivalis* releases large amounts of LPS in outer membrane vesicles, which have been shown to be able to penetrate periodontal tissue and elicit an innate immune response (Darveau *et al.*, 2004). The LPS of *P. gingivalis* is believed to cause local bone resorption via the direct activation of osteoclasts and by the stimulation of TNF-α and IL1-β release from macrophages, monocytes and fibroblasts, thus contributing to the tooth loss associated with advanced periodontal disease (Lamont & Jenkinson, 1998). In addition to the induction of bone resorption, *P. gingivalis* LPS has been shown to inhibit bone formation via the inhibition of bone collagen synthesis (Millar *et al.*, 1986). LPS from oral bacteria also stimulates antibody production by lymphocytes, thus exacerbating the immune response (Wilson, 1995).

1.3.2.3 Management of infections

Mechanical removal of supra- and subgingival plaque by scaling and root planing and periodontal surgery is generally the first-line treatment for periodontal disease. Although the microbial burden can be reduced by 90% by such methods, it has been shown that the number of microorganisms associated with the tooth surface returns to near-baseline levels within three months of the procedure; in fact, it has been suggested that this recolonisation may occur as soon as 4-8 days post-treatment (Socransky & Haffajee, 2002).

Topical antiseptic agents such as chlorhexidine are commonly used for the reduction of plaque accumulation. Chlorhexidine is available in a number of formulations, including mouth rinses, gels and varnishes, and demonstrates activity against Gram positive and Gram negative microorganisms due to membrane damage and loss of structural organization (Sreenivasan & Gaffar, 2002). A significant side effect of chlorhexidine is staining of teeth, and resistance to the agent has also been observed among oral microorganisms (Slots, 2002; Sweeney *et al.*, 2004). It has been proposed that outer membrane vesicles produced by *P. gingivalis* may provide protection against chlorhexidine by binding to the agent (Grenier *et al.*, 1995).

Topical antimicrobial agents are also widely used for the prevention and/or treatment of periodontitis; however, the development of microbial resistance to these agents is a cause for concern (Sweeney *et al.*, 2004; Wilson, 2004). A recent survey found periodontal microorganisms, including *P. gingivalis*, which were resistant to antibiotics commonly used for the treatment of periodontal disease. Approximately 25% of *P. gingivalis* isolates were resistant to amoxicillin and clindamycin, with resistance to metronidazole observed in 21% of isolates (Ardila *et al.*, 2010).

A further issue associated with antibiotic treatment for periodontitis is the challenge of maintaining therapeutic drug concentrations in the periodontal pocket, due to the high flow conditions associated with saliva and gingival crevicular fluid. Disturbance of the commensal oral microbiota and consequent opportunistic infection is also a problem of antibiotic therapy (Wilson, 2004). Delivery systems, for example for the local delivery of tetracycline, minocycline and metronidazole, have also been developed in order to achieve sustained concentrations of the antibiotic; however, their variable efficacy has resulted in limited uptake by clinicians (Krayer *et al.*, 2010).

Compounding the issue of antibiotic delivery is the resistance of bacterial biofilms to antibiotics. Periodontal diseases are particularly associated with biofilm formation; when forming part of a biofilm, microorganisms are typically more resistant to antibiotics than planktonic cells. Some authors have suggested that biofilm-associated organisms have 1000- to 1500-fold greater resistance compared with planktonic cells. It has been proposed that this greater level of resistance may be due to the slower growth rate seen in biofilms, which consequently reduces the susceptibility to some antibiotics. In addition, slow-growing biofilm-associated microorganisms often overexpress defence mechanisms such as shock proteins, multidrug efflux pumps and are associated with increased exopolymer production (Socransky & Haffajee, 2002). Treatment options for periodontal disease should therefore have activity against bacterial biofilms, not just planktonic cells.

1.4 Aims and objectives

In the face of increasing antibiotic resistance and unfavourable adverse event profiles of existing antibiotic therapies, there exists a need for novel therapeutic strategies for both superficial staphylococcal infections and periodontal disease. A desirable feature for a novel antimicrobial strategy would be the ability to reduce or inactivate microbial virulence factors and undesirable inflammatory responses that contribute to the morbidity of the disease. PDT has emerged as a promising alternative to conventional antibiotic treatment, and may present solutions to these problems.

Whilst the susceptibility of microorgansisms to photosensitisation has been demonstrated, there is relatively little published data on the effect of light-activated antimicrobials on the virulence factors of pathogenic microorganisms or their effect on inflammatory mediators. This thesis therefore has three main aims:

1. To assess the effect of light-activated antimicrobial agents on the viability of *P. gingivalis* and meticillin-sensitive and meticillin-resistant strains of *S. aureus*

2. To assess the effect of light-activated antimicrobial agents on key virulence mechanisms of *S. aureus* and *P. gingivalis*

3. To assess the effect of light-activated antimicrobial agents on key modulators of inflammation

2 General materials and methods

2.1 Light sources

A Periowave™ diode laser (Ondine Biopharma Inc., Canada), which emits light with a wavelength of 665 nm was used for all methylene blue experiments. The laser system was set up so that the laser beam covered the entire well of a microtitre plate in which the experiments were performed. The power output of the laser was measured using a thermopile power meter (TPM-300CE, Genetic, Canada) and was found to be 73 mW. The beam diameter was measured and found to be 1.7 cm.

A Helium/Neon (HeNe) gas laser (NEC Corporation, Japan) with a measured power output of 19 mW and a wavelength of 633 nm was used for all tin chlorin e6 experiments. The diameter of the beam was 1.5 cm.

For all experiments, the sample to be irradiated was placed in a well of a microtitre plate and the surrounding wells filled with tin foil in order to prevent leakage of laser light into adjacent wells (see Figure 2.1). In addition, the microtitre plates were covered in tin foil during the experiment so that only the well being irradiated was exposed to laser light.

The light dose delivered during a specified period of irradiation was calculated using the following formula:

Light dose (J) = power output (W) x irradiation time (seconds)

For the 665 nm laser, light doses of 4.38, 8.76 and 21.9 J corresponded to irradiation times of 1.0, 2.0 and 5.0 minutes, respectively. For the 633 nm laser, light doses of 4.38, 8.76 and 21.9 J corresponded to irradiation times of 3.8, 7.7 and 19.2 minutes, respectively.

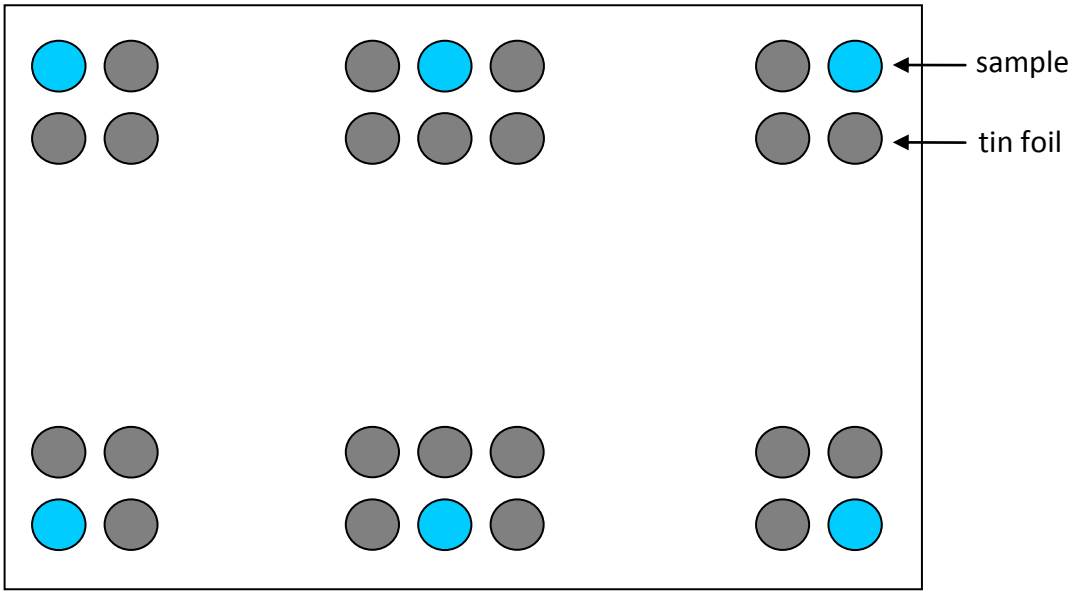

Figure 2.1 Layout of microtitre plate for photosensitisation studies

2.2 Photosensitisers

2.2.1 Methylene blue

Methylene blue ($C_{16}H_{18}ClN_3S.3H_2O$) was purchased from Sigma-Aldrich (UK). Stock solutions of 0.1 mg/ml were prepared in phosphate buffered saline (PBS) and kept in the dark at room temperature. Further dilutions were also made in PBS.

2.2.2 Tin chlorin e6

Tin chlorin e6 (SnCe6) ($C_{34}H_{31}Cl_2N_4O_6Sn.Na_3$) was purchased from Frontier Scientific. Stock solutions of 0.1 mg/mL were prepared in PBS and kept in the dark at room temperature. Further dilutions were also made in PBS.

2.3 Target organisms

2.3.1 *Staphylococcus aureus*

Staphylococcus aureus NCTC 8325-4, *S. aureus* LS-1, *S. aureus* LS-1ΔhemB and EMRSA-16 (gifts from Dr Derren Ready, UCL Eastman Dental Institute, except for LS-1ΔhemB, which was constructed by Dr John Wright, UCL) were maintained by subculture on blood agar (Oxoid Ltd, UK), supplemented with 5% horse blood (E & O Laboratories Ltd, UK). *S. aureus* D1324 (a gift from Professor Richard Proctor) was maintained by subculture on blood agar (Oxoid Ltd, UK) containing 5 μg/ml erythromycin. Cultures were incubated aerobically at 37°C. For experimental purposes, a few colonies were inoculated into 10 mL brain heart Infusion broth (plus 5 μg/ml erythromycin for *S. aureus* D1324) (Oxoid Ltd, UK) and the cultures incubated aerobically overnight at 37°C, with shaking, at 200 rpm.

2.3.2 *Porphyromonas gingivalis*

Porphyromonas gingivalis W50 (a gift from Dr Derren Ready, UCL Eastman Dental Institute) was maintained by subculture on Wilkins Chalgren agar (Oxoid Ltd, UK) supplemented with 5% horse blood (E & O Laboratories Ltd, UK) and incubated at 37°C in an anaerobic cabinet (10% carbon dioxide, 10% hydrogen and 80% nitrogen, Don Whitley Scientific Inc.). For experimental purposes, several colonies were placed into 10 mL liquid medium (BM broth) and incubated as previously for 48 hours.

BM broth was made according to the following recipe:

Tryptone soya broth	10 g
Proteose peptone	10 g
Yeast extract	5 g
Glucose	5 g
Sodium chloride	5 g
Cysteine-HCl	0.75 g
Distilled water	1 L

The pH of the broth was adjusted to 7.5 and the medium was sterilised by autoclaving at 121°C for 15 minutes. Prior to use, the medium was supplemented with haemin (Sigma-Aldrich, UK) and menadione (Sigma-Aldrich, UK) so that the final concentrations were 5 µg/mL and 0.5 µg/mL respectively.

2.4 Preparation of P. gingivalis W50 culture supernatant

One hundred millilitres of BM broth (see section 2.3.2) was inoculated with several colonies of *P. gingivalis* W50 and incubated anaerobically at 37°C for 48 hours. After 48 hours, 90 mL supplemented BM broth was inoculated with the 10 ml culture and incubated anaerobically at 37°C for 3 days. The culture was then centrifuged at 1370 x *g* for 15 minutes and the supernatant removed. The supernatant was centrifuged again at 1370 x *g* for 15 minutes, following which the supernatant was collected and filtered through a 0.45 µM filter. Culture supernatants were stored at -20°C until use.

2.5 Photosensitisation studies

2.5.1 Light dose experiments

For all light dose experiments, light doses of 4.38 J, 8.76 J and 21.9 J were used. A photosensitiser concentration of 20 µM (final concentration) was used unless otherwise stated. Controls were performed to investigate the effect of laser light alone (L+S-) and photosensitiser alone (L-S+). The reduction in activity/viability was calculated relative to untreated samples (L-S-).

2.5.2 Photosensitiser dose experiments

For all photosensitiser dose experiments, final photosensitiser concentrations of 1, 5, 10 and 20 µM were used. A light dose of 4.38 J was used unless otherwise stated. Controls were performed to investigate the effect of laser light alone (L+S-) and photosensitiser alone (L-S+). The reduction in activity/viability was calculated relative to untreated samples (L-S-).

2.6 Lethal photosensitisation of target organisms

The target organisms were grown as described in sections 2.3.1 for *S. aureus* and 2.3.2 for *P. gingivalis*. After incubation, cultures were centrifuged and the cells resuspended in PBS to an optical density of 0.05 at 600 nm, corresponding to approximately $1x10^7$ colony forming units/mL (cfu/mL). Aliquots of 50 µL of the photosensitiser were added to an equal volume of the inoculum in triplicate wells of a sterile, flat-bottomed, untreated 96-well plate ,and irradiated with laser light of the appropriate wavelength (see section 2.1), with stirring. Three additional wells containing 50 µL photosensitiser and 50 µL of the bacterial suspension were kept in the dark to assess the toxicity of the photosensitiser alone (L-S+). Fifty microlitres of PBS was also added to 50 µL of the inoculum in a further six wells, three of which

were irradiated with laser light as above (L+S-) and the remaining three were kept in the dark (L-S-).

After irradiation/dark incubation, samples were diluted using serial 10-fold dilutions to a dilution factor of 10^{-4} and 10 µL of each dilution was spotted onto blood agar (*S. aureus*) or Wilkins Chalgren agar (*P.* gingivalis) supplemented with 5% horse blood in triplicate. For *S. aureus*, plates were incubated aerobically at 37°C for 18 to 24 hours and for *P. gingivalis*, plates were incubated anaerobically at 37°C for 5 days, following which the surviving colonies were counted and the number of surviving cfu/mL calculated.

2.7 Azocasein hydrolysis assay

2.7.1 Azocasein hydrolysis assay to assess V8 protease activity

Endoproteinase Glu-C (also known as V8 protease) from *Staphylococcus aureus* V8 was purchased from Sigma-Aldrich (UK) and stored at -20°C at a concentration of 1 mg/mL in dH_2O. A final concentration of 5µg/mL was obtained by diluting the enzyme in PBS. An equal volume of V8 protease was added to 50 µL of either photosensitiser (S+) or PBS (S-) in triplicate wells of a 96-well plate and samples were irradiated with laser light of the appropriate wavelength for each photosensitiser (L+) or incubated in the dark (L-).

After irradiation, samples were removed added to 50 µL of 6% azocasein (w/v) in 0.5 M Tris buffer, pH 7 (Sigma-Aldrich, UK) in 0.5 mL Eppendorf tubes. Samples were incubated in the dark for one hour at 37°C. The reaction was stopped with an equal volume of 20% acetic acid and the samples centrifuged for 10 minutes at 5590 x *g*. Seventy five microlitres of the supernatant was removed in duplicate from each sample and the optical density read at 450 nm using a Dynex plate reader. The

enzyme activity at one hour was calculated; one unit of activity was determined as that which caused a chance in absorbance of 0.001 in one hour at 450 nm.

2.7.2 Azocasein hydrolysis assay to assess *P. gingivalis* W50 protease activity

In 24-well plates, 300 µL of *P. gingivalis* W50 culture supernatant (see section 2.) was added to either PBS (S-) or photosensitiser (S+) in sextuplicate. Three wells were exposed to laser light, with stirring (L+) and the remaining 3 wells were incubated at room temperature in the dark (L-). 500 µL of the test sample was then added to 250 µL 0.6% azocasein (w/v) in 0.5M Tris, pH 7.3 and incubated for 4 hours, with shaking, in the dark. Two hundred and fifty microlitres of BM plus 250 µL of PBS was used as a blank and treated as above. After 4 hours, 750 µL 20% acetic acid was added to stop the reaction and the tubes centrifuged for 10 minutes at 5590 x *g*. The optical density of 1 mL supernatant at 420 nm was read using a UNICAM UV500 UV-Visible spectrophotometer (ThermoSpectronic, Rochester, NY, USA) and the units of activity determined as that which caused a chance in absorbance of 0.001 in one hour at 420 nm

2.8 The effect of a singlet oxygen enhancer and scavenger on the efficacy of photosensitisation

2.8.1 The effect of a singlet oxygen enhancer and scavenger on the lethal photosensitisation of target organisms

S. aureus 8325-4 was maintained and grown for experimental purposes as described in section 2.3.1, with the following modifications. Cultures were centrifuged and resuspended in an equal volume of either sterile distilled water (H_2O), deuterium oxide (D_2O) or 10 mM L-tryptophan (Sigma Aldrich, UK). Photosensitisation experiments were carried out according to section 2.6, using methylene blue diluted

in PBS to give a final concentration of 5 µM for the D_2O experiments and 10 µM for the L-tryptophan experiments. A light dose of 4.38 J of 665 nm laser light was used.

2.8.2 The effect of a singlet oxygen enhancer and scavenger on the photodynamic inactivation of *S. aureus* V8 protease

The V8 protease was suspended in PBS, deuterium oxide or 10 mM L-tryptophan (Sigma-Aldrich, UK). Photosensitisation was carried out using a 665 nm laser light dose of 4.38 J. For D_20 experiments, a methylene blue concentration of 1 µM was used and for L-tryptophan experiments, a methylene blue concentration of 5 µM was used. Following photosensitisation, the azocasein hydrolysis assay performed as described in section 2.7.1.

2.8.3 The effect of a singlet oxygen enhancer and scavenger on the photodynamic inactivation of *P. gingivalis* proteases

Methylene blue was suspended in PBS, deuterium oxide or 10 mM L-tryptophan (Sigma-Aldrich, UK), and photosensitisation was carried out using 5 µM methylene blue and 21.9 J of 665 nm laser light. The azocasein hydrolysis assay performed as described in section 2.7.2.

2.9 Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis

A 15% resolving gel was used for all sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) experiments and was made as follows:

Distilled water	4.6 mL
30% acrylamide	10 mL
1.5M Tris, pH8.8	5 mL
10% SDS	0.2 mL
10% ammonium persulfate	0.2 mL
TEMED	0.008 mL

A 5% stacking gel was added once the resolving gel had polymerised and was made as follows:

Distilled water	3.4 mL
30% acrylamide	0.83 mL
1.0M Tris, pH 6.8	0.63 mL
10% SDS	0.05 mL
10% ammonium persulfate	0.05 mL
TEMED	0.005 mL

Sample buffer (Pierce, UK) was diluted 1 in 5 with sample and boiled for 5 minutes before being loaded onto the gel. A pre-stained protein marker (7-175 KDa) (New England Biolabs) was also run on each gel.

Approximately 500 mL Tris glycine electrophoresis buffer was added to the electrophoresis apparatus. A 5X stock solution was made and diluted to 1 in 5 prior to use.

5X Tris glycine electrophoresis buffer:

Tris base	15.1 g
Glycine	94g

Nine hundred millilitres of distilled water was added, followed by 50 mL 10% SDS. The volume was then adjusted to 1 litre with distilled water.

The apparatus was connected to a power supply and run at a constant 40 mA until the dye front neared the bottom of the gel. Gels were fixed for 10 minutes in 40% methanol and 15% acetic acid, following which they were stained overnight with Brilliant Blue G (Sigma-Aldrich, UK). After overnight staining, gels were destained by placing in distilled water for approximately one hour and then visualised.

2.10 Statistical analysis

Data are expressed as means ± standard error. All statistical analyses were performed using SPSS 14.0 (LEAD Technologies, Inc.). For data with a standard distribution, results were analysed using the univariate ANOVA test with post-hoc Bonferroni (equal variance) or Games-Howell (unequal variance) analysis. A *P* value of less than 0.05 was considered statistically significant. For data with a non-standard distribution, the Mann-Whitney *U* test was used to compare groups. A *P* value of less than 0.05 was considered statistically significant.

2.11 Chemical structures

All chemical structures were drawn using ChemDraw Ultra 12.0 (CambridgeSoft Corporation).

3 The susceptibility of target organisms to lethal photosensitisation

3.1 Introduction

As a result of the global increase in antibiotic resistance, there is an unmet clinical need for novel therapeutic strategies. In addition to the burden of antibacterial resistance, the current methods of treatment may be invasive, cause host toxicity and damage to the normal microbiota (Feaster & Singer, 2010; Wilson, 2004). The use of light-activated antimicrobial agents has been proposed as a promising alternative treatment for a number of bacterial infections, in particular localised infections such as wounds, burns and periodontitis (Jori *et al.*, 2006).

Superficial skin infections are ideally suited to PDT as the site of infection is generally easily accessible for topical application of the photosensitiser and subsequent irradiation (Wainwright *et al.*, 1998). The most frequently isolated pathogen from skin and skin structure infections is *S. aureus*, which is isolated in almost 50% of cases (Lee *et al.*, 2005). The sensitivity of *S. aureus* to photodynamic killing using a variety of light sources and photosensitisers has been demonstrated (Omar *et al.*, 2008; Wainwright *et al.*, 1997; Wilson & Pratten, 1994; Zeina *et al.*, 2001). Recent *in vivo* studies have confirmed the potential of PDT as a potent antibacterial strategy active against both MSSA and MRSA (Dai *et al.*, 2010; Zolfaghari *et al.*, 2009).

Periodontitis is also well-suited to treatment with PDT due to the accessibility of the periodontal pocket (Wilson, 2004). The use of PDT in periodontal disease is well documented, and the first antibacterial PDT systems have been licensed for this indication (Jose *et al.*, 2010). Periodontitis is a complex polymicrobial disease, in which *P. gingivalis* is one of the major aetiological agents (Dumitrescu, 2010). In the disease state, the numbers of *P. gingivalis* present in the oral microbiota have been

observed to increase significantly (Holt *et al.*, 1999). This pathogen also produces a number of virulence factors that play an important role in the pathology of periodontitis (Yilmaz, 2008).

This chapter describes the effect of the photosensitisers methylene blue and tin chlorin e6 in combination with laser light of the appropriate wavelength on the viability of *S. aureus* and *P. gingivalis* W50. These bacterial species were chosen as target organisms due to their association with infections that are potentially treatable with antimicrobial PDT. For *S. aureus*, the susceptibilities of a meticillin-sensitive strain (*S. aureus* 8325-4) and a meticillin-resistant strain (EMRSA-16) were investigated. Preliminary studies to investigate the mechanism of photodynamic killing were carried out using the singlet oxygen scavenger L-tryptophan and the enhancer of singlet oxygen lifetime, D_2O.

3.2 Materials and methods

3.2.1 The effect of photosensitiser dose on the lethal photosensitisation of *S. aureus* 8325-4 and EMRSA-16

S. aureus 8325-4 and EMRSA-16 were maintained and grown for experimental purposes as described in section 2.3.1. Following overnight incubation, experiments to assess the effect of photosensitiser dose on the lethal photosensitisation of *S. aureus* 8325-4 and EMRSA-16 were performed as described in sections 2.5.2 and 2.6. Experiments were performed three times in triplicate.

3.2.2 The effect of laser light dose on the lethal photosensitisation of *S. aureus* 8325-4 and EMRSA-16

Bacterial strains were grown and harvested as described in section 2.3.1. Following harvesting of the cells, the effect of 665 nm laser light dose on the lethal photosensitisation of *S. aureus* 8325-4 and EMRSA-16 was assessed as described in sections 2.5.1 and 2.6. Experiments were performed three times in triplicate.

3.2.3 The effect of a singlet oxygen enhancer and scavenger on the lethal photosensitisation of *S. aureus* 8325-4

Experiments to examine the effect of deuterium oxide and L-tryptophan were performed according to section 2.8.1. Experiments were performed twice in triplicate.

3.2.4 The effect of photosensitiser dose on the lethal photosensitisation of *P. gingivalis* W50

P. gingivalis W50 was maintained and grown for experimental purposes as described in section 2.3.2. Experiments to assess the effect of photosensitiser dose on the lethal photosensitisation of *P. gingivalis* W50 were performed as described in sections 2.5.2 and 2.6. Experiments were performed three times in triplicate.

3.2.5 The effect of laser light dose on the lethal photosensitisation of *P. gingivalis* W50

P. gingivalis W50 was grown and harvested for experimental purposes as described in section 2.3.2. The effect of 665 nm laser light dose on the lethal photosensitisation of *P. gingivalis* W50 was assessed; experiments were performed according to sections 2.5.1 and 2.6. Experiments were performed three times in triplicate.

3.2.6 The effect of laser light dose on sample temperature

In order to determine whether laser light dose had an effect on the temperature of samples, either PBS, 20 µM photosensitiser or 20 µM photosensitiser plus 12.5% human serum were exposed to laser light doses of 0, 4.38, 8.76 and 21.9 J, and the temperature recorded using a Fluke 179 True RMS multimeter. Experiments were performed in triplicate.

3.3 Results

3.3.1 The effect of photosensitiser dose on the lethal photosensitisation of *S. aureus* 8325-4

3.3.1.1 Methylene blue

Figure 3.1 shows the effect of an increasing methylene blue dose on the lethal photosensitisation of *S. aureus* 8325-4. Photosensitisation of *S. aureus* 8325-4 using methylene blue and 665 nm diode laser light resulted in significant, photosensitiser dose-dependent killing of the microorganism. Photosensitisation using the highest concentration of methylene blue tested (20 μM) and 4.38 J of laser light resulted in a 99.99% kill. This reduction in the number of viable bacteria was highly significant compared with the control (P <0.001; ANOVA). There was no effect of either photosensitiser or laser light alone.

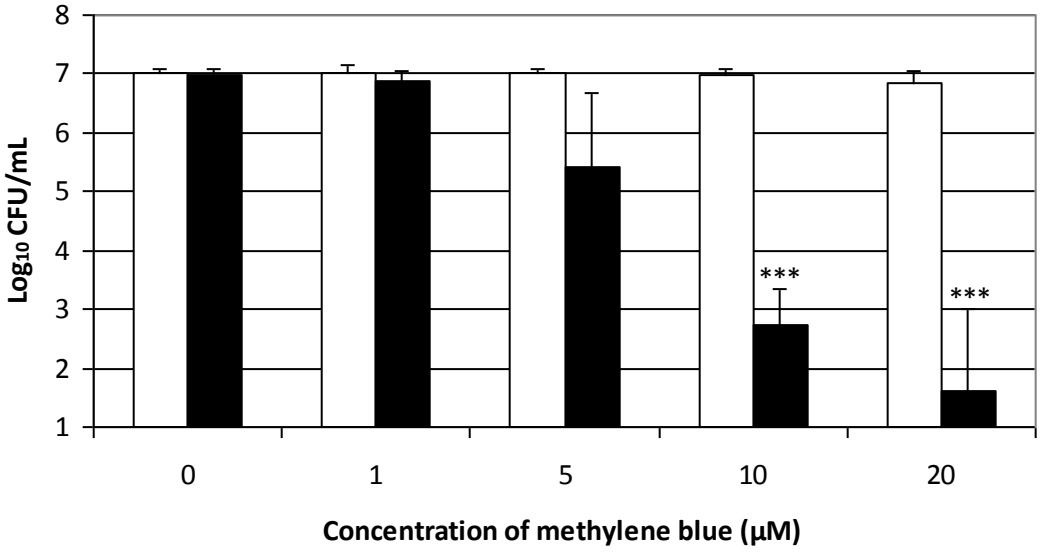

Figure 3.1 Lethal photosensitisation of *S. aureus* 8325-4 with 1, 5, 10 and 20 µM methylene blue and 4.38 J of 665 nm laser light.

An equal volume of either PBS or methylene blue (final concentrations ranging from 1-20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

3.3.1.2 Tin chlorin e6

Killing of *S. aureus* 8325-4 using tin chlorin e6 and 633 nm laser light was less effective than methylene blue and 665 nm laser light, as can be seen in Figure 3.2. A significant reduction in viability was only achieved using the highest concentration of tin chlorin e6 tested (20 µM), corresponding to a 92% reduction in the number of viable bacteria compared with the control (** $P < 0.01$; ANOVA). As seen previously,

there was no effect of either photosensitiser or laser light alone on the viability of *S. aureus* 8325-4.

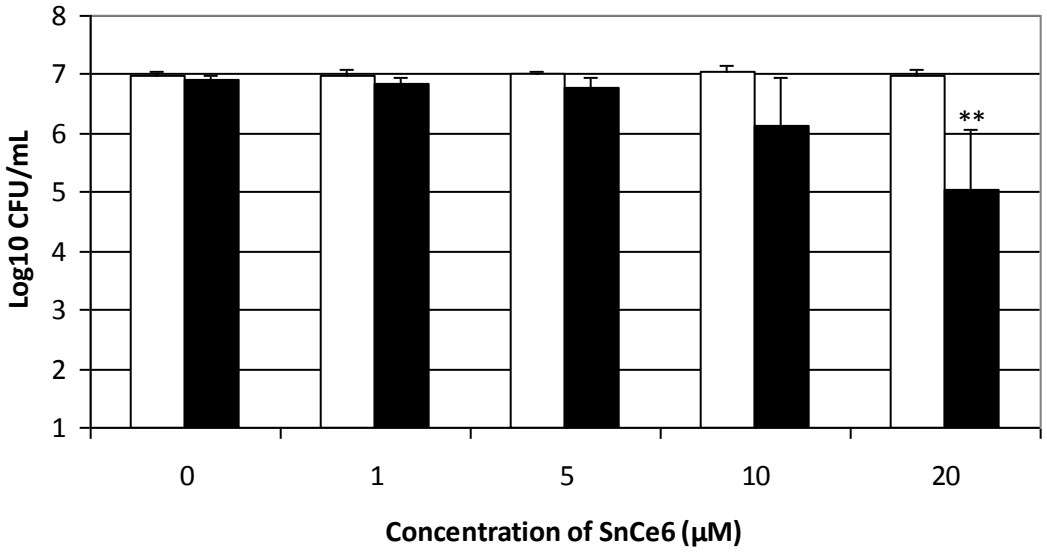

Figure 3.2 Lethal photosensitisation of *S. aureus* 8325-4 with 1, 5, 10 and 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light.

An equal volume of either PBS or tin chlorin e6 (final concentrations ranging from 1-20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 633 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. ** $P < 0.01$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

3.3.2 The effect laser light dose on the lethal photosensitisation of *S. aureus* 8325-4

3.3.2.1 Laser light of 665 nm

Figure 3.3 shows the effect of 665nm laser light dose on the photodynamic killing of *S. aureus* 8325-4. Laser light alone had no effect on the viability of the bacteria; however, 20 µM methylene blue and laser light doses of 4.38 J, 8.76 J and 21.9 J all resulted in significant, light dose-dependent killing of *S. aureus* 8325-4. A 99.999% kill was achieved after treatment with 21.9 J laser light and 20 µM methylene blue.

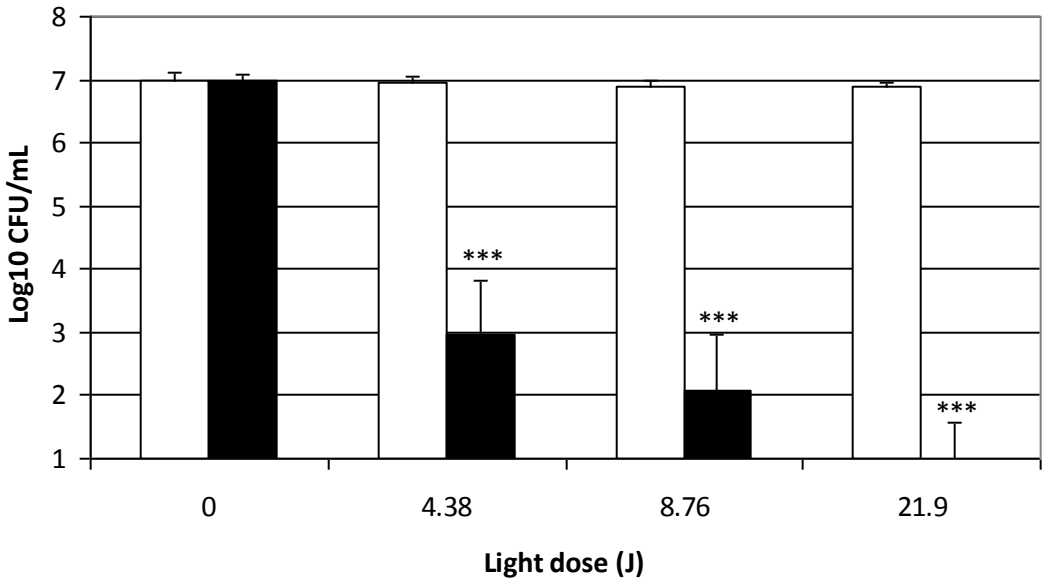

Figure 3.3 Lethal photosensitisation of *S. aureus* 8325-4 using 20 µM methylene blue and laser light doses of 4.38 J, 8.76 J and 21.9 J.

An equal volume of either PBS (□) or 20 µM methylene blue (■) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 665 nm laser light doses of 4.38 J, 8.76 J and 21.9 J respectively. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.2.2 Laser light of 633 nm

The effect of photosensitisation of *S. aureus* 8325-4 with tin chlorin e6 and laser light of 633 nm is shown in Figure 3.4. Treatment with 20 µM tin chlorin e6 and 633 nm laser light resulted in a highly significant reduction in the number of viable bacteria, with a 99.93% kill being achieved after irradiation with 21.9 J of laser light ($P < 0.001$; ANOVA). Interestingly, irradiation of the bacteria with laser light in the absence of photosensitiser also resulted in significant killing when light doses of 8.76 J and 21.9 J were used ($P < 0.01$ and < 0.001 for 8.76 J and 21.9, respectively).

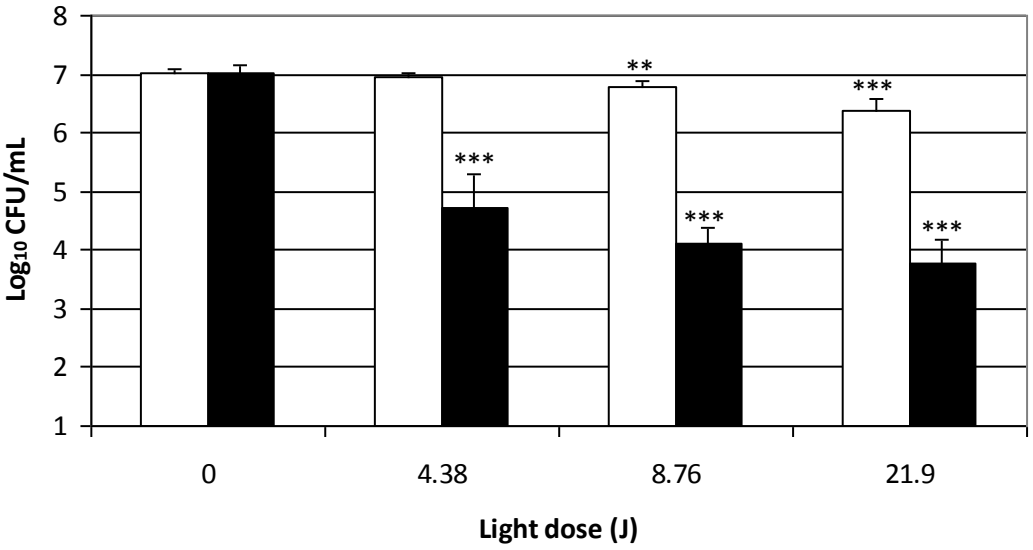

Figure 3.4 Lethal photosensitisation of *S. aureus* 8325-4 using 20 µM tin chlorin e6 and laser light doses of 4.38 J, 8.76 J and 21.9 J.

An equal volume of either PBS (□) or 20 µM tin chlorin e6 (■) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 633 nm laser light doses of 4.38 J, 8.76 J and 21.9 J. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. ** $P < 0.01$, *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.3 The effect of a singlet oxygen enhancer on the lethal photosensitisation of *S. aureus* 8325-4

It can be seen from Figure 3.5 that suspension of bacteria in D_2O rather than distilled water resulted in a highly significant enhancement of lethal photosensitisation using 5 µM methylene blue and laser light ($P < 0.001$; ANOVA). Killing was increased from 92.11% to 99.96% in the presence of D_2O, approximately equivalent to a 2-Log_{10} reduction in the number of viable bacteria. D_2O alone did not have an effect on the viability of *S. aureus* 8325-4.

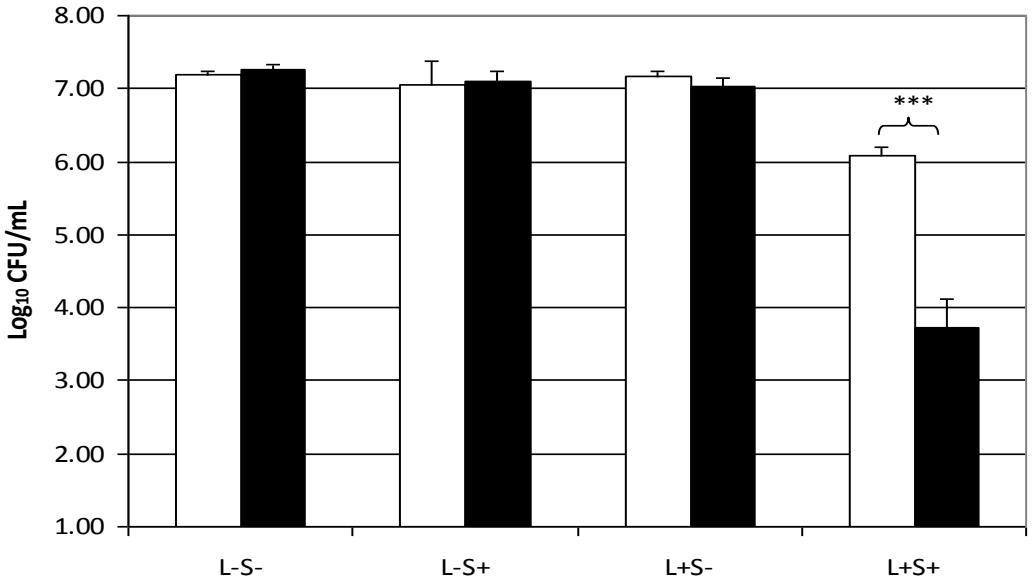

Figure 3.5 The effect of a singlet oxygen enhancer on the lethal photosensitisation of *S. aureus* 8325-4.

An equal volume of either PBS (S-) or 5 µM methylene blue (S+) was added to 50 µL of the bacterial suspension in either H_2O (□) or D_2O (■) and either exposed to 4.38 J of 665 nm laser light (L+) or incubated in the dark (L-). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed twice in triplicate and the combined data are shown.

3.3.4 The effect of a singlet oxygen scavenger on the lethal photosensitisation of *S. aureus* 8325-4

Figure 3.6 shows that the singlet oxygen scavenger L-tryptophan inhibited the killing of *S. aureus* 8325-4 by methylene blue and laser light. Killing of *S. aureus* 8325-4 was reduced by approximately 2-Log_{10} in the presence of L-tryptophan; this reduction was found to be highly significant ($P < 0.001$; ANOVA).

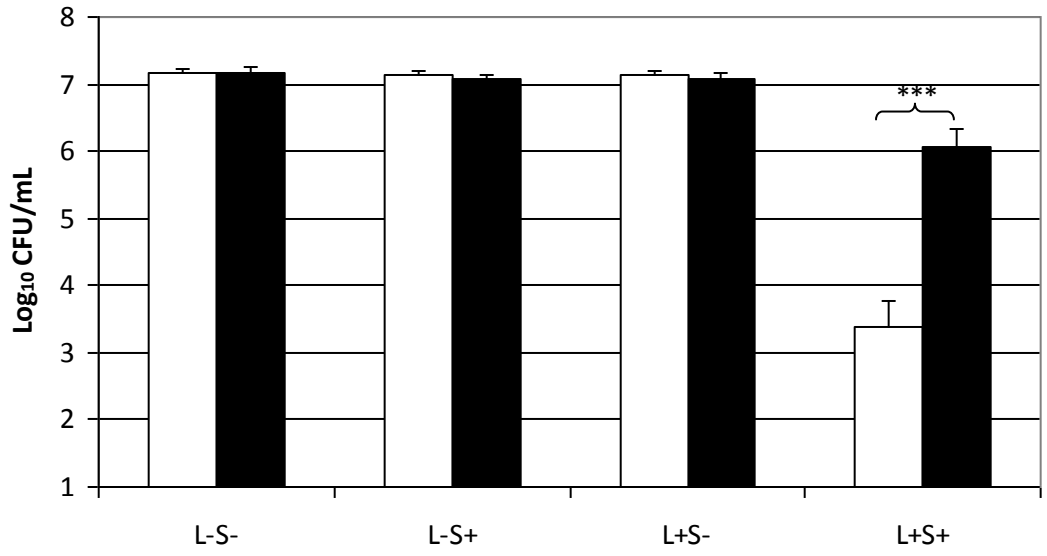

Figure 3.6 The effect of a singlet oxygen scavenger on the lethal photosensitisation of *S. aureus* 8325-4.

An equal volume of either PBS (S-) or 10 µM methylene blue (S+) was added to 50 µL of the bacterial suspension in either H_2O (□) or 10 mM L-tryptophan (■) and either exposed to 4.38 J of 665 nm laser light (L+) or incubated in the dark (L-). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.5 The effect of photosensitiser dose on the lethal photosensitisation of EMRSA-16

3.3.5.1 Methylene blue

Figure 3.7 shows that treatment of EMRSA-16 with 20 µM methylene blue and 4.38 J of 665 nm laser light resulted in approximately a 4-$\log_{10}$ reduction in viability (99.98% kill). This was equivalent to the kills observed with *S. aureus* 8325-4 using the same parameters, showing that this regimen was as effective against a meticillin-resistant strain of *S. aureus* as a meticillin-sensitive strain.

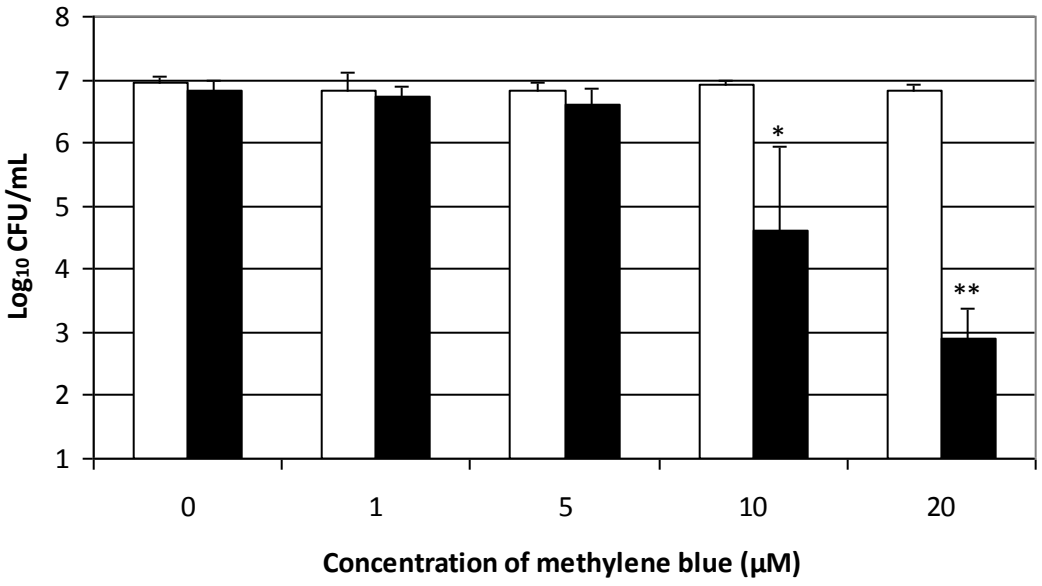

Figure 3.7 Lethal photosensitisation of EMRSA-16 with 1, 5, 10 and 20 µM methylene blue and 4.38 J of 665 nm laser light.

An equal volume of either PBS or methylene blue (final concentrations ranging from 1-20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. * $P < 0.05$, *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

3.3.5.2 Tin chlorin e6

The effect of tin chlorin e6 dose on the lethal photosensitisation of EMRSA-16 is shown in Figure 3. 8. It can be seen that killing of EMRSA-16 was dependent on photosensitiser dose, with significant reduction in viability being achieved with doses of tin chlorin e6 of 5 µM and above. Treatment of EMRSA-16 with 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light resulted in a 94% kill, comparable to the kills achieved for the meticillin-sensitive strain 8325-4 (92%).

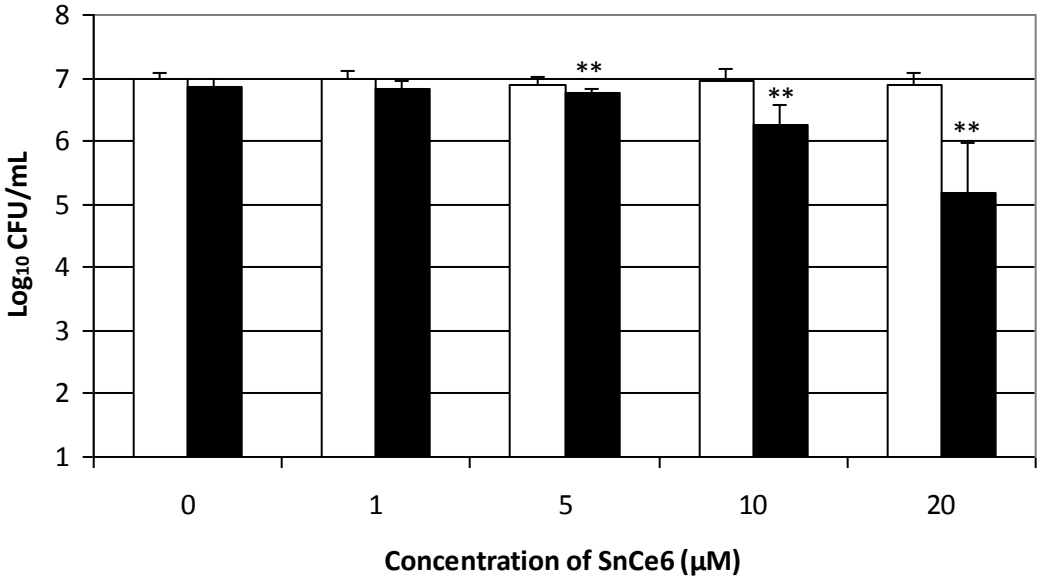

Figure 3. 8 Lethal photosensitisation of EMRSA-16 with 1, 5, 10 and 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light.

An equal volume of either PBS or tin chlorin e6 (concentrations ranging from 1-20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 633 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. ** $P < 0.01$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

3.3.6 The effect of laser light dose on the lethal photosensitisation of EMRSA-16

3.3.6.1 Laser light of 665 nm

Figure 3.9 shows the effect of 665 nm laser light dose on the viability of EMRSA-16. Light doses of 4.38 J and above all resulted in a highly significant reduction in the number of viable bacteria when EMRSA-16 was irradiated in the presence of methylene blue ($P < 0.001$; ANOVA). After irradiation with 21.9 J laser light in the presence of 20 μM methylene blue an approximate 6-$\log_{10}$ reduction in viability was achieved, corresponding to a 99.999% kill, demonstrating the effectiveness of this regimen against MRSA. This was equivalent to the reduction in viability observed for the meticillin-sensitive strain.

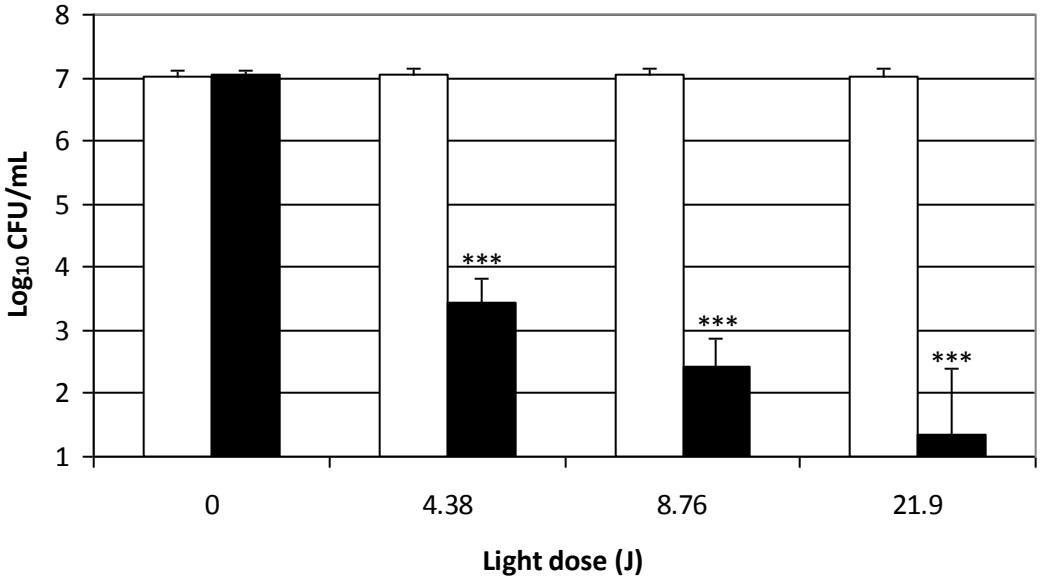

Figure 3.9 The effect of 20 µM methylene blue and 665 nm laser light doses of 4.38 J, 8.76 J and 21.9 J on the lethal photosensitisation of EMRSA-16.

An equal volume of either PBS (□) or 20 µM methylene blue (■) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 665 nm laser light doses of 4.38 J, 8.76 J and 21.9 J. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.6.2 Laser light of 633 nm

The effect of 633 nm laser light dose on the lethal photosensitisation of EMRSA-16 can be seen in Figure 3.10. The results were similar to those observed with *S. aureus* 8325-4; treatment of EMRSA-16 with 20 µM tin chlorin e6 and 21.9 J of laser light resulted in a 99.83% kill, comparable to the 99.93% kill observed for the meticillin-sensitive strain. As also observed for *S. aureus* 8325-4, irradiation of EMRSA-16 with either 8.76 J or 21.9 J laser light in the absence of photosensitiser resulted in significant killing of bacteria; however, significantly less than the kills achieved with the same light dose in the presence of photosensitiser ($P < 0.001$; ANOVA).

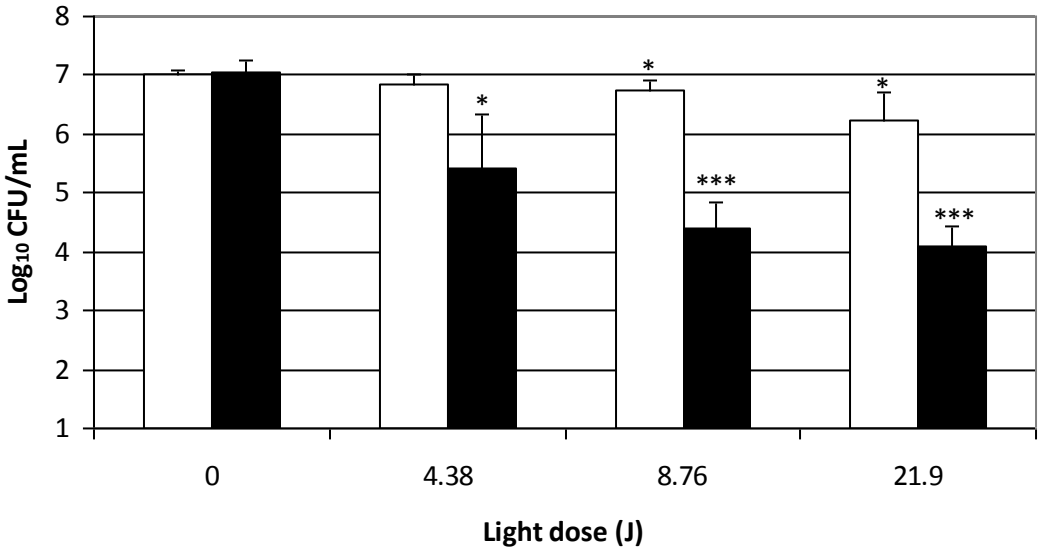

Figure 3.10 The effect of 20 µM tin chlorin e6 and 633 nm laser light doses of 4.38 J, 8.76 J and 21.9 J on the lethal photosensitisation of EMRSA-16.

An equal volume of either PBS (□) or 20 µM tin chlorin e6 (■) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 633 nm laser light doses of 4.38 J, 8.76 J and 21.9 J. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. * $P < 0.05$, *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.7 The effect of photosensitiser dose on the lethal photosensitisation of *P. gingivalis* W50

3.3.7.1 Methylene blue

The effect of methylene blue dose on the lethal photosensitisation of *P. gingivalis* W50 is shown in Figure 3.11. Killing of this microorganism using methylene blue and 665 nm laser light was found to be highly effective and dependent on photosensitiser dose. Photosensitisation using 20 μM methylene blue in combination with 665 nm laser light achieved a highly significant reduction in the number of viable bacteria, corresponding to a 99.999% kill ($P < 0.001$; ANOVA). There was no effect of either laser light or photosensitiser alone on the viability of *P. gingivalis* W50. Statistically significant killing was achieved at a lower photosensitiser dose (5 μM) than that observed for *S. aureus*.

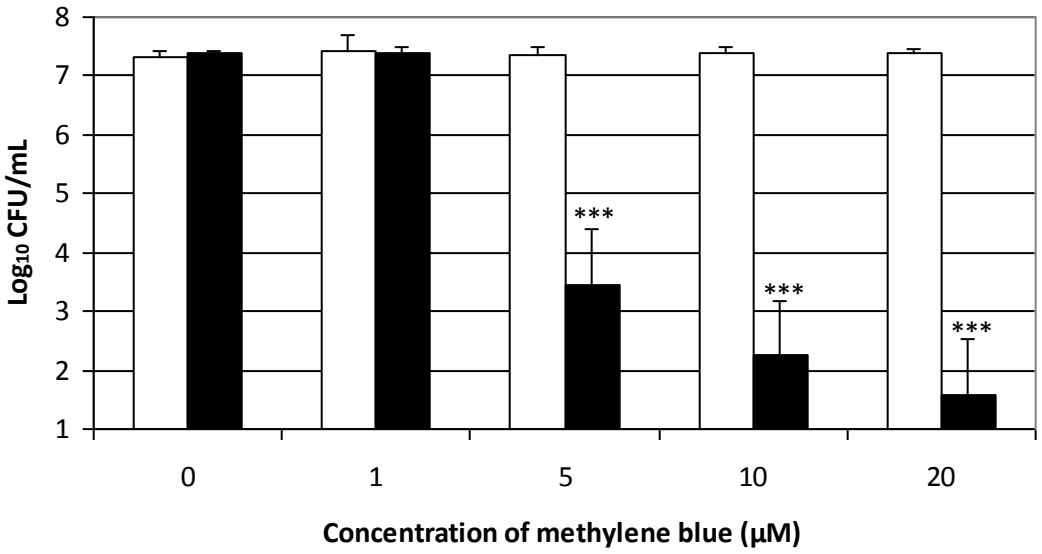

Figure 3.11 Lethal photosensitisation of *P. gingivalis* W50 with 1, 5, 10 and 20 µM methylene blue and 4.38 J of 665 nm laser light.

An equal volume of either PBS or methylene blue (concentrations ranging from 1-20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown

3.3.7.2 Tin chlorin e6

Significant, photosensitiser dose-dependent killing of *P. gingivalis* W50 was achieved following treatment with tin chlorin e6 and 633 nm laser light, as can be seen in Figure 3.12. As observed for methylene blue, significant killing was achieved using a lower photosensitiser dose than necessary for *S. aureus* 8325-4 and EMRSA-16. A 99.95% reduction in the number of viable bacteria was achieved following photosensitisation with 4.38 J of 633 nm laser light and 20 µM tin chlorin e6. Again,

there was no effect of either laser light or photosensitiser alone on the viability of *P. gingivalis* W50.

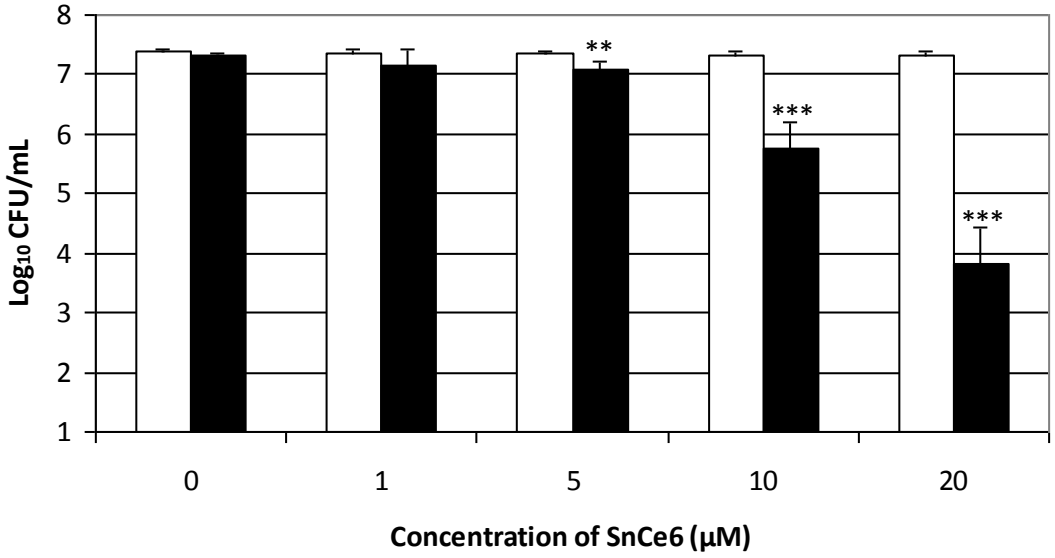

Figure 3.12 Lethal photosensitisation of *P. gingivalis* W50 with 1, 5, 10 and 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light.

An equal volume of either PBS or tin chlorin e6 (concentrations ranging from 1-20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J 633 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. ** $P < 0.01$, *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

3.3.8 The effect of laser light dose on the lethal photosensitisation of *P. gingivalis* W50

3.3.8.1 Laser light of 665 nm

Figure 3.13 shows the effect of 665 nm laser light dose on the photodynamic killing of *P. gingivalis* W50 in the presence or absence of 20 µM methylene blue. As can be seen, highly significant reductions in the number of viable bacteria were achieved following irradiation with light doses of 4.38 J and above. Percentage kills above or equal to 99.99% were observed for all three light doses ($P < 0.001$; ANOVA).

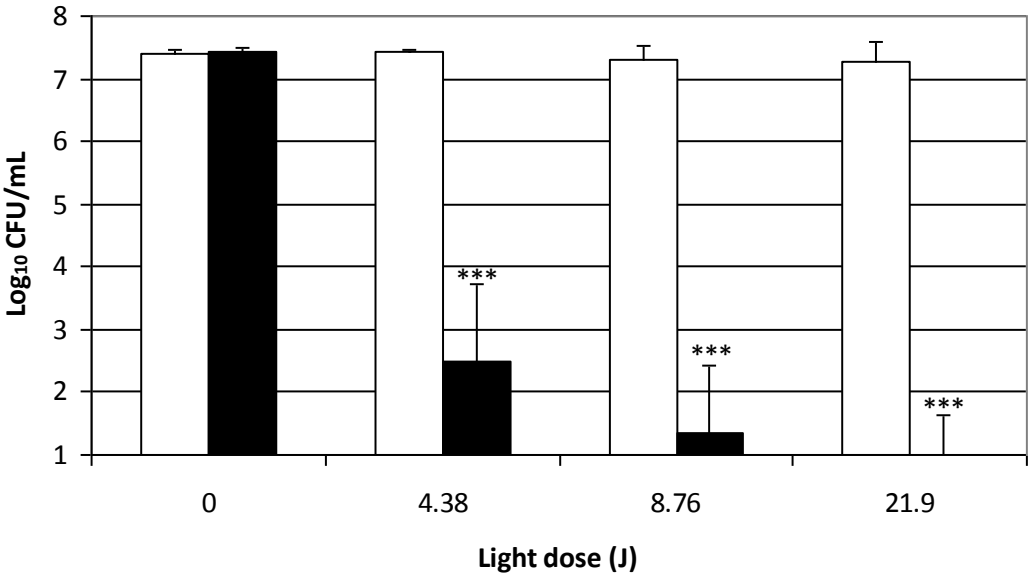

Figure 3.13 The effect of 20 µM methylene blue and laser light doses of 4.38 J, 8.76 J and 21.9 J on the lethal photosensitisation of *P. gingivalis* W50.

An equal volume of either PBS (□) or 20 µM methylene blue (■) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 665 nm laser light doses of 4.38 J, 8.76 J and 21.9 J. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.8.2 Laser light of 633 nm

The effect of 633 nm laser light dose on the lethal photosensitisation of *P. gingivalis* W50 is shown in Figure 3.14. Similar to the results for 665 nm laser light, light doses of 4.38 J and above resulted in highly significant reductions in the number of viable bacteria in the presence of tin chlorin e6 ($P < 0.001$; ANOVA). Kills of greater than 99.99% were observed for all three light doses. Unlike *S. aureus* 8325-4 and EMRSA-16, laser light alone did not cause a significant reduction in the number of viable *P. gingivalis* W50.

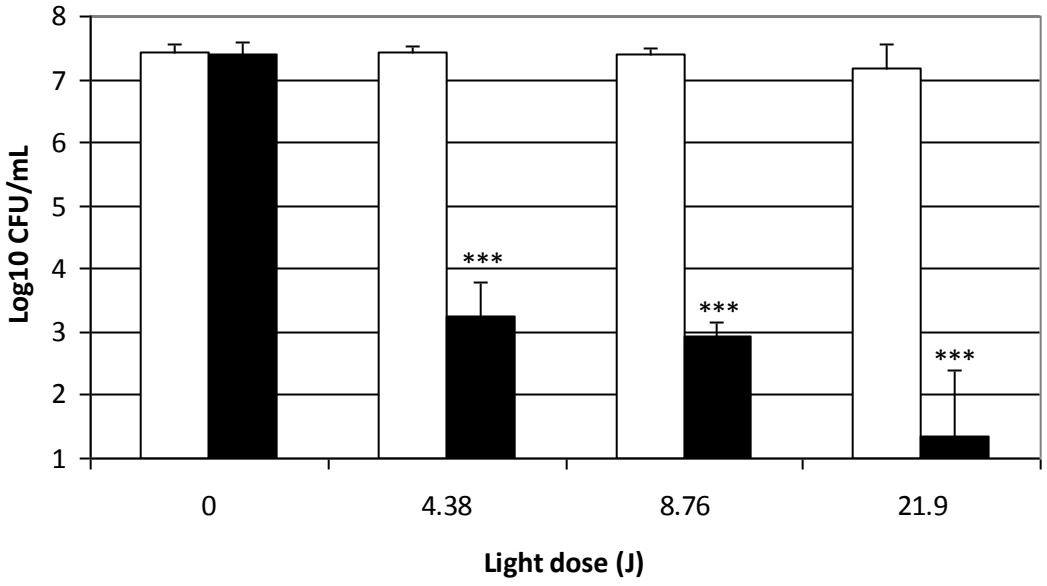

Figure 3.14 The effect of 20 µM tin chlorin e6 and laser light doses of 4.38 J, 8.76 J and 21.9 J on the lethal photosensitisation of P. gingivalis W50.

An equal volume of either PBS (□) or 20 µM tin chlorin e6 (■) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 633 nm laser light doses of 4.38 J, 8.76 J and 21.9 J. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data are shown.

3.3.9 The effect of laser light dose on sample temperature

3.3.9.1 Laser light of 665 nm

Following irradiation of PBS with 0, 4.38, 8.76 and 21.9 J 665 nm laser light, the average temperature of the samples was 25.3, 25.7, 26.1 and 26.7°C, respectively.

For 20 µM methylene blue, the average temperature following irradiation with 0, 4.38, 8.76 and 21.9 J 665 nm laser light was 26.0, 26.2, 27.0 and 27.6°C, respectively. For 20 µM methylene blue in the presence of 12.5% human serum (final concentration of 6.25%), the average temperature following irradiation with 0, 4.38, 8.76 and 21.9 J 665 nm laser light was 25.6, 26.3, 26.6 and 28.0°C, respectively.

The average room temperature was recorded as 24.3°C.

3.3.9.2 Laser light of 633 nm

Following irradiation of PBS with 0, 4.38, 8.76 and 21.9 J 633 nm laser light, the average temperature of the samples was 27.0, 28.1, 28.3 and 28.8°C, respectively.

For 20 µM tin chlorin e6, the average temperature following irradiation with 0, 4.38, 8.76 and 21.9 J 665 nm laser light was 25.4, 26.6, 27.4 and 28.8°C, respectively.

The average room temperature was recorded as 23.3°C.

3.4 Discussion

The successful photodynamic killing of *S. aureus* (including MRSA) *in vitro* using a range of photosensitisers has been well documented (Bertoloni *et al.*, 2000; Wilson & Yianni, 1995; Zeina *et al.*, 2001) and the results presented here support these findings. Both a meticillin-sensitive and meticillin-resistant strain of *S. aureus* were shown to be susceptible to lethal photosensitisation by both regimens tested, with highly significant kills achieved for the two strains. The photodynamic killing of both *S. aureus* 8325-4 and EMRSA-16 was found to be dependent on photosensitiser dose and laser light dose. The combination of methylene blue and 665 nm laser light was found to be the most effective photosensitisation strategy, resulting in kills of 99.999% for both the meticillin-sensitive and meticillin-resistant strain after treatment with 20 μM methylene blue and 21.9 J laser light. This light dose corresponds to a short irradiation time (5 minutes), which would be beneficial in a clinical setting.

Laser light of 633 nm alone (i.e. in the absence of photosensitiser) was found to cause a significant reduction in the number of viable MSSA and MRSA. The antistaphylococcal effect of 633 nm laser light alone has been previously observed and may be due to the presence of endogenous photosensitisers that absorb light of this wavelength (Wilson & Pratten, 1994). *S. aureus* is known to produce a number of light-absorbing compounds including staphyloxanthin (Pelz *et al.*, 2005) and porphyrins (Nitzan & Kauffman, 1999). As the average temperature recorded following the highest light dose used (21.9 J) was 28.8°C, it can be concluded that the killing effect observed is not due to thermal killing of the bacteria.

Investigation of the mechanism of photodynamic killing revealed that singlet oxygen is likely to be involved as lethal photosensitisation of *S. aureus* 8325-4 in the presence of an enhancer of singlet oxygen lifetime. Deuterium oxide significantly

enhanced the efficacy of photodynamic killing using methylene blue and 665 nm laser light. Equally, photosensitisation of *S. aureus* 8325-4 in the presence of a quencher of singlet oxygen (L-tryptophan) resulted in significantly lower killing of the microorganism. These findings are consistent with those of Omar *et al*., who reported similar results using indocyanine green and near-infrared laser light for the photodynamic killing of *S. aureus* 8325-4 (Omar *et al.*, 2008). Singlet oxygen is generally thought to be responsible for the majority of oxidative damage sustained during lethal photosensitisation of microorganisms (Wainwright, 2000).

Although a concentration of 20 μM methylene blue in combination with 665 nm laser light achieved a kill of 99.99% for both strains when irradiated with 4.38 J of laser light, EMRSA-16 was less susceptible than *S. aureus* 8325-4 at lower concentrations (5 and 10 μM). Grinholc *et al*. reported the reduced susceptibility of clinical meticillin-resistant strains of *S. aureus* compared with meticillin-sensitive strains to sensitisation with protoporphyrin diarginate and laser light of 624 nm. The reduced susceptibility of MRSA was proposed to be due to the presence of capsular polysaccharides that may affect penetration of the photosensitiser (Grinholc *et al.*, 2008). High-level meticillin resistance in *S. aureus* has also been correlated with increased cell wall cross-linking (Higashi *et al.*, 1999). Thicker cell walls have been observed in MRSA compared with MSSA (Hiramatsu *et al.*, 1997); therefore it is possible that these differences in the cell wall between MSSA and MRSA may contribute to the difference in susceptibility to lethal photosensitisation observed at lower photosensitisation concentrations. Higher concentrations of photosensitiser may overcome the protection afforded by these structural differences, as similar susceptibility to lethal photosensitisation was observed following treatment of MSSA and MRSA with 20 μM methylene blue and 665 nm laser light.

Previous studies have shown that significant killing of *S. aureus*, including meticillin-resistant strains, can be achieved in the presence of serum, designed to more closely

resemble wound conditions (Omar *et al.*, 2008; Soncin *et al.*, 2002; Wilson & Pratten, 1995). MRSA has also been shown to be successfully killed by methylene blue *in vivo* (Zolfaghari *et al.*, 2009), albeit less effectively than *in vitro*; however this is to be expected, considering the complexity of the wound environment. It has been proposed that the efficacy of photodynamic killing *in vivo* may be enhanced by the use of targeted photosensitisers, thus increasing the specificity of PDT; indeed, the selective killing of MRSA using a tin chlorin e6 conjugate has been shown to be successful in preliminary tests (Embleton *et al.*, 2002). The potential for the development of a targeted photosensitiser is extremely relevant as selective killing would reduce disruption of the commensal microbiota and damage to host tissues.

The study reported herein has demonstrated the effectiveness of lethal photosensitisation for the photodynamic inactivation of MSSA and MRSA. Considering the increasing problem of antibiotic resistance among both nosocomial and community-acquired strains of *S. aureus*, PDT is an extremely relevant and promising alternative antimicrobial strategy. More research is required to examine the effectiveness of light-activated antimicrobials against MRSA *in vivo*, and there is much scope for the development of targeted light-activated antimicrobial agents. Topical antimicrobials are important in the treatment of infections caused by *S. aureus* (Wilson & Pratten, 1995) and due to their accessibility, superficial MRSA infections are ideally suited to treatment using PDT (Maisch, 2007).

The Gram-negative bacterium *P. gingivalis* has previously been shown to be susceptible to photosensitisation using a range of photosensitisers (Meisel & Kocher, 2005). In this study, *P. gingivalis* W50 was shown to be highly susceptible to lethal photosensitisation with both methylene blue and tin chlorin e6, with greater kills being observed than those seen with *S. aureus* for both photosensitisers. Gram-negative bacteria are generally considered to be less susceptible to photodynamic killing than Gram-positive bacteria as the outer membrane of Gram-negative

bacteria acts as a permeability barrier (Maisch *et al.*, 2004). In Gram-positive bacteria, due to the absence of the outer membrane, the relatively porous cell wall readily allows diffusion of small molecules such as some photosensitisers (< 60 kDa) through to the cell membrane where the formation of reactive oxygen species causes damage (Jori *et al.*, 2006). It is therefore interesting that *P. gingivalis* W50 was found to be more susceptible to lethal photosensitisation than the Gram-positive *S. aureus* in this study. It has previously been postulated that uptake of cationic compounds may be higher in Gram-negative bacteria via binding to lipopolysaccharide (LPS) and consequent self-promoted uptake. In addition, the LPS is highly negatively charged, and therefore cationic photosensitisers may localise to the LPS and on irradiation cause damage to the outer membrane (Maisch *et al.*, 2004).

A highly significant reduction in the number of viable bacteria was achieved at lower photosensitiser concentrations than observed for *S. aureus* (i.e. 5 µM). Bhatti *et al*. (Bhatti *et al.*, 1997) reported highly successful photodynamic killing of this organism using toluidine blue O (TBO) and 4.4 J of 633 nm laser light; in fact, 100% killing of *P. gingivalis* was claimed to be achieved under these conditions. TBO, a phenothiazinium compound similar to methylene blue, has also been successfully employed in the killing of *P. gingivalis* in subgingival plaque samples (Wilson, 2004). It is thought that cationic photosensitisers (i.e. methylene blue) may be taken up by Gram-negative bacteria by a self-promoted uptake pathway (Jori *et al.*, 2006). As methylene blue is cationic, this may account for the efficacy of photodynamic killing using this photosensitiser. It is also possible that the bacterium's anaerobic requirements render it more susceptible to the effects of reactive oxygen species and this overcomes any protective effects provided by the Gram-negative cell envelope.

Photodynamic therapy is ideally suited to the treatment of periodontal diseases, of which *P. gingivalis* is a major aetiological agent, as "traditional" treatments such as antibiotics are difficult to maintain at a suitable concentration in the periodontal pocket (Jori *et al.*, 2006) and may not penetrate bacterial biofilms (Maisch, 2007). The use of photosensitisers for the killing of bacteria within plaque biofilms is well documented in the literature (Konopka & Goslinski, 2007). Highly significant killing of *P. gingivalis* is possible in the presence of serum (Bhatti *et al.*, 1997) and blood (Matevski *et al.*, 2003), indicating that photodynamic killing of this organism may be possible *in vivo*. Photodynamic therapy has been shown to be effective in animal models of periodontitis (Komerik *et al.*, 2003). Preliminary clinical studies have shown PDT to be as effective as scaling and root planing for the non-surgical treatment of aggressive periodontitis, and has the advantages of not requiring anaesthesia and a relatively short treatment time (de Oliveira *et al.*, 2007).

3.5 Summary

Treatment of MSSA, MRSA and *P. gingivalis* with the light-activated antimicrobials methylene blue and tin chlorin e6 in combination with laser light of the appropriate wavelength resulted in a highly significant reduction in the viability of all strains. In addition, HeNe laser light alone is able to kill *S. aureus* species. Reports in the literature state that photodynamic killing of these organisms is possible in conditions designed to mimic the wound environment; therefore PDT shows great promise for the eradication of these microorganisms and the treatment of infections of which they are the causative organism.

4 Inactivation of key staphylococcal virulence factors using light-activated antimicrobial agents

4.1 Introduction

Staphylococcus aureus is an important opportunistic human pathogen, and has been found to be the most common cause of skin and soft tissue infections in Europe and the Americas (Moet *et al.*, 2007). Due to the accessibility of skin and soft tissue infections, these infections have become a prime target for antimicrobial PDT (Wainwright, 2010). There has been much interest in the development of PDT for the treatment of staphylococcal skin infections, especially those caused by MRSA (Embleton *et al.*, 2002; Grinholc *et al.*, 2008; Wilson & Yianni, 1995; Zolfaghari *et al.*, 2009).

The pathogenic potential of *S. aureus* is dependent on production of an extensive array of over 40 multifunctional virulence factors, which have been implicated in host colonisation and tissue damage, and modulation of the host immune response (Arvidson & Tegmark, 2001). Consequently, the ability of an antimicrobial therapy to inactivate or reduce the biological activity of *S. aureus* virulence factors would be a highly desirable property.

S. aureus produces a number of cell surface-anchored binding proteins that mediate attachment to host extracellular matrix (ECM) proteins. Fibronectin is involved in wound healing and thrombosis, and is found in a soluble form in body fluids and in an insoluble form in the ECM (Potts & Campbell, 1994). The fibronectin-binding proteins A and B have been implicated in the adherence of *S. aureus* to host cells, plasma clots and biomaterials, and are also believed to act as invasions by facilitating the entry of *S. aureus* into host cells (Foster & Höök, 1998; Menzies, 2003). In addition to the classic fibronectin-binding proteins, *S. aureus* also

expresses the cell envelope-associated ECM-binding protein homologue, which specifically binds human fibronectin (Clarke *et al.*, 2002). Fibronectin-binding proteins are also thought to play a role in the binding of *S. aureus* to atopic skin, which is frequently colonised by this organism, and have been proposed as a therapeutic target for the reduction of staphylococcal colonisation in atopic skin disorders (Cho *et al.*, 2001).

The binding of *S. aureus* to fibrinogen has also been implicated in colonisation of atopic skin (Cho *et al.*, 2001). *S. aureus* binds to fibrinogen via cell wall-associated clumping factors A and B and fibronectin-binding protein A, and also secretes extracellular fibrinogen-binding protein (Efb) and extracellular adherence protein (Eap), which bind to fibrinogen in addition to several other plasma proteins, in the case of Eap (Foster & Höök, 1998; Götz, 2004; Rivera *et al.*, 2007). Eap also mediates internalisation of *S. aureus* by host cells and also has immunomodulatory properties (Harraghy *et al.*, 2003). Another staphylococcal protein with immunomodulatory functions is Protein A, which exists in both membrane-associated and secreted forms (Graille *et al.*, 2000). Protein A binds the Fc region of IgG, binding the antibody in the wrong orientation and disabling recognition by neutrophils, thus escaping opsonisation and phagocytosis (Foster, 2005). Protein A also stimulates the release of proinflammatory cytokines from monocytes and fibroblasts and is believed to play a role in sepsis (Fournier & Philpott, 2005).

S. aureus also secretes a number of toxins that are implicated in virulence. The bacterium produces several extracellular proteolytic enzymes, including serine-, cysteine- and metalloproteases, which are not affected by human plasma protease inhibitors (Dubin, 2002). These proteases are multifunctional enzymes, and have been proposed to be involved in host tissue destruction, avoidance of the host immune response, inactivation of host enzymes and regulation of bacterial adhesion (Dubin, 2003). The *S. aureus* serine protease, or V8 protease after the strain it was

first isolated from, was the first proteolytic enzyme to be purified from *S. aureus* (Drapeau *et al.*, 1972). V8 protease is produced by the majority of human isolates of *S. aureus*, and as well as providing nutrients for the bacterium via the cleavage of host proteins, the V8 protease also enhances bacterial survival in the host by cleavage of immunoglobulins and inactivation of human α_1-proteinase inhibitor (Arvidson, 2000).

S. aureus produces several cytotoxins: the α-, β-, δ- and γ-haemolysins and the Panton-Valentine leukocidin. The α-haemolysin is thought to be important in infection as it has a number of detrimental effects on host cells due to the disruption of ion transport across host cell membranes, ultimately leading to apoptotic cell death and oedema (Dinges *et al.*, 2000). Alpha-haemolysin can cause cell death in different ways depending on the concentration of the toxin. At high concentrations, α-haemolysin forms large pores in lipid bilayers that result in massive necrosis, whilst low doses result in the formation of small pores that result in apoptosis and DNA fragmentation (Essmann *et al.*, 2003). *S. aureus* α-haemolysin has recently been shown to be critical for dermonecrosis in an animal model of community-acquired MRSA skin infection (Kennedy *et al.*, 2010). In addition, the toxin is believed to play a role in facilitating escape of the bacterium from endocytic vesicles, and can also induce the expression of proinflammatory cytokines (Jarry *et al.*, 2008; Rose *et al.*, 2002).

The role of sphingomyelinase (also known as β toxin or β-haemolysin) is not yet fully understood (Dinges *et al.*, 2000); however, the haemolysin has several proposed functions in human infection, particularly in protection against host defences. Selective killing of monocytes by β-haemolysin has been reported and believed to be due to the generation of defects in the monocyte membrane (Walev *et al.*, 1996). The toxin has been shown to be important for defence against T cell-mediated killing and inhibits production of chemokines and neutrophil migration (Collins *et al.*, 2008;

Tajima *et al.*, 2009). Sphingomyelinase also acts synergistically with *S. aureus* δ-toxin to enable escape of the bacterium from phagosomes and thus avoid killing (Giese *et al.*, 2010).

S. aureus produces a multitude of virulence factors, which have a wide range of activities and functions in the infection process. The photosensitisation of selected *S. aureus* virulence factors with the light-activated antimicrobial agents methylene blue and tin chlorin e6 in the presence of laser light of 665 nm and 633 nm respectively is described in the following chapter. The effect of concentrations of photosensitiser ranging from 1 to 20 µM and laser light on a selection of secreted enzymes and surface proteins was investigated.

4.2 Materials and methods

4.2.1 Crystal violet assay to assess the effect of photosensitisation on the detachment of *S. aureus* from fibronectin

The following assay was performed to assess the ability of photosensitisation to cause detachment of *S. aureus* from fibronectin-coated surfaces. Maxisorp™ 96-well plates (Nunc, UK) were coated with 200 µL of 0.1 mg/ml fibronectin in PBS (from human plasma; Sigma-Aldrich, UK) and incubated overnight at 4°C. The plates were washed four times with 200 µL PBS and the test wells blocked with 200 µL 1% bovine serum albumin (Sigma-Aldrich, UK) in PBS for one hour at 37°C. The plates were then washed four times with 200 µL PBS.

S. aureus 8325-4 was grown aerobically in BHI broth (Oxoid Ltd, UK) at 37°C for 16 hours in a shaking incubator at 200 rpm. Cultures were centrifuged and resuspended in an equal volume of PBS and the optical density was adjusted to 1 at 590 nm. The bacteria were titred by making serial dilutions in PBS and spotting 10 µL of the dilutions in triplicate onto triplicate 5% horse blood agar plates. Plates were incubated overnight at 37°C and the viable counts determined.

Aliquots of 200 µL of the bacterial inoculum were added to duplicate wells of a Maxisorp™ 96-well plate pre-coated with fibronectin as described above, and incubated for 2 hours at 37°C. After incubation, plates were washed three times with 200 µL PBS to remove any unbound bacteria. One hundred microlitres of methylene blue (S+) or 100 µL PBS (S-) was added to the test wells and either exposed to 4.38 J of 665 nm laser light (L+) or incubated in the dark (L-), without stirring. After irradiation, the plates were washed three times with 200 µL PBS to remove any unbound bacteria. Each well was stained with 200 µL 0.1% crystal violet solution (Pro-Lab Diagnostics) for 10 minutes at room temperature and then washed

five times with 200 µL PBS. Plates were then dried at 37°C for approximately one hour. Following drying, 200 µL of ≥ 99.5% ethanol was added to each well and incubated at 4°C for four hours. The optical density of the wells at 590 nm was recorded using a Dynex plate reader.

4.2.2 Viable count assay to examine the effect of sub-lethal photosensitisation on the detachment of *S. aureus* from fibronectin

The following assay was performed to determine the effect of photosensitisation with sub-lethal concentrations of methylene blue on the fibronectin-binding properties of *S. aureus*. Maxisorp™ plates were coated, blocked and washed as described in section 4.2.1. Cultures of *S. aureus* 8325-4 (grown as previously described) were centrifuged and resuspended in an equal volume of PBS and the optical density was adjusted to 0.1 at 600 nm, giving an inoculum of approximately $5x10^7$ cfu/mL. The bacteria were titred by making serial dilutions in PBS and spotting 10 µL of the dilutions in triplicate onto triplicate 5% horse blood agar plates. Plates were incubated overnight at 37°C and the viable counts determined.

100 µL of the inoculum was added to the coated wells and the plates were incubated at 37°C for one hour. Unbound bacteria were removed by washing the plates four times with 200 µL PBS. One hundred microlitres of 0.25, 0.5 or 1 µM methylene blue (final concentration) (S+) or PBS (S-) was added to the wells, which were then irradiated with 4.38 J of 665 nm laser light (L+) or incubated at room temperature in the dark (L-). Any bacteria that had detached from the surface were removed by washing the plate three times with 200 µL PBS. One hundred microlitres of 0.25% trypsin in PBS was added to each well and the plates were incubated at 37°C for five minutes, with shaking. Serial dilutions were made in PBS and 10 µL of each dilution was spotted in triplicate onto 5% horse blood agar plates, which were incubated overnight at 37°C and the viable counts subsequently determined.

4.2.3 LIVE/DEAD® staining to assess the effect of photosensitisation on the detachment of *S. aureus* from fibronectin, fibrinogen and IgG

To investigate the effect of lethal concentrations of methylene blue on the detachment of *S. aureus* from fibronectin, fibrinogen and IgG-coated surfaces, LIVE/DEAD® staining was performed using the L7007 *Bac*Light™ Bacterial Viability Kit (Invitrogen, UK). Cultures of *S. aureus* 8325-4 were grown as previously described and the inoculum adjusted to an optical density of 0.1 at 600 nm. Bacteria were allowed to bind to the coated wells of a Maxisorp™ plate as described in section 4.2.2 and exposed to methylene blue and 665 nm laser light as described in section 4.2.1. Unbound bacteria were removed by washing the plates four times with 200 µL PBS. Two hundred microlitres of 0.25% trypsin was added to remove the bound bacteria from the surface and the released bacteria were then stained with LIVE/DEAD® stain and visualised as described below. The LIVE/DEAD® staining procedure utilises a mixture of the SYTO 9 green fluorescent nucleic acid stain and the red fluorescent propidium iodide nucleic acid stain. SYTO 9 stains all bacteria regardless of membrane integrity, whereas propidium iodide can only penetrate bacteria with damaged membranes, causing a reduction in SYTO 9 fluorescence when both dyes are present. Consequently, bacteria with intact cell membranes fluoresce green and bacteria with damaged membranes fluoresce red (Molecular Probes, 2004).

Equal volumes of Component A (containing 1.67 mM SYTO 9 dye and 1.67 mM propidium iodide) and Component B (containing 1.67 mM SYTO 9 and 18.3 mM propidium iodide) of the L7007 *Bac*Light™ Bacterial Viability Kit were mixed thoroughly and 0.6 µL of the dye mixture was added to each well. The plates were incubated in the dark at room temperature for 15 minutes and then 5 µL of the stained bacterial suspension was trapped between a microscope slide and an 18 mm

square coverslip. The stained bacterial suspensions were observed using a fluorescence microscope and the live and dead cells at five different locations were enumerated to allow a mean value to be determined. Live bacteria fluoresce green whereas dead bacteria fluoresce red when visualised under the fluorescence microscope. The total number of cells bound (live plus dead) per mL was then calculated.

4.2.4 Modified viable counting method to examine the fibronectin-binding capacity of remaining viable bacteria following photosensitisation

To investigate the effect of photosensitisation on the capacity of any remaining viable bacteria to bind to fibronectin, the following assay was devised. Maxisorp™ plates were coated with either 0.1 mg/ml fibronectin (from human plasma; Sigma-Aldrich, UK), fibrinogen (from human plasma; Sigma-Aldrich, UK) or IgG (from human serum; Sigma-Aldrich, UK), blocked and washed as previously described. Cultures of *S. aureus* 8325-4 were grown and the inoculum titred according to section 4.2.2.

In duplicate wells of an uncoated 96-well microtitre plate, 150 µL of inoculum was added to either an equal volume of methylene blue (final concentrations of 1, 5, 10 and 20 µM) (S+) or PBS (S-) and samples were either irradiated with 4.38 J of 665 nm laser light with stirring, or kept in the dark (L-). The surviving bacteria were titred by viable counting. One hundred microlitres was removed for the binding assay as detailed in section 4.2.2. Following incubation to allow binding, the bound bacteria were trypsinised so as to release them and titred by viable counting.

The percentage of surviving bacteria bound to the surface was calculated using the following formula:

$$\left(\frac{\text{CFU/mL (viable count from binding assay)}}{\text{Surviving CFU/mL (viable count post-photosensitisation)}} \right) \text{X } 100$$

4.2.5 Azocasein hydrolysis assay to assess the effect of photosensitisation on the activity of *S. aureus* V8 protease

Endoproteinase Glu-C (also known as V8 protease) from *Staphylococcus aureus* V8 was purchased from Sigma-Aldrich (UK) and stored at -20°C at a concentration of 1 mg/mL in dH_2O. A final concentration of 5µg/mL was obtained by diluting the enzyme in PBS. An equal volume of V8 protease was added to 50 µL of either photosensitiser (S+) or PBS (S-) in triplicate wells of a 96-well plate and photosensitisation experiments were performed according to sections 2.5.1 and 2.5.2. After irradiation, the azocasein hydrolysis assay was performed according to section 2.7.1.

4.2.6 The effect of deuterium oxide and L-tryptophan on the photodynamic inactivation of V8 protease

Experiments to assess the effect of an enhancer of singlet oxygen lifetime (deuterium oxide) and a singlet oxygen scavenger (L-tryptophan) were performed according to section 2.8.2.

4.2.7 SDS PAGE analysis

After photosensitisation or dark incubation as previously described, the V8 protease and α-haemolysin were analysed by SDS PAGE. V8 protease samples were incubated on ice with 100 mM phenylmethanesulfonyl fluoride for 30 minutes prior to SDS

PAGE in order to minimise self-digestion. SDS PAGE was performed according to section 2.9. The expected molecular masses of the V8 protease and α-haemolysin were given as 29 and 33 kDa, respectively, as specified by the manufacturer.

4.2.8 Haemolytic titration to determine the effect of photosensitisation on the haemolytic activity of *S. aureus* α-haemolysin

Alpha-haemolysin from *S. aureus* (Sigma-Aldrich, UK) was reconstituted at a concentration of 0.5 mg/mL in sterile, deionised water plus sodium citrate buffer and stored at 2-8°C. For experimental purposes, □-haemolysin was diluted in sterile PBS to a final concentration of 100 μg/mL. An equal volume of photosensitiser was added to 50 μL of α-haemolysin in duplicate wells of a sterile, flat-bottomed, untreated 96-well plate and laser light dose and photosensitiser dose experiments were performed according to sections 2.5.1 and 2.5.2, respectively. Following photosensitisation, samples were removed and aliquoted into round-bottomed 96-well plates for the haemolytic titration assay.

Samples were serially diluted using doubling dilutions in PBS. Sterile, deionised water was used as a positive haemolysis control and sterile PBS as a negative haemolysis control. Defibrinated rabbit blood (E & O Laboratories, UK) was centrifuged at 503 x *g* for 10 minutes and the supernatant discarded. The cells were washed and resuspended in sterile PBS to a final concentration of 2%. Aliquots of 50 μL of the erythrocyte solution were added to the serially diluted toxin and control wells and incubated in the dark at 37°C for 1 hour. After incubation, the haemolytic titre for each sample was determined as the reciprocal of the highest dilution giving rise to lysis.

4.2.9 The effect of human serum on the photosensitisation of *S. aureus* α-haemolysin

Alpha-haemolysin was diluted to a final concentration of 100 µg/mL in either PBS or PBS plus 12.5% human serum (final concentration of 6.25%) (Sigma Aldrich, UK) in order to determine the effect of serum on the photoinactivation of the toxin. Human serum was added at a final concentration of 6.25 % to model *in vivo* conditions as this concentration provided a protein concentration similar to that found in an acute wound (Lambrechts *et al.*, 2005). Alpha-haemolysin in either the presence or absence of human serum was exposed to 20 µM methylene blue and 665 nm laser light doses of 4.38 J, 8.76 J and 21.9 J and the haemolytic titration assay was performed as previously described.

4.2.10 Spectrophotometric assay to assess the effect of photosensitisation on *S. aureus* sphingomyelinase activity

Sphingomyelinase (also known as β-haemolysin or β-toxin) from *S. aureus* was purchased from Sigma-Aldrich (UK) in buffered aqueous glycerol containing 0.25 M phosphate buffer, pH 7.5. For experimental purposes, the enzyme was diluted to a final concentration of 0.5 Units/mL in 250 mM Tris-HCl buffer with 10 mM magnesium chloride, pH 7.4 at 37°C. An equal volume of sphingomyelinase was added to either 25 µL of (S+) or 25 µL PBS (S-) and irradiation of the enzyme suspension was carried according to sections 2.5.1 and 2.5.2 for light-dose and photosensitiser-dose experiments, respectively.

Following irradiation/dark incubation, 10 µL from each sample was removed and added to 190 µL of incubation buffer containing 0.02mg Trinitrophenylaminolauroyl-Sphingomyelin (TNPAL-Sphingomyelin; Sigma-Aldrich, UK), 250 mM Tris-HCl, 10 mM $MgCl_2$ and 1% Triton X-100 in 0.5 mL Eppendorf tubes and incubated in the dark at 37°C for 5 minutes, with shaking. 150 µL of isopropanol:heptane:H_2SO_4 (40:10:1) was

added to stop the reaction and the tubes were placed on ice immediately after addition. One hundred microlitres of n-heptane (Sigma-Aldrich, UK) and 80 µL deionised water were then added and the samples were centrifuged for ten minutes at 5000 rpm. Following centrifugation, the tubes were left to settle at room temperature for 5 minutes, after which 60 µL of the upper layer was removed and the optical density at 330 nm recorded using a UV-visible spectrophotometer. A blank sample containing 10 µL incubation buffer instead of sphingomyelinase was used as a reference. The enzyme activity was calculated, with one unit of activity determined as that which caused a chance in absorbance of 0.001 in one minute at 330 nm

4.2.11 The effect of human serum on the photosensitisation of *S. aureus* sphingomyelinase

Sphingomyelinase was diluted to a final concentration of 0.5 Units/mL in either 250 mM Tris-HCl buffer with 10 mM $MgCl_2$, pH 7.4 at 37°C or the buffer with the addition of 12.5% human serum (for a final concentration of 6.25%) (Sigma Aldrich, UK) in order to model acute wound conditions as previously described. Samples were exposed to 20 µM methylene blue and 665 nm laser light with energy densities of 4.38 J or 21.9 J. The spectophotometric assay for sphingomyelinase activity was performed as previously described.

4.3 Results

4.3.1 Crystal violet assay to assess the effect of photosensitisation on the detachment of *S. aureus* from fibronectin

Colonisation of host tissues is an important step in the infection process and therefore the binding of *S. aureus* to human proteins represents a relevant target for photodynamic inactivation. The crystal violet assay was thought to be a straightforward method for assessing the effect of lethal photosensitisation on the fibronectin-binding properties of *S. aureus* 8325-4; however the results obtained were unexpected and rendered this assay unsuitable for further investigation.

As can be seen from Figure 4.1, the exposure of bacteria to 20 μM methylene blue and laser light resulted in an increase in the optical density at 590 nm compared with the control. As a known number of bacteria were bound to the fibronectin-coated surface prior to irradiation, an increase in the numbers of bacteria binding to this surface was not possible as no more bacteria were added during the experiment. It is possible that binding of methylene blue to the bacteria was sufficiently high enough to alter the optical density at 590nm, although increases in the optical density were also observed following irradiation of the bacteria in the absence of photosensitiser.

Due to the nature of the mechanism of killing by lethal photosensitisation, it is possible that in the presence of light and methylene blue, the process of photodynamic killing makes bacterial membranes more “leaky”. Consequently, the bacteria may take up more of the dye, resulting in an increase in optical density rather than the decrease expected if the process negatively affected fibronectin-binding and hence detachment of the cells from the surface. Laser light alone has previously been shown to have a killing effect on *S. aureus,* possibly due to the

presence of endogenous photosensitisers (Wilson & Pratten, 1994). Such endogenous photosensitisers may account for the increase in optical density observed following irradiation with laser light alone.

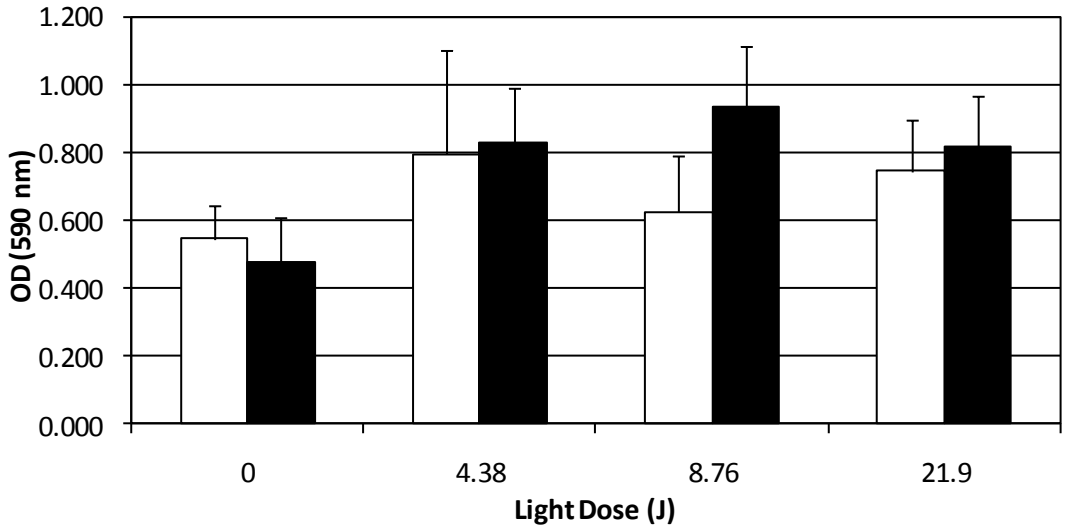

Figure 4.1 The effect of light dose on the detachment of *S. aureus* 8325-4 from fibronectin as determined by the crystal violet assay.

An equal volume of either PBS (□) or methylene blue (■) was added to fibronectin-coated wells that had been incubated with *S. aureus* to allow binding and wells were then either exposed to laser light of 665 nm or kept in the dark. Following irradiation, wells were washed to remove unbound bacteria and stained with crystal violet. Error bars represent the standard deviation from the mean. Experiments were performed twice in duplicate and the combined data are shown.

4.3.2 Viable count assay to examine the effect of sub-lethal photosensitisation on the detachment of *S. aureus* from fibronectin

In this part of the study the ability of photosensitisation to detach *S. aureus* which had bound to fibronectin was examined. Since the viable count assay relies on the retrieval and culture of bacteria attached to a surface, the cells must be viable to be enumerated in this manner. This presents a problem when combined with lethal photosensitisation as if a lethal dose is used, it will not be possible to distinguish between kill and detachment using this assay; therefore the effect of sub-lethal concentrations on the detachment of *S. aureus* 8325-4 from fibronectin-coated surfaces was investigated. Figure 4.2 shows that sub-lethal concentrations of the photosensitiser in combination with laser light did not cause detachment of bound *S. aureus* from fibronectin.

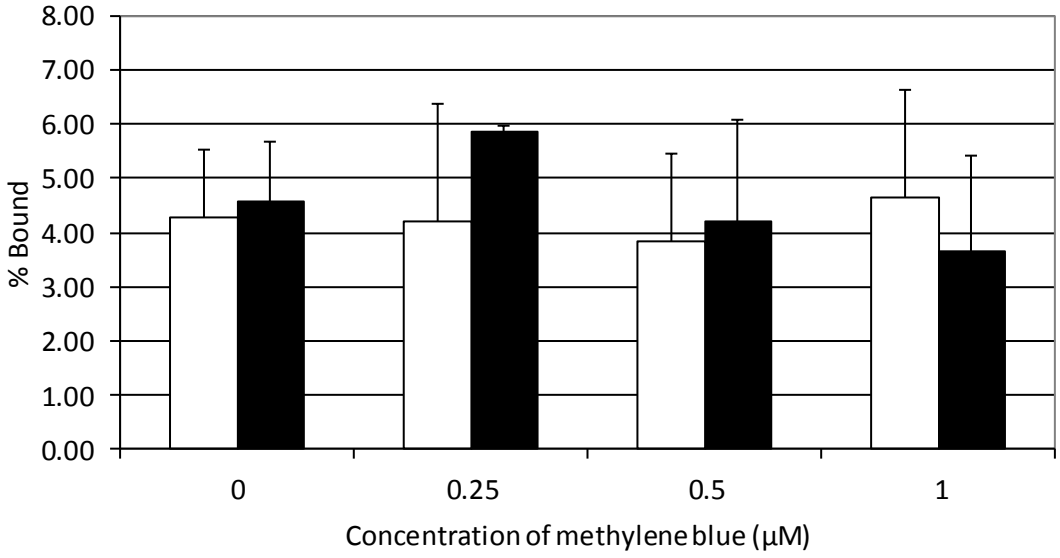

Figure 4.2 The ability of sub-lethal doses of methylene blue and 4.38 J laser light to detach bound *S. aureus* 8325-4 from fibronectin using the viable count method.

An equal volume of either methylene blue or PBS was added to fibronectin-coated wells that had been incubated with *S. aureus* to allow binding and wells were then either exposed to laser light of 665 nm (■) or kept in the dark (□). Following irradiation, wells were washed to remove unbound bacteria and stained with crystal violet. Error bars represent the standard deviation from the mean. Experiments were performed twice and the combined data are shown.

4.3.3 LIVE/DEAD® staining to assess the effect of photosensitisation on the detachment of *S. aureus* from fibronectin, fibrinogen and IgG

As the viable count assay was limited by its ability to only detect viable cells and could not be used for determining the effect of methylene blue and laser light under lethal conditions on the detachment of bacterial cells from ligand-coated surfaces, LIVE/DEAD® staining using the BacLight™ Bacterial Viability Kit was performed. This method enabled the detection of all bacteria regardless of their viable state and

therefore could be utilised to enumerate cells bound to a surface, even if the lethal photosensitisation process successfully killed the organism. Using this method, the effect of lethal photosensitisation on the detachment of *S. aureus* 8325-4 from fibronectin-, fibrinogen- and IgG-coated surfaces was assessed, as shown in Figure 4.3, Figure 4.4 and Figure 4.5, respectively. Irradiation of the ligand-coated surfaces with laser light and methylene blue had no significant effect on the total detachment of viable and non-viable *S. aureus* 8325-4 from these ligands.

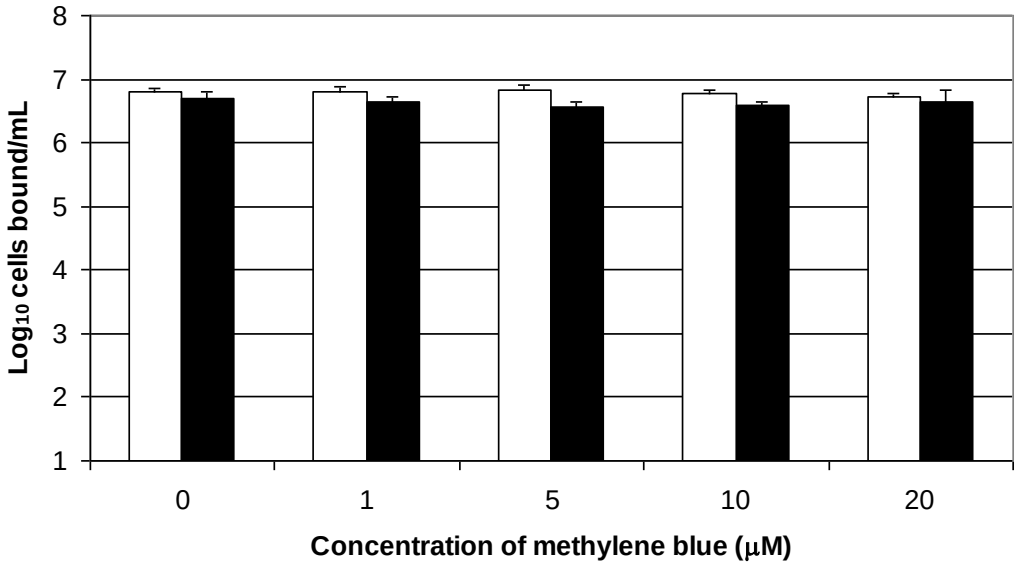

Figure 4.3 The effect of methylene blue and 4.38 J of 665 nm laser light on the detachment of *S. aureus* 8325-4 from fibronectin-coated surfaces.

An equal volume of either methylene blue or PBS was added to ligand-coated wells that had been incubated with *S. aureus* 8325-4 to allow binding and wells were then either exposed to 4.38 J of laser light (■) or kept in the dark (□). Following irradiation/dark incubation, unbound bacteria were removed by washing the wells with PBS and the total bound bacteria enumerated by LIVE/DEAD® staining. Error bars represent the standard deviation from the mean. Experiments were performed twice and the combined data are shown.

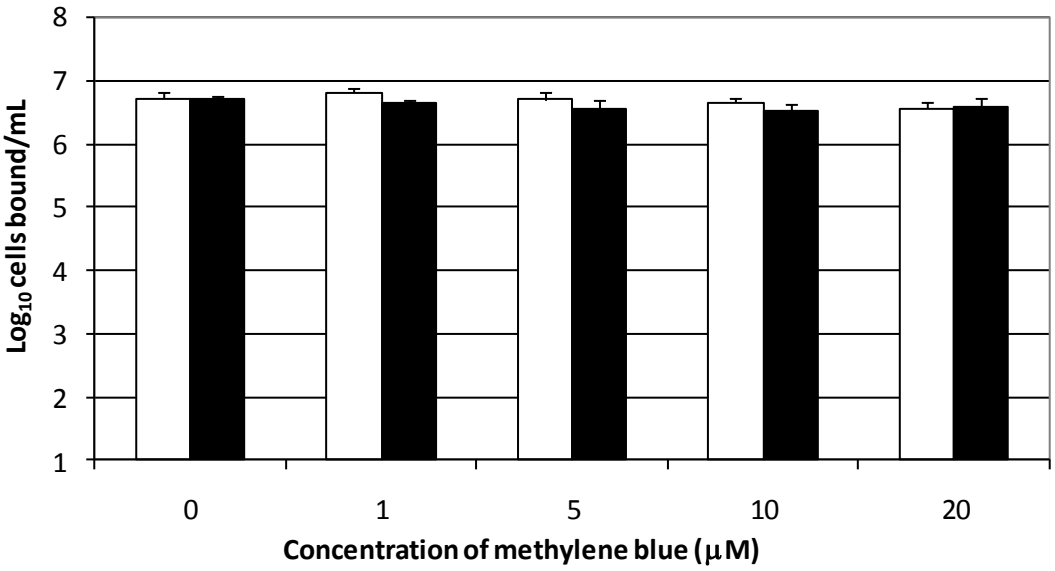

Figure 4.4 The effect of methylene blue and 4.38 J of 665 nm laser light on the detachment of *S. aureus* 8325-4 from fibrinogen-coated surfaces.

An equal volume of either methylene blue or PBS was added to ligand-coated wells that had been incubated with *S. aureus* 8325-4 to allow binding and wells were then either exposed to 4.38 J of 665 nm laser light (■) or kept in the dark (□). Following irradiation/dark incubation, unbound bacteria were removed by washing the wells with PBS and the total bound bacteria enumerated by LIVE/DEAD® staining. Error bars represent the standard deviation from the mean. Experiments were performed twice and the combined data are shown.

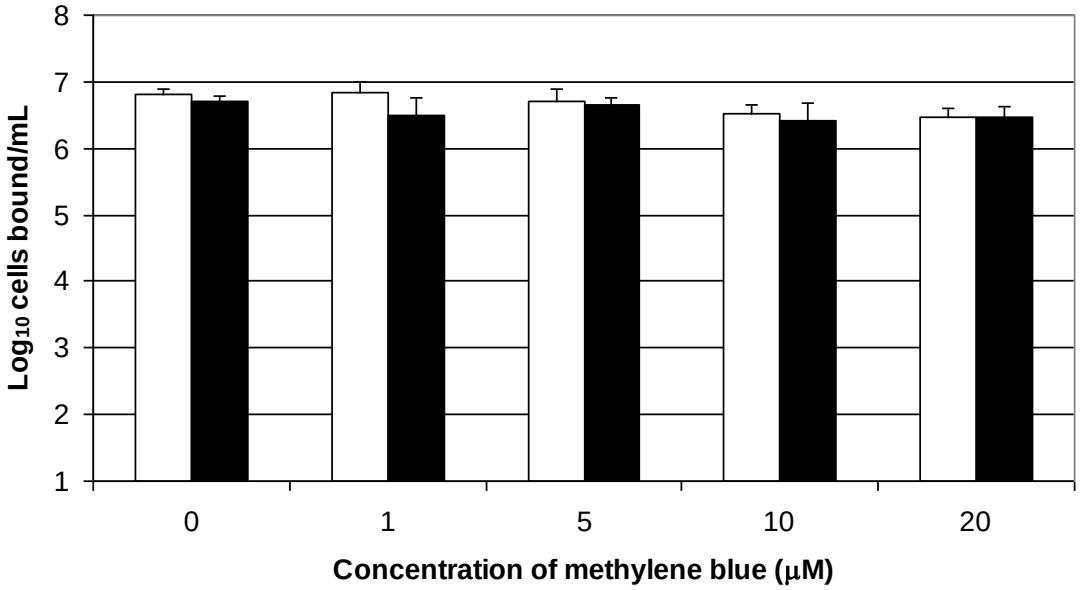

Figure 4.5 The effect of methylene blue and 4.38 J of 665 nm laser light nm on the detachment of *S. aureus* 8325-4 from IgG-coated surfaces.

An equal volume of either methylene blue or PBS was added to ligand-coated wells that had been incubated with *S. aureus* 8325-4 to allow binding and wells were then either exposed to laser light (■) or kept in the dark (□). Following irradiation/dark incubation, unbound bacteria were removed by washing the wells with PBS and the total bound bacteria enumerated by LIVE/DEAD® staining. Error bars represent the standard deviation from the mean. Experiments were performed twice and the combined data are shown.

4.3.4 Modified viable counting method to examine the fibronectin-binding capacity of remaining viable bacteria following photosensitisation

In this part of the study, the effect of pre-exposing *S. aureus* to photosensitisation on the capacity of remaining viable bacteria to bind to fibronectin was investigated. In order to be able to assess the effect of lethal concentrations of methylene blue on the capacity of *S. aureus* to bind to fibronectin, a new strategy was devised that was also based on the viable count assay. It was important to be able to distinguish between any decrease in binding from a reduction in the viable count due to lethal photosensitisation. Viable counts were taken after lethal photosensitisation and also after the binding assay, making it possible to calculate the percentage of viable bacteria bound compared with the number of viable bacteria remaining after photosensitisation. It is important to note that this modified method was used to evaluate the capacity of viable *S. aureus* post-lethal photosensitisation to bind to fibronectin, rather than the detachment of bacteria from fibronectin, which involved irradiation of bacteria already bound to human proteins.

It can be seen from Figure 4.6 that whilst a dose-dependent decrease in fibronectin-binding was seen when *S. aureus* was exposed to methylene blue and laser light, the photosensitiser alone also has a significant inhibitory effect on the binding of *S. aureus* to fibronectin-coated surfaces.

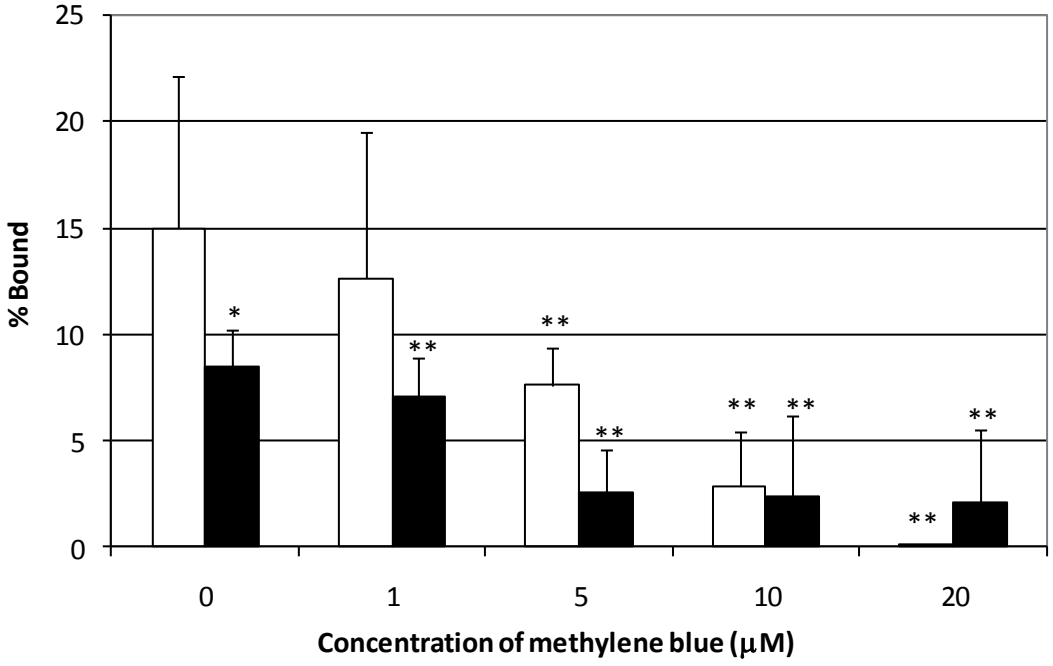

Figure 4.6 The effect of lethal doses of methylene blue and 4.38 J 665 nm laser light on the capacity of *S. aureus* 8325-4 to bind to fibronectin post-photosensitisation as determined by viable counting.

An equal volume of methylene blue or PBS was added to *S. aureus* 8325-4 and samples either exposed to 4.38 J of 665 nm laser light (■) or kept in the dark (□). Following irradiation/dark incubation, bacteria were allowed to bind to a fibronectin-coated surface and the bound bacteria enumerated by viable counting. Error bars represent the standard deviation from the mean. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney *U* Test). Experiments were performed three times and the combined data are shown.

4.3.5 The effect of photosensitisation on the capacity of remaining viable *S. aureus* to bind to fibrinogen and IgG-coated surfaces

Once a suitable method for determining the effect of lethal concentrations of methylene blue on the capacity of any remaining viable *S. aureus* to bind to fibronectin-coated surfaces had been established, the effect of lethal photosensitisation on fibrinogen and IgG-binding was also investigated. The results are shown in Figure 4.7 and Figure 4.8, respectively. Again, a photosensitiser dose-dependent inhibition of binding was observed in both the presence and absence of laser light, although it was only statistically significant at methylene blue concentrations of 10 μM and above in the absence of laser light.

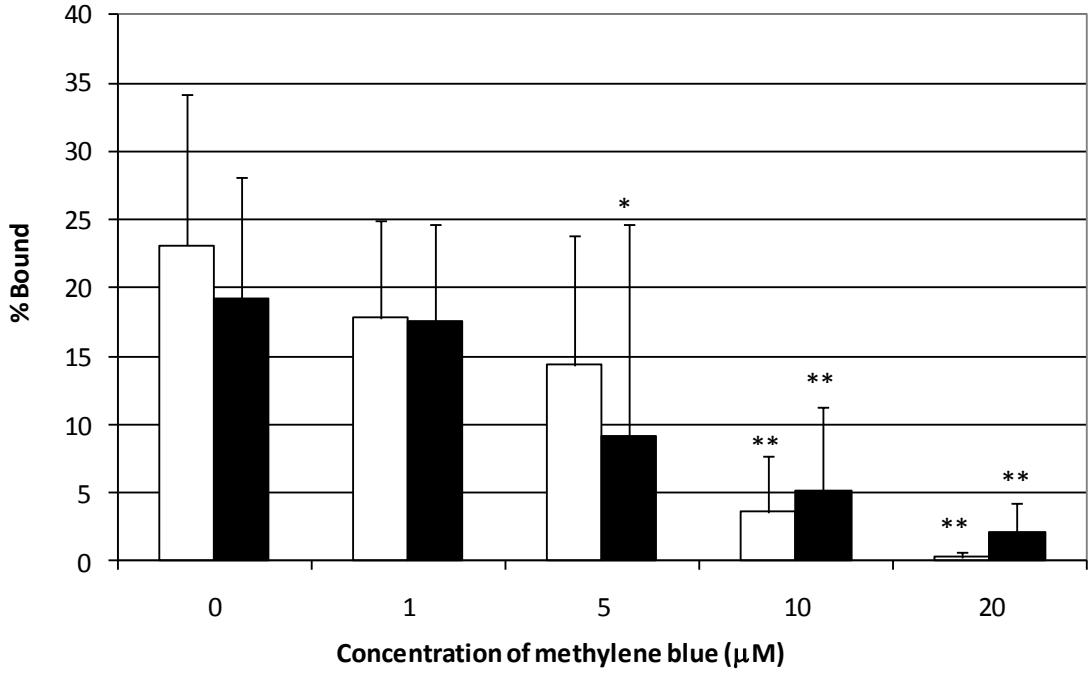

Figure 4.7 The effect of methylene blue and 4.38 J of 665 nm laser light on the capacity of the remaining viable *S. aureus* 8325-4 to bind to fibrinogen.

An equal volume of methylene blue or PBS was added to *S. aureus* 8325-4 and samples either exposed to 4.38 J of 665 nm laser light (■) or kept in the dark (□). Following irradiation/dark incubation, bacteria were allowed to bind to the ligand-coated surface and the bound bacteria enumerated by viable counting. Error bars represent the standard deviation from the mean. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney *U* Test). Experiments were performed three times and the combined data are shown.

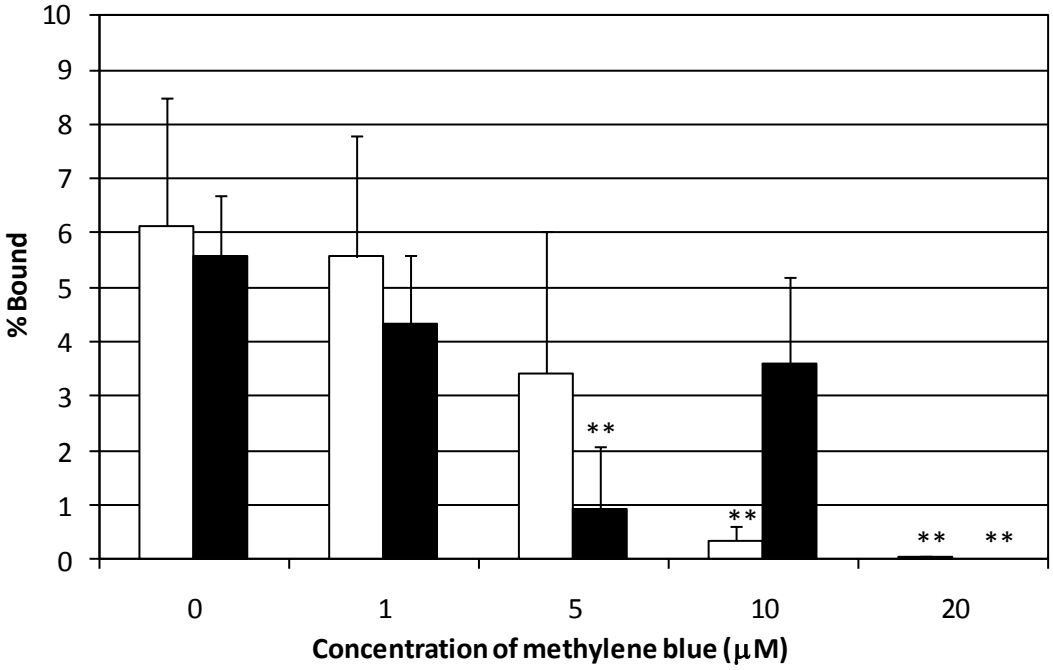

Figure 4.8 The effect of methylene blue and 4.38 J of 665 nm laser light on the capacity of the remaining viable *S. aureus* 8325-4 to bind to IgG.

An equal volume of methylene blue or PBS was added to *S. aureus* 8325-4 and samples either exposed to 4.38 J of 665 nm laser light (■) or kept in the dark (□). Following irradiation/dark incubation, bacteria were allowed to bind to the ligand-coated surface and the bound bacteria enumerated by viable counting. Error bars represent the standard deviation from the mean. * $P < 0.05$, ** $P < 0.01$ (Mann-Whitney *U* Test). Experiments were performed three times and the combined data are shown.

4.3.6 The effect of photosensitiser dose on the photodynamic inactivation of V8 protease

4.3.6.1 Methylene blue

The effect of methylene blue and 4.38 J of 665 nm laser light on the proteolytic activity of *S. aureus* V8 protease as determined by the azocasein-hydrolysis assay is shown in Figure 4.9. One unit of activity was defined as that which caused a change in absorbance of 0.001 in one hour at 450 nm. It can be seen that the activity of the V8 protease was inhibited in a photosensitiser concentration-dependent manner. A decrease in proteolytic activity of 75% was achieved with the highest concentration of methylene blue tested (20 μM) upon irradiation with 4.38 J of 665 nm laser light. Photosensitisation of EMRSA-16 using the same conditions resulted in an approximate 4-log reduction in viability, showing that inactivation of this enzyme is effective within the parameters required to kill a meticillin-resistant strain of *S. aureus in vitro*.

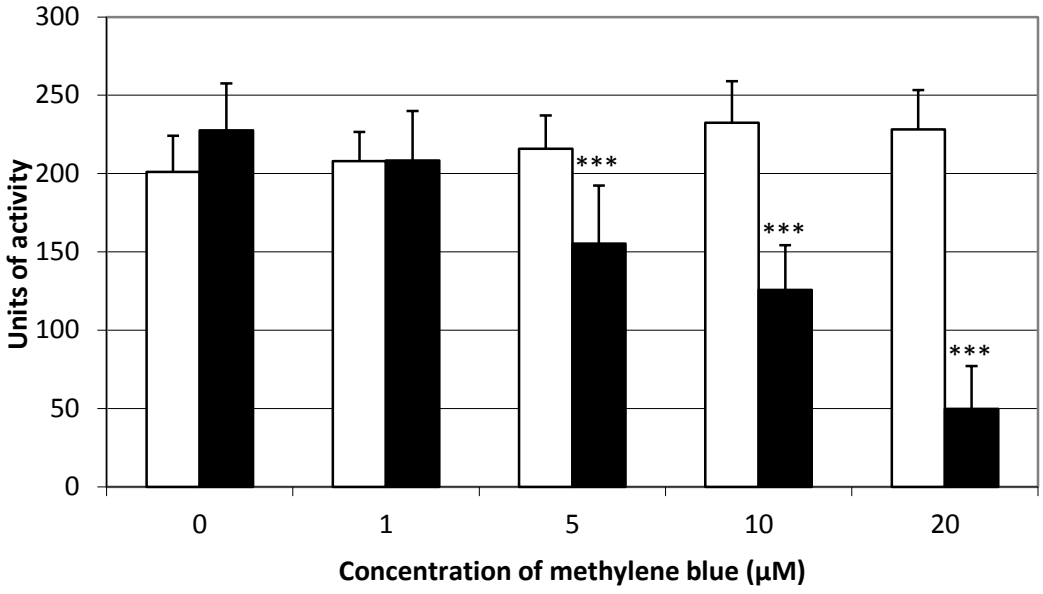

Figure 4.9 The effect of methylene blue and 4.38 J of 665 nm laser light on the proteolytic activity of V8 protease.

An equal volume of either methylene blue or PBS was added to V8 protease and samples were either exposed to 4.38 J of 665 nm laser light (■) or kept in the dark (□). The activity of the V8 protease was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.6.2 Tin chlorin e6

The effect of tin chlorin e6 dose on the photodynamic inactivation of V8 protease is shown in Figure 4.10. As observed with methylene blue, tin chlorin e6 in the presence of laser light inhibits the proteolytic activity of V8 protease in a dose-dependent manner. Photosensitisation with tin chlorin e6 resulted in a higher level of inactivation than that achieved with methylene blue; irradiation of the enzyme with 4.38 J in the presence of 20 µM tin chlorin e6 resulted in complete inhibition of

proteolytic activity as detectable by the azocasein hydrolysis assay ($P < 0.001$; ANOVA). Indeed, all concentrations of tin chlorin e6 tested causing a statistically significant reduction in activity compared with untreated samples.

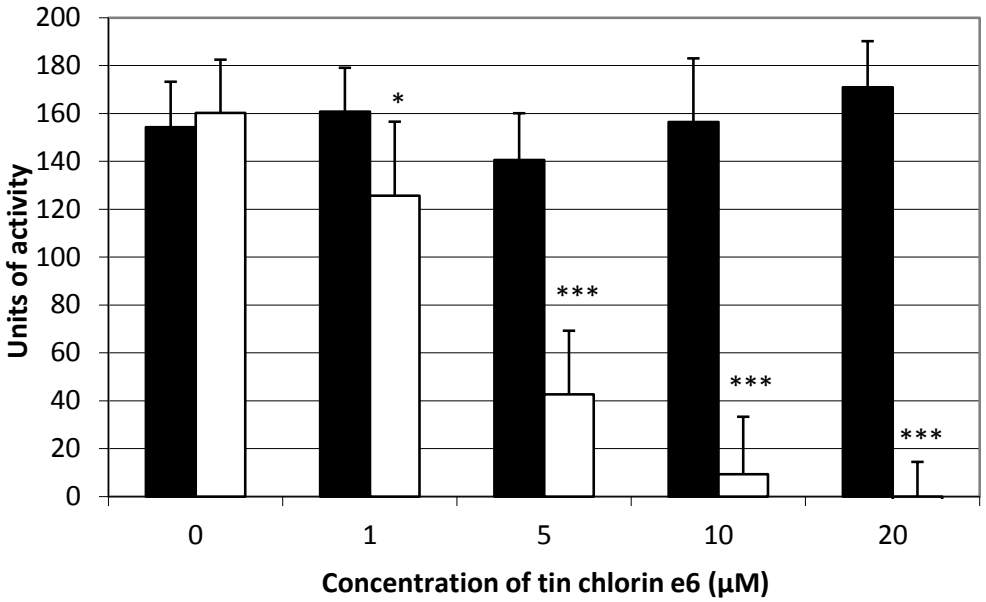

Figure 4.10 The effect of tin chlorin e6 and 4.38 J of 633 nm laser light on the proteolytic activity of V8 protease.

An equal volume of either tin chlorin e6 or PBS was added to V8 protease and samples were either exposed to 4.38 J of 633 nm laser light (■) or kept in the dark (□). The activity of the V8 protease was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. * $P < 0.05$, *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.7 The effect of laser light dose on the photodynamic inactivation of V8 protease

4.3.7.1 Laser light of 665 nm

Figure 4.11 shows the effect of 665 nm laser light doses of 4.38, 8.76 and 21.9 J on the activity of the V8 protease. Inactivation was light dose-dependent; complete inhibition of proteolytic activity was observed following irradiation with 21.9 J of 665 nm laser light in the presence of 20 μM methylene blue. Irradiation of the V8 protease in the absence of methylene blue did not have a significant effect on the activity of the enzyme.

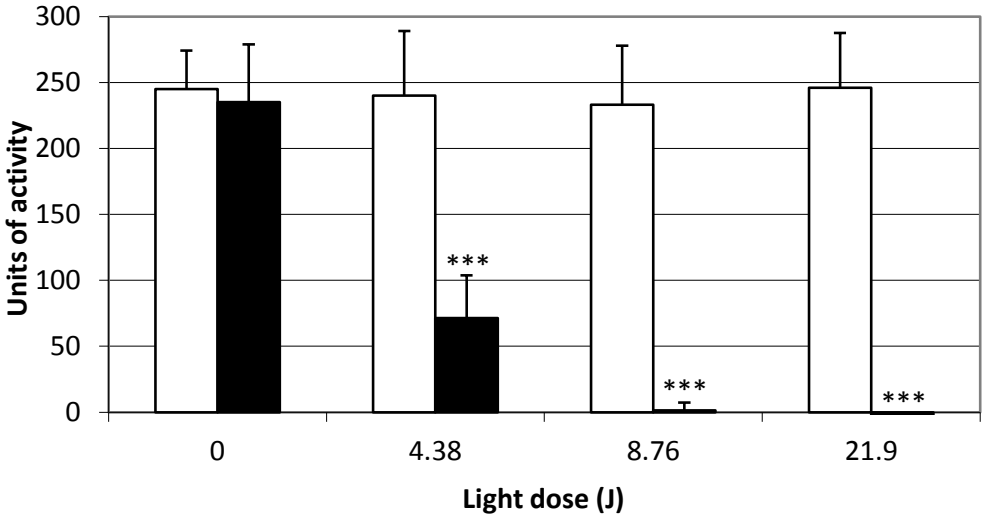

Figure 4.11 The effect of 20 µM methylene blue and 665 nm laser light on the proteolytic activity of V8 protease.

An equal volume of V8 protease added to either PBS (□) or 20 µM methylene blue (■) and samples were either irradiated with 4.38 J of 665 nm laser light or kept in the dark. Following irradiation, the activity of the enzyme was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.7.2 Laser light of 633 nm

As treatment of V8 protease with 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light completely inhibited the proteolytic activity of the enzyme, the photosensitiser and light doses were lowered in order to investigate the effect of light dose on the photodynamic inactivation of the enzyme. As shown by Figure 4.12, treatment of V8 protease with 5 µM tin chlorin e6 and as little as 1.5 J of 633 nm laser light resulted in a highly significant reduction in activity, demonstrating the susceptibility of this enzyme to photodynamic inactivation using tin chlorin e6 ($P < 0.001$; ANOVA).

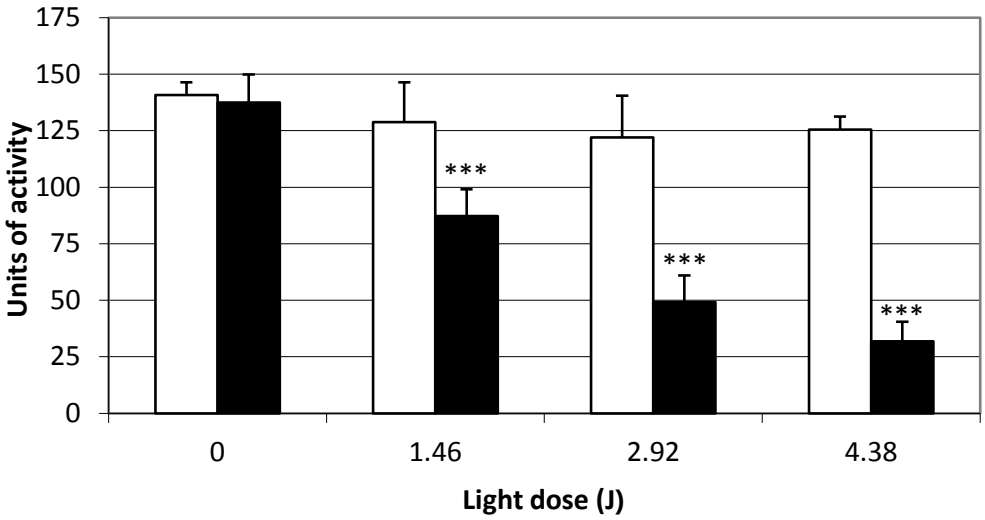

Figure 4.12 The effect of 5 µM tin chlorin e6 and 633 nm laser light on the proteolytic activity of V8 protease.

V8 protease was irradiated with 633 nm laser light in the presence of an equal volume of either PBS (□) or 5 µM tin chlorin e6 (■). Following irradiation, the activity of the enzyme was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.8 The effect of a singlet oxygen enhancer on the photodynamic inactivation of V8 protease

Deuterium oxide did not appear to enhance the photodynamic inactivation of V8 protease by methylene blue and 4.38 J of 665 nm laser light, as can be seen in Figure 4.13. As D_2O prolongs the lifetime of singlet oxygen, this may suggest that the lifetime of singlet is unimportant in the reaction, or that other reactive oxygen species other than singlet oxygen are also involved.

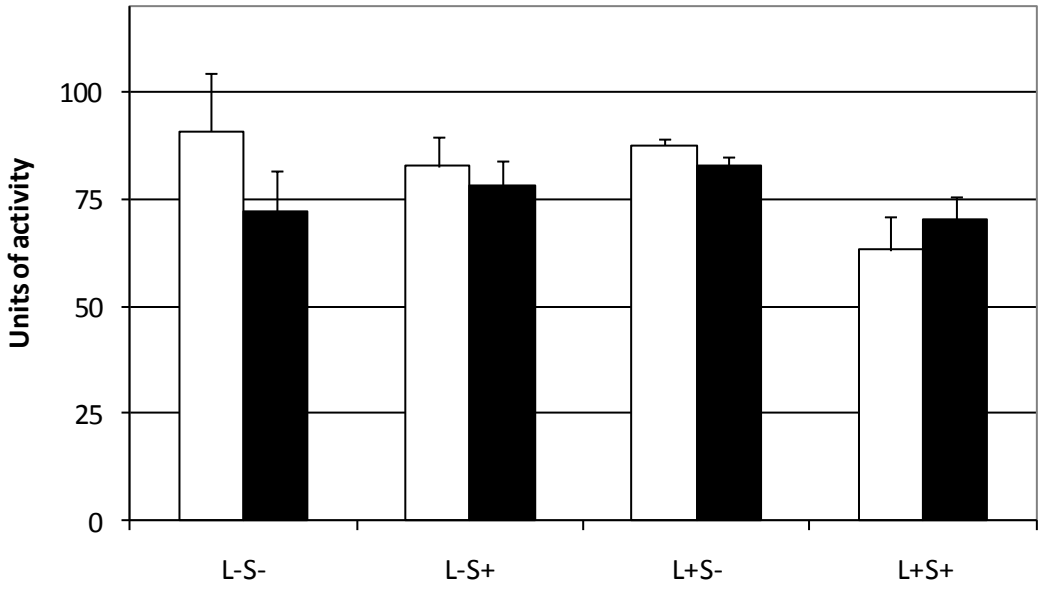

Figure 4.13 The effect of deuterium oxide on the photodynamic inactivation of *S. aureus* V8 protease.

V8 protease suspended in either H_2O (□) or D_2O (■) was irradiated with 4.38 J laser light (L+) in the presence of either 1 µM methylene (S+) or PBS (S-) or incubated in the dark (L-). Following irradiation, the activity of the enzyme was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. Experiments were performed twice in triplicate and a representative experiment is shown.

4.3.9 The effect of a singlet oxygen scavenger on the photodynamic inactivation of V8 protease

The singlet oxygen scavenger L-tryptophan was found to have a protective effect against the effects of methylene blue and laser light. Figure 4.14 shows that the addition of L-tryptophan completely inhibited the photodynamic inactivation of V8 protease, indicating the importance of reactive oxygen species in the photodynamic inactivation of this enzyme.

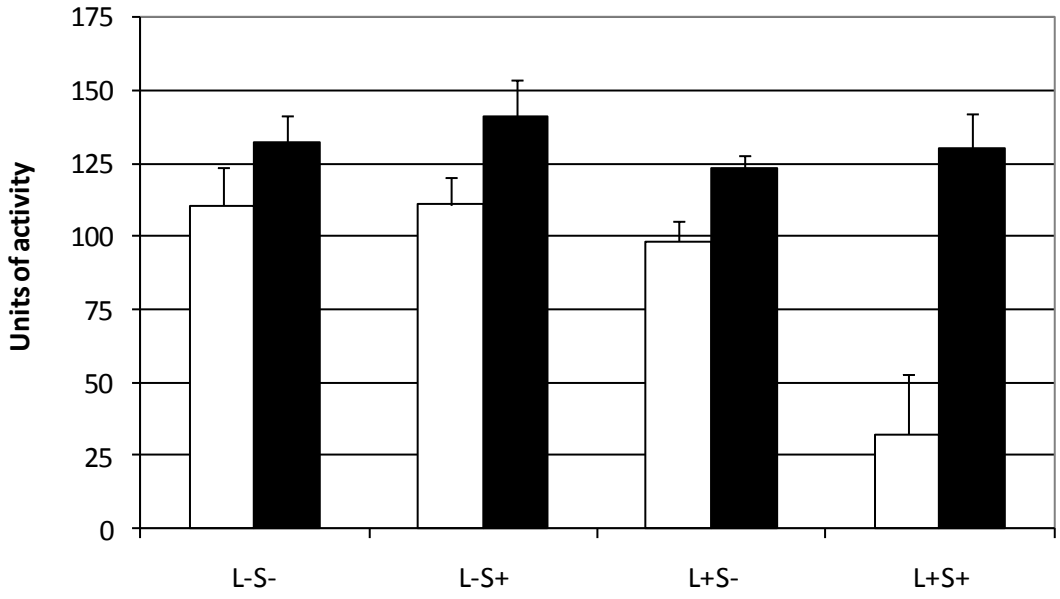

Figure 4.14 The effect of L-tryptophan on the photodynamic inactivation of *S. aureus* V8 protease.

V8 protease suspended in either PBS (□) or 10 mM L-tryptophan (■) and was irradiated with 4.38 J of 665 nm laser light (L+) in the presence of either 5 µM methylene (S+) or PBS (S-) or incubated in the dark (L-). Following irradiation, the activity of the enzyme was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. Experiments were performed twice in triplicate and a representative experiment is shown.

4.3.10 SDS PAGE analysis of V8 protease

SDS PAGE analysis (Figure 4.15) showed that after exposure to laser light and methylene blue, the bands derived from the V8 protease appeared to be progressively more smeared and of lower intensity with increased irradiation time, demonstrating that photosensitisation may cause a change in the protein, perhaps due to oxidation of the protein. A band of 29 kDa was expected for the V8 protease; however the gel showed some degradation of the V8 protease that could not be inhibited by the addition of a protease inhibitor.

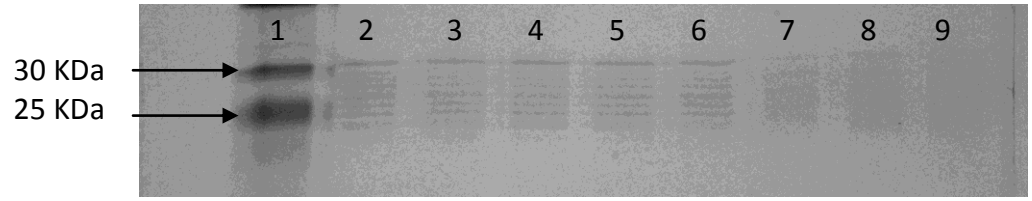

Figure 4.15 SDS PAGE analysis of V8 protease irradiated with methylene blue and 665 nm laser light doses of 4.38 J, 8.76 J and 21.9 J.

V8 protease was either kept in the dark (L-) or irradiated with 665 nm laser light doses of 4.38, 8.76 and 21.9 J (L+) in the presence of an equal volume of either PBS (S-) or 20 µM methylene blue (S+). Following irradiation, samples were analysed by SDS PAGE using a 5% stacking gel and 15% resolving gel under denaturing conditions. Lane 1: molecular weight marker, lane 2: L-S-, lane 3: L-S+, lane 4: L+S- (4.4 J), lane 5: L+S- (8.8 J), lane 6: L+S- (22 J), lane 7: L+S+ (4.4 J), lane 8: L+S+ (8.8 J), lane 9: L+S+ (22 J). The expected molecular mass of V8 protease was 29 kDa.

4.3.11 The effect of photosensitiser dose on the photodynamic inactivation of *S. aureus* α-haemolysin

In this assay, rabbit erythrocytes were incubated with serially diluted toxin and its ability to lyse the cells was determined by recording the haemolytic titre. The haemolytic titre was defined as the highest dilution giving rise to haemolysis. PBS does not lyse rabbit erythrocytes, and the intact cells settle at the bottom of the wells upon incubation at 37°C, acting as a negative control. When the cells are incubated with sterile, distilled water, disruption of their osmotic balance results in lysis, acting as a positive control. Treatment with α-haemolysin also results in the osmotic lysis of the cell.

4.3.11.1 Methylene blue

Table 4.1 shows the effect of photosensitisation of α-haemolysin with 1, 5, 10 and 20 μM methylene blue and 4.38 J of 665 nm laser light. Concentrations of 5, 10 and 20 μM methylene blue completely inhibited the haemolytic activity (as detectable by this assay) of the enzyme, when exposed to laser light (L+); therefore inactivation of the toxin occurs even at photosensitiser doses that are sub-inhibitory to EMRSA-16 (i.e. 5 μM). There was no effect on the activity of the haemolysin when the enzyme was incubated with the photosensitiser in the absence of laser light (L-).

Concentration of methylene blue (μM)	**Haemolytic Titre**	
	L-	**L+**
1	1/1024	1/256
5	1/1024	1/2
10	1/1024	< 1/2
20	1/512	1/2

Table 4.1 The effect of photosensitiser dose on the activity of α-haemolysin when treated with methylene blue and 4.38 J of 665 nm laser light

An equal volume of either methylene blue or PBS was added to *S. aureus* α-haemolysin and samples were either exposed to 4.38 J of 665 nm laser light (L+) or kept in the dark (L-). After irradiation/dark incubation, samples were serially diluted and an equal volume of 4% rabbit erythrocytes was added. Following incubation in the dark at 37°C for one hour, the haemolytic titre was recorded. The haemolytic titre was defined as the reciprocal of the highest dilution giving rise to haemolysis. Experiments were performed twice and a representative experiment is shown.

4.3.11.2 Tin chlorin e6

The effect of tin chlorin e6 dose on the photodynamic inactivation of α-haemolysin is shown in Table 4.2. Concentrations of tin chlorin e6 of 5 µM and above completely inhibited the haemolytic activity of the toxin when exposed to 4.38 J of 633 nm laser light. Neither tin chlorin e6 nor laser light alone had an effect on the activity of the toxin. Treatment of α-haemolysin with 1 µM tin chlorin e6 in the presence of laser light resulted in a four-fold reduction in the toxin's activity.

Concentration of tin chlorin e6 (µM)	**Haemolytic Titre**	
	L-	**L+**
0	1/128	1/128
1	1/128	1/32
5	1/128	< 1/2
10	1/128	< 1/2
20	1/128	< 1/2

Table 4.2 The effect of photosensitiser dose on the activity of α-haemolysin when treated with tin chlorin e6 and laser light of 633 nm

An equal volume of either tin chlorin e6 (S+) or PBS (S-) was added to *S. aureus* α-haemolysin and samples were either exposed to 4.38 J of 633 nm laser light (L+) or kept in the dark (L-). After irradiation/dark incubation, samples were serially diluted and an equal volume of 4% rabbit erythrocytes was added. Following incubation in the dark at 37°C for one hour, the haemolytic titre was recorded. The haemolytic titre is the highest dilution giving rise to haemolysis. Experiments were performed three times and a representative experiment is shown.

4.3.12 The effect of laser light dose on the photodynamic inactivation of *S. aureus* α-haemolysin

4.3.12.1 Laser light of 665 nm

To investigate the effect of 665 nm laser light dose on the activity of α-haemolysin, the enzyme was exposed to 20 µM methylene blue and light doses of 1.5 J, 2.9 J and 4.4 J, corresponding to 20, 40 and 60 seconds exposure to 665 nm laser light respectively. Table 4.3 shows that the activity of the enzyme was completely inhibited after exposure to a light dose as small as 1.5 J in the presence of 20 µM methylene blue. As previously observed, laser light alone had no appreciable effect on the activity of the α-haemolysin.

Light Dose (J)	Haemolytic Titre	
	S-	S+
0	1/1024	1/1024
1.5	1/1024	< 1/2
2.9	1/1024	< 1/2
4.4	1/1024	< 1/2

Table 4.3 The effect of light dose on the activity of α-haemolysin when treated with 20 µM methylene blue

An equal volume of either 20 µM methylene blue (S+) or PBS (S-) was added to *S. aureus* α-haemolysin and samples were either exposed to 665 nm laser light (L+) or kept in the dark (L-). After irradiation/dark incubation, samples were serially diluted and an equal volume of 4% rabbit erythrocytes was added. Following incubation in the dark at 37°C for one hour, the haemolytic titre was recorded. The haemolytic titre is the highest dilution giving rise to haemolysis. Experiments were performed twice and a representative experiment is shown.

4.3.12.2 Laser light of 633 nm

Table 4.4 shows the effect of light dose on the inactivation of α-haemolysin by 5 µM tin chlorin e6 and 633 nm laser light. It can be seen that even the lowest light dose tested (1.5 J) had an inhibitory effect on the α-haemolysin, reducing its activity by eight-fold, whilst light doses of 2.9 J and 4.4 J in the presence of 5 µM tin chlorin e6 completely inhibited the haemolytic activity of the toxin as detectable by this assay.

Light Dose (J)	**Haemolytic Titre**	
	S-	**S+**
0	1/128	1/128
1.5	1/128	1/16
2.9	1/128	< 1/2
4.4	1/128	< 1/2

Table 4.4 The effect of 633 nm laser light dose on the activity of α-haemolysin when treated with 5 µM tin chlorin e6

An equal volume of either 5 µM tin chlorin e6 (S+) or PBS (S-) was added to *S. aureus* α-haemolysin and samples were either exposed to laser light of 633 nm (L+) or kept in the dark (L-). After irradiation/dark incubation, samples were serially diluted and an equal volume of 4% rabbit erythrocytes was added. Following incubation in the dark at 37°C for one hour, the haemolytic titre was recorded. The haemolytic titre is the highest dilution giving rise to haemolysis. Experiments were performed three times and a representative experiment is shown.

4.3.13 The effect of human serum on the photosensitisation of α-haemolysin

The addition of 6.25% human serum did not affect the ability of photosensitisation to inactivate the α-haemolysin, and complete inhibition of haemolytic activity was observed after treatment of the toxin with 20 μM methylene blue and a laser light dose of 4.38 J in the presence of serum. This finding is consistent with the inactivation of the toxin in the absence of serum.

4.3.14 SDS PAGE analysis of α-haemolysin

SDS PAGE analysis (Figure 4.16) showed that bands derived from the α-haemolysin after photosensitisation with 20 μM methylene blue and 665 nm laser light became less well defined and smeared with increasing irradiation time compared with untreated samples. This result is similar to that observed for the V8 protease.

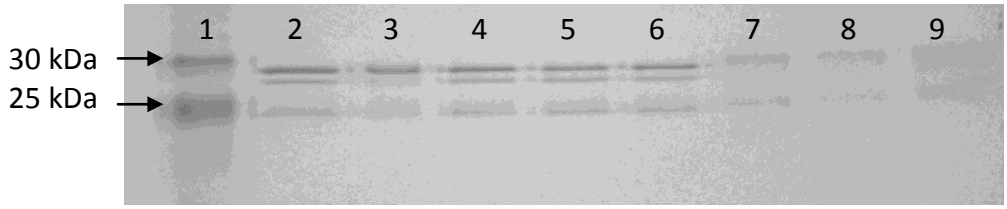

Figure 4.16 SDS PAGE analysis of α-haemolysin irradiated with 20 μM methylene blue and laser light doses of 4.38 J, 8.76 J and 21.9 J

Alpha-haemolysin was either kept in the dark (L-) or irradiated with 665 nm laser light doses of 4.38, 8.76 and 21.9 J (L+) in the presence of an equal volume of either PBS (S-) or 20 μM methylene blue (S+). Following irradiation, samples were analysed by SDS PAGE using a 5% stacking gel and 15% resolving gel under denaturing conditions. Lane 1: molecular weight marker, lane 2: L-S-, lane 3: L-S+, lane 4: L+S- (4.38 J), lane 5: L+S- (8.76 J), lane 6: L+S- (21.9 J), lane 7: L+S+ (4.38 J), lane 8: L+S+ (8.76 J), lane 9: L+S+ (21.9 J). The expected molecular mass of α-haemolysin was 33 kDa.

4.3.15 The effect of photosensitiser dose on the photodynamic inactivation of sphingomyelinase

4.3.15.1 Methylene blue

The activity of *S. aureus* sphingomyelinase was inhibited by treatment with methylene blue and laser light of 665 nm in a dose-dependent manner, as shown in Figure 4.17. One unit of activity was defined as that which caused a chance in absorbance of 0.001 in one minute at 330 nm. Interestingly, laser light alone appeared to have a slight effect on the activity of the enzyme, causing a decrease in activity of 10%, although this was not statistically significant ($P > 0.05$; ANOVA). Irradiation with 4.38 J of 665 nm laser light in the presence of 20 μM methylene blue achieved a 76% decrease in the activity of sphingomyelinase, which was comparable to the decrease in activity seen for the V8 protease (75%). These photosensitisation conditions correspond to an approximate 4-log reduction in viable EMRSA-16 and therefore inactivation of the sphingomyelinase was effective with light and energy doses required for the effective killing of bacteria.

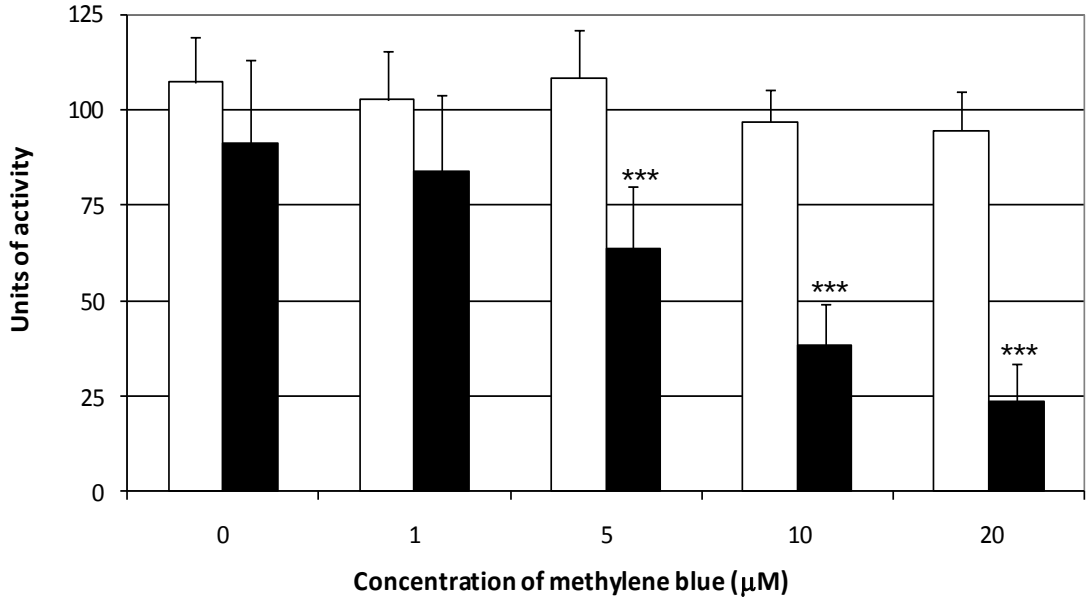

Figure 4.17 The effect of methylene blue dose and 4.38 J of 665 nm laser light on the activity of *S. aureus* sphingomyelinase.

An equal volume of either methylene blue or PBS was added to sphingomyelinase and samples were either exposed to laser light (■) or kept in the dark (□). Following irradiation, the activity of the enzyme was assessed spectrophotometrically using the substrate TNPAL-Sphingomyelin. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.15.2 Tin chlorin e6

Treatment with 4.38 J of 633 nm laser light and tin chlorin e6 was found to inhibit the activity of *S. aureus* sphingomyelinase in a dose-dependent manner as shown by Figure 4.18. Treatment of sphingomyelinase with 20 μM tin chlorin e6 and 4.38 J of 633 nm laser light resulted in a highly significant decrease in activity of 93%, which was higher than that seen following treatment of the enzyme with the same doses of methylene blue and laser light of 665 nm (76%).

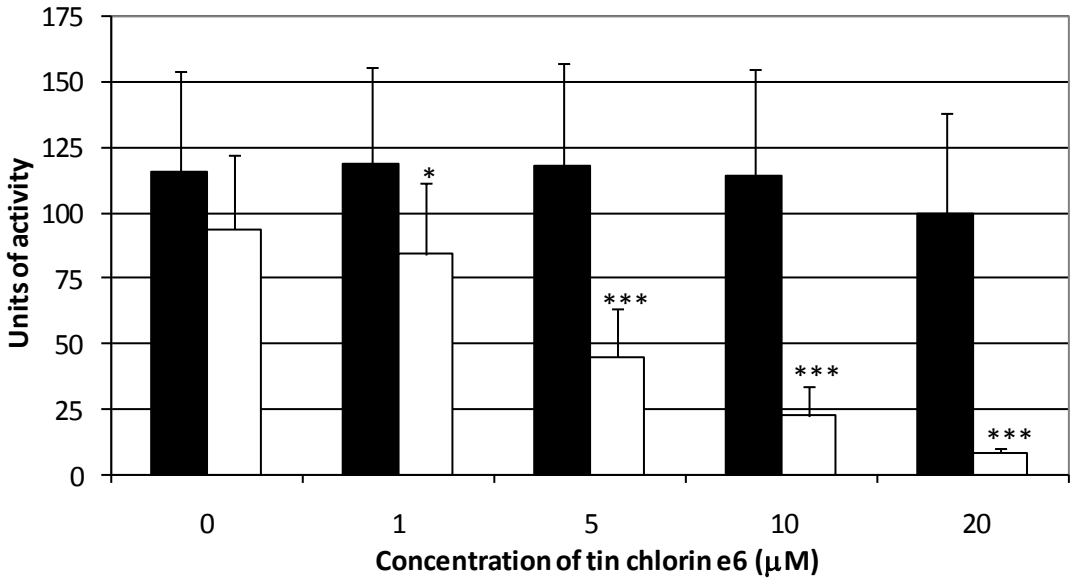

Figure 4.18 The effect of tin chlorin e6 dose and 4.38 J of 633 nm laser light on the activity of S. aureus sphingomyelinase.

An equal volume of either tin chlorin e6 or PBS was added to sphingomyelinase and samples were either exposed to 4.38 J of 633 nm laser light (■) or kept in the dark (□). Following irradiation, the activity of the enzyme was assessed spectrophotometrically using the substrate TNPAL-Sphingomyelin. Error bars represent the standard deviation from the mean. * $P < 0.05$, *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.16 The effect of laser light dose on the photodynamic inactivation of sphingomyelinase

4.3.16.1 Laser light of 665 nm

The effect of 665 nm laser light dose on the photodynamic inactivation of sphingomyelinase is shown in Figure 4.19. After irradiation with 21.9 J of 665 nm laser light in the presence of 20 μM methylene blue, a 94% decrease in activity was observed ($P < 0.001$; ANOVA). Neither photosensitiser nor 665 nm laser light alone had an effect on the activity of the sphingomyelinase.

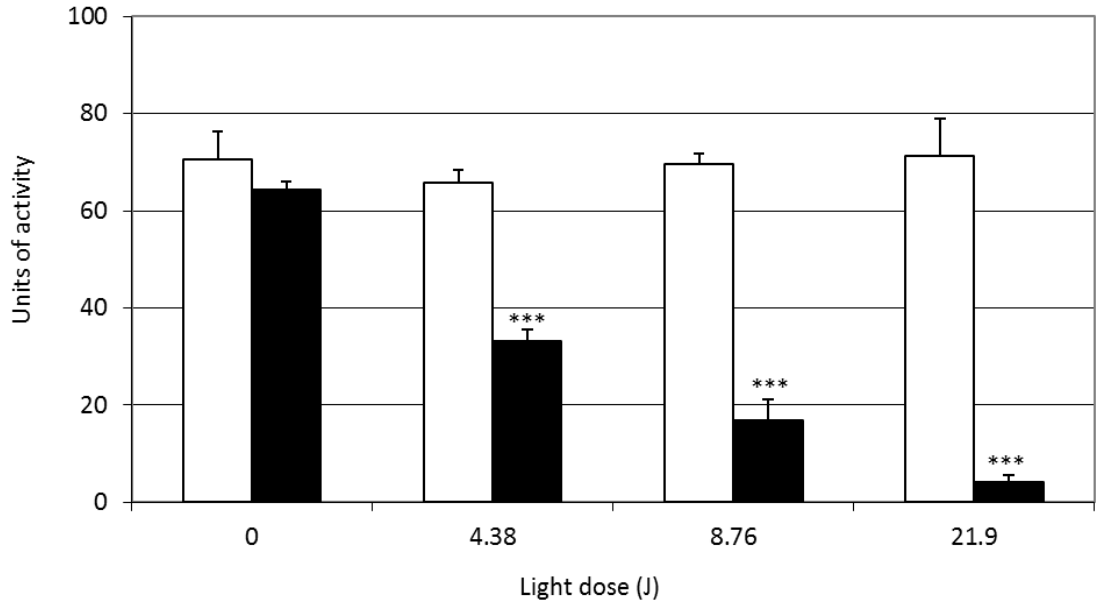

Figure 4.19 The effect of 665 nm laser light dose on the activity of *S. aureus* sphingomyelinase.

An equal volume of either methylene blue (■) or PBS (□) was added to sphingomyelinase and samples were either exposed to laser light or kept in the dark. Enzyme activity was assessed spectrophotometrically using the substrate TNPAL-Sphingomyelin. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed twice and the combined data are shown.

4.3.16.2 Laser light of 633 nm

Figure 4.20 shows the effect of 633 nm laser light dose on the photodynamic inactivation of *S. aureus* sphingomyelinase. All laser light doses resulted in a significant reduction in the activity of the enzyme when it was irradiated in the presence of 20 μM tin chlorin e6 ($P < 0.001$; ANOVA). Neither laser light or tin chlorin e6 alone had a significant effect on the activity of sphingomyelinase ($P > 0.05$; ANOVA).

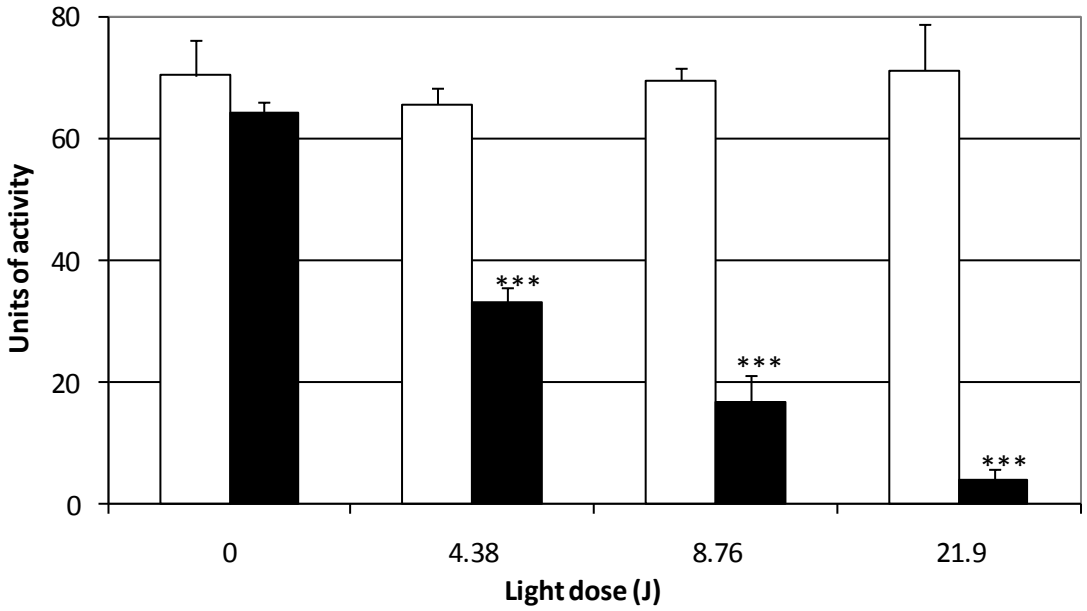

Figure 4.20 The effect of 633 nm laser light dose and 20 μM tin chlorin e6 on the activity of *S. aureus* sphingomyelinase.

An equal volume of either tin chlorin e6 (■) or PBS (□) was added to sphingomyelinase and samples were either exposed to 633 nm laser light or kept in the dark. Following irradiation, the activity of the enzyme was assessed spectrophotometrically using the substrate TNPAL-Sphingomyelin. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

4.3.17 The effect of human serum on the photosensitisation of sphingomyelinase

Irradiation of the sphingomyelinase in the presence of 6.25% human serum did not have an effect on the ability of photosensitisation to inactivate the enzyme. Photosensitisation using 20 µM methylene blue and the lowest laser light dose (4.4 J) resulted in a decrease in the enzyme's activity of 70% ± 12% in the presence of human serum, compared with a decrease of 76% ± 10% in the absence of serum. This difference was not found to be statistically significant ($P > 0.05$, ANOVA).

4.4 Discussion

Treatment of *S. aureus* which had been allowed to bind to a fibronectin-coated surface with sub-inhibitory doses of methylene blue (i.e. $\leq$ 1 µM) and 4.38 J of 665 nm laser light did not cause detachment of viable bacteria from fibronectin. It was hypothesised that photodynamic treatment of the bacteria may disrupt fibronectin-binding protein-mediated adhesion of the bacterial cells and thus cause them to detach from fibronectin. However, no detachment of the cells was observed in this assay (Figure 4.2).

The LIVE/DEAD® staining procedure was performed to examine the ability of photosensitisation to induce the detachment of *S. aureus* from fibronectin-, fibrinogen- and IgG-coated surfaces. This assay allowed the quantification of both live and dead cells and was used to determine whether photosensitisation of bacteria caused detachment of viable and non-viable cells from ligand-coated surfaces. As the bacterium binds to these ligands via cell wall-associated proteins, it is possible that the reactive oxygen species formed during photosensitisation could disrupt the interaction of these binding proteins with their associated ligands and consequently cause the detachment of bacterial cells. However, no detachment of bacterial cells from fibronectin, fibrinogen or IgG was observed. It is possible that higher doses of either photosensitiser or laser light may be required to cause damage to the binding proteins involved in these processes and thus cause detachment of *S. aureus* from these ligands.

The next set of experiments examined the effect of treatment with methylene blue and 665 nm laser light on the ability of any remaining viable bacteria to bind to host proteins post-sensitisation. Methylene blue in both the presence and absence of laser light was found to have a significant, dose-dependent inhibitory effect on the

binding of the remaining viable *S. aureus* to fibronectin-, fibrinogen- and IgG-coated surfaces. Methylene blue is a positively-charged dye, which is considered beneficial as it enables the targeting of the negatively-charged bacterial membrane. It is therefore possible that this photosensitiser-dependent inhibition of binding was due to a charge interaction between the bacterium and the photosensitiser, thus preventing binding of the organism to these proteins. This inhibition of binding to host proteins would be advantageous as if lethal photosensitisation was unsuccessful in killing the organism, the photosensitiser alone may be able to inhibit the binding of *S. aureus* to these proteins.

The inhibitory effect of the methylene blue alone was most marked in the case of fibronectin-coated surfaces, for which significant inhibition of binding was seen when *S. aureus* 8325-4 was treated with 5 µM methylene blue in the absence of laser light. For fibrinogen and IgG-coated surfaces, inhibition of binding by the photosensitiser in the absence of laser light was only significant for concentrations of 10 µM and above. In addition, a significant inhibition of binding to fibronectin was observed when *S. aureus* 8325-4 was treated with 1 µM methylene blue, the lowest concentration tested, and irradiated with 4.38 J of 665 nm laser light, which was not observed for the fibrinogen and IgG-coated surfaces. A decrease in the percentage of surviving bacteria binding to fibronectin was also observed after treatment with 4.38 J laser light in the absence of photosensitiser. This finding places particular emphasis on the ability of photosensitisation, and indeed methylene blue alone, to inhibit the binding of pre-photosensitised *S. aureus* to fibronectin.

The binding of *S. aureus* to host extracellular matrix components represents an important step in both colonisation and infection. Both fibronectin and fibrinogen-binding proteins have been implicated in the enhanced adhesion of *S. aureus* to atopic skin, where colonisation is thought to play an important role in the exacerbation of atopic dermatitis. Mutants deficient in either fibronectin-binding

proteins A and B or clumping factor A and B both demonstrated a significant reduction in binding to atopic skin compared with the parent strain (Cho *et al.*, 2001). PDT using the combination of methylene blue and laser light of 665 nm may therefore be of use in the treatment of skin disorders by decontaminating atopic skin. In fact, PDT has long been shown to be beneficial in the treatment of atopic disorders, for example the use of ultraviolet light and 8-methoxypsoralen for the treatment of atopic dermatitis (Morison *et al.*, 1978). Clearly, the combination of the elimination of disease-exacerbating microorganisms and neutralisation of virulence factors would be extremely advantageous to the treatment of these diseases.

The role of fibronectin-binding proteins A and B in the colonisation of nasal epithelia has also been suggested (Nashev *et al.*, 2004). Consequently, the ability of photosensitisation to prevent new bacterial cells binding to fibrinogen may be beneficial in the decontamination of the nares. Nasal colonisation by *S. aureus* is believed to be a complex process involving a number of bacterial binding proteins. *S. aureus* wall teichoic acids have been shown to be essential for nasal colonisation in an animal model (Weidenmaier *et al.*, 2004; Weidenmaier *et al.*, 2008). The fibrinogen-binding protein clumping factor B has also been proposed as playing a key role in the process and identified as a target for decolonisation strategies (Schaffer *et al.*, 2006; Wertheim *et al.*, 2008). MRSA is an ever-increasing problem; elimination of the organism from the anterior nares using such a treatment could present an important step in the control of MRSA infections, particularly in the hospital setting. Eradication of MRSA from the anterior nares using a methylene blue-based PDT regimen has recently been shown to be successful in preliminary clinical studies (Street *et al.*, 2009).

S. aureus expresses several proteins with fibrinogen-binding abilities, such as fibronectin binding protein A, clumping factor A, extracellular adherence protein and extracellular fibrinogen binding protein, which are considered virulence

determinants due to their involvement in platelet and fibrin-platelet clot binding; deletion mutants also have reduced virulence in animal models of endocarditis and septic arthritis (Josefsson *et al.*, 2001; Rivera *et al.*, 2007; Sullam *et al.*, 1996). Fibronectin-binding protein A and clumping factor A belong to the MSCRAMM family of cell wall-associated proteins and have been found to have similar mechanisms of binding to fibrinogen (Rivera *et al.*, 2007). A dose-dependent inhibition of fibrinogen-binding was found when *S. aureus* 8325-4 was treated with methylene blue in both the presence and absence of 665 nm laser light, although significant inhibition was seen at lower concentrations of methylene blue (i.e. 5 μM) when irradiated with laser light compared with non-irradiated samples, suggesting photodynamic inhibition of binding may occur.

Protein A is expressed on the surface of *S. aureus* and interacts with the Fc region of IgG molecules, binding them in the wrong orientation and thereby preventing phagocytosis of the organism by neutrophils (Foster, 2005). This antiphagocytic effect is believed to play a role in the virulence of *S. aureus*, and wild-type strains have been shown to cause more severe infections compared with protein A-deficient mutants in an animal model (Palmqvist *et al.*, 2002). Protein A-deficient mutants also show attenuated intracellular persistence (Kubica *et al.*, 2008). As previously noted for fibronectin and fibrinogen-coated surfaces, methylene blue in the absence of laser light also had an inhibitory effect on the binding of *S. aureus* to IgG-coated surfaces, although again, significant inhibition of binding was achieved at a lower concentration of methylene blue when irradiated with laser light. The treatment of *S. aureus* 8325-4 with 20 μM methylene blue and 4.4 J laser light resulted in the complete inhibition of binding to IgG-coated surfaces, whilst treatment with 20 μM methylene blue in the absence of laser light resulted in almost complete inhibition of binding. The dose-dependent inhibition of IgG-binding by methylene blue and laser light suggests that as well as inactivating toxins, PDT (and indeed methylene blue alone) may also be able to counteract the evasion of

the immune response by staphylococci by preventing the interaction of protein A with the Fc portion of IgG. In contrast, methylene blue and laser light of 665 nm was not found to promote the detachment of pre-bound *S. aureus* from host proteins, suggesting that PDT may only be able to prevent colonisation at an early stage.

Packer *et al*. demonstrated that proteolytic enzymes of the periodontal pathogen *Porphyromonas gingivalis* could be inactivated using the photosensitiser TBO and red laser light with a wavelength of 633 nm (Packer *et al.*, 2000). The results presented in this chapter demonstrate that photodynamic inactivation of a proteolytic enzyme from a different bacterial species is possible, with a highly significant reduction in the activity of *S. aureus* V8 protease being achieved with a 665 nm laser light dose as low as 4.38 J in combination with 20 μM methylene blue. This inactivation was found to be dose-dependent, with the highest concentration of methylene blue tested (20 μM) and irradiation with 21.9 J of 665 nm laser light achieving a 100 % reduction in activity compared with non-treated samples. Treatment of EMRSA-16 under the same conditions resulted in a 99.999% kill, indicating that inactivation of secreted proteases may be possible as well as eradicating infecting bacteria. The photodynamic inactivation of V8 protease using tin chlorin e6 and 633 nm laser light was also found to be photosensitiser and light dose-dependent. The combination of tin chlorin e6 and 633 nm laser light was more effective than equivalent doses of methylene blue and 665 nm laser light, as irradiation of the enzyme in the presence of 20 μM tin chlorin e6 resulted in the complete inactivation of proteolytic activity. The lowest concentration of tin chlorin tested (1 μM) had a significant inhibitory effect when irradiated with 4.38 J of 633 nm laser light, which was not observed with the same concentration of methylene blue.

Proteases are responsible for the destruction of host tissues both directly and indirectly; inactivation of these enzymes using PDT could therefore limit the damage

to the host, as well as killing the infecting organism. It has been suggested that the V8 protease plays an important role in the pathogenesis of *S. aureus* infections, as strains lacking this enzyme show reduced virulence in a number of infection models (Cheung *et al.*, 1994; Shaw *et al.*, 2004; Sifri *et al.*, 2003). Of particular relevance is a murine abscess model, in which inactivation of V8 protease resulted in significant attenuation of virulence (Shaw *et al.*, 2004); therefore the photodynamic inactivation of this enzyme may be able to reduce the virulence potential of *S. aureus* in other hosts.

As staphylococcal proteases and the colonisation of atopic skin by *S. aureus* have been implicated in the pathogenicity of atopic dermatitis (Miedzobrodzki *et al.*, 2002), the inactivation of proteolytic enzymes could have particular relevance for the decontamination of infected lesions using PDT. Photosensitisation using the combination of methylene blue and laser light of 665 nm may therefore be of use in the treatment of atopic skin disorders, an area in which PDT has a successful history (Morison *et al.*, 1978). Clearly, the combination of elimination of disease-exacerbating microorganisms and neutralisation of virulence factors would be extremely advantageous to the treatment of these diseases.

The treatment of α-haemolysin with methylene blue and laser light resulted in an effective inhibition of haemolytic activity. Concentrations of methylene blue ranging from 1-20 µM all had an inhibitory effect on α-haemolysin when irradiated with laser light, and α-haemolysin was shown to be inactivated following photosensitisation with 4.38 J of 665 nm laser light in the presence of 20 µM methylene blue. Irradiation of α-haemolysin with 633 nm laser light in combination with tin chlorin e6 yielded similar results. The results shown here demonstrate that α-haemolysin is the most susceptible of the virulence factors tested, perhaps due to the nature of its amino acid composition, which may leave it more vulnerable to attack by reactive oxygen species. These data indicate that photodynamic

inactivation of this toxin is highly effective and as such, could significantly attenuate the virulence of *S. aureus* due to the multiple functions of α-haemolysin as a virulence factor.

The role of α-haemolysin in the virulence of *S. aureus* has been demonstrated in a number of infection models such as mastitis (Jonsson *et al.*, 1985) and pneumonia (Wardenburg *et al.*, 2007). It has also been proposed that α-haemolysin may play a role in colonisation of epithelia by attenuating bacterial clearance from the epithelial surface (Eichstaedt *et al.*, 2009); this could therefore be of relevance to the decontamination of nasal epithelia using PDT. In addition, α-haemolysin has immunomodulatory properties, notably its ability to trigger the release of pro-inflammatory cytokines such as interleukin-1β (Bhakdi & Tranum-Jensen, 1991); thus inactivation of α-haemolysin by PDT may also protect against harmful inflammatory processes as well as eliminating infecting organisms.

The treatment of *S. aureus* sphingomyelinase with 665 nm laser light and methylene blue resulted in a significant, dose-dependent reduction in the enzyme's activity. Laser light alone also appeared to reduce the activity of sphingomyelinase; however this was found to be not statistically significant. Irradiation of sphingomyelinase with 4.4 J laser light in the presence of the highest concentration of methylene blue tested (20 μM) achieved a highly significant reduction in the activity of the enzyme (76%), which was comparable to the reduction in activity observed for the V8 protease when irradiated for the same time period. This reduction in activity was increased to 94% after irradiation of the enzyme for 5 minutes with laser light of 665 nm in the presence of 20 μM methylene blue. The combination of tin chlorin e6 and 633 nm laser light was also found to be highly effective in the inactivation of sphingomyelinase activity, with a 93% reduction in activity being achieved with 20 μM tin chlorin e6 and a light dose as low as 4.4 J.

Production of sphingomyelinase (β-haemolysin) has been associated with severe, chronic skin infections, and strains of *S. aureus* producing high levels of this enzyme have been shown to cause more intense skin lesions than low-producing strains (Hedström & Malmqvist, 1982). Inactivation of these toxins may therefore be of relevance to the treatment of superficial staphylococcal skin infections. More recently, sphingomyelinase has been shown to be involved in protection against the host immune response by interfering with host signalling (Tajima *et al.*, 2009). Sphingomyelinase has also been shown to kill proliferating T lymphocytes, supporting a role for this toxin in evasion of the host immune response (Huseby *et al.*, 2007), Consequently, the photodynamic inactivation of sphingomyelinase during PDT could also reduce the immunomodulatory properties of *S. aureus*.

The photodynamic inactivation of α-haemolysin and sphingomyelinase was shown to be unaffected by the presence of human serum at concentrations resembling the protein content of an acute wound (Lambrechts *et al.*, 2005), indicating that photodynamic therapy may be effective in inactivating these virulence factors *in vivo*. Together with the data showing that photodynamic treatment with methylene blue or tin chlorin e6 is an effective means of killing *S. aureus*, this supports the potential of PDT as a treatment for superficial staphylococcal infections.

The precise mechanism of inhibition of these virulence factors has not yet been determined; however it is possible that the reactive oxygen species formed during photosensitisation can oxidise proteins, thereby disrupting their function (Hamblin & Hasan, 2004). Oxidation of active site groups has been suggested as the mechanism of action for the photodynamic inactivation of the proteolytic enzymes of *P. gingivalis* (Packer *et al.*, 2000). SDS PAGE analysis of the V8 protease and α-haemolysin demonstrated that photosensitisation caused changes to the proteins which resulted in smearing of the protein bands. We propose that singlet oxygen may play a role in the inactivation of V8 protease as a protective effect is observed

when photosensitisation is performed in the presence of the singlet oxygen scavenger L-tryptophan; however an enhancer of the lifetime of singlet oxygen (D_2O) does not increase inactivation, suggesting that other reactive oxygen species may be involved, and/or that inactivation of V8 protease may be independent of the lifetime of singlet oxygen.

4.5 Summary

Treatment with the light-activated antimicrobials methylene blue and tin chlorin e6 in combination with laser light of the appropriate wavelength successfully inhibited the activity of a number of staphylococcal virulence factors. Considering the extensive damage virulence factors can cause to host tissues, the ability to inhibit their activity would be a highly desirable feature for any antimicrobial treatment regimen and would represent a significant advantage over conventional antibiotic strategies. Inactivation was shown to occur under conditions required to kill both a meticillin-sensitive and meticillin-resistant strain of *S. aureus* and was not found to be inhibited by the presence of human serum, suggesting that inactivation of these virulence factors may be possible *in vivo*.

5 Investigation of possible methods for the enhancement of photodynamic inactivation of *S. aureus* and staphylococcal virulence factors

5.1 Introduction

PDT causes oxidative damage via two distinct mechanisms, Type I and Type II reactions. Type I reactions occur when a photosensitiser reacts with substrates to form radicals or radical ions, whereas Type II reactions occur when the photosensitiser reacts with oxygen to produce singlet oxygen (Foote, 1991). The two reactions may occur simultaneously, although there is generally believed to be a shift from Type II to Type I reactions as a result of decreasing oxygen concentration in the course of PDT (Jakus & Farkas, 2005; Martins *et al.*, 2004).

The efficacy of photosensitisation is dependent on a number of factors, including the chemistry and formulation of the photosensitiser, the localisation of the photosensitiser in the target tissue, the light dose delivered, and the degree of oxygenation of the tissue (Konan *et al.*, 2002). There has been considerable interest in various methods of enhancing the efficacy of PDT, including targeted photosensitisers, increasing the susceptibility of target cells, and formulations to increase accumulation of the photosensitiser at a specific site (Verma *et al.*, 2007). The formulation of the photosensitiser can also be modified in order to enhance the photosensitiser's photophysical properties (George & Kishen, 2008). The half-life of singlet oxygen generated is dependent on the photosensitiser solvent; the half-life of singlet oxygen is five-fold greater in ethanol compared with water (Meisel & Kocher, 2005). Therefore, it may be possible to enhance the lifetime of singlet oxygen and therefore increase the photooxidative damage and efficacy of PDT treatment by incorporating ethanol into the photosensitiser formulation.

Solvents incorporating ethanol and glycerol have been suggested for use in endodontic applications, as an aqueous formulation of methylene blue containing ethanol and glycerol (glycerol, ethanol and water in a 30:20:50 ratio; referred to as MIX) demonstrated greater *in vitro* penetration into dentinal tubules compared with water and glycerol alone. This formulation also demonstrated enhanced photodynamic killing of *Enterococcus faecalis* and *Aggregatibacter actinomycetemcomitans* (George & Kishen, 2007). The MIX formulation has also been shown to cause greater damage to the cell wall of *E. faecalis* compared with methylene blue dissolved in water alone, potentially due to the MIX formulation inhibiting the aggregation of methylene blue and resulting in better penetration of the photosensitiser into the bacterial cell (George & Kishen, 2008).

The oxidative damage produced by photosensitisation may also be enhanced by the generation of additional reactive oxygen species via the use of additives such as ascorbate (Kramarenko *et al.*, 2006b). Although typically protective against reactive oxygen species, antioxidants such as ascorbic acid (vitamin C) may also exhibit pro-oxidant activity in the presence of certain catalytic metals. Type II reactions generate singlet oxygen, which can then interact with membrane lipids to form lipid hydroperoxides (Jakus & Farkas, 2005). The addition of ascorbic acid and iron (Fe^{2+}) has been shown to enhance the Photofrin-induced photosensitisation of murine leukaemia cells. It was proposed that the enhancement was a result of a Fenton-type chemical reaction between lipid hydroperoxides and iron that resulted in lipid radical formation ($LO^{\bullet}$):

Fe^{2+} + lipid hydroperoxide $\rightarrow$ Fe^{3+} + OH- + $LO^{\bullet}$

The formation of lipid radicals may then initiate a free radical chain reaction, and consequently further lipid peroxidation and cell membrane damage (Buettner *et al.*,

1993). Free radicals generated in this manner may then react with oxygen to form a superoxide radical anion or other reactive oxygen species (Jakus & Farkas, 2005).

Type I reactions may result in the generation of cytotoxic hydrogen peroxide as a result of hydroxyl radicals reacting with biomolecules (Wainwright, 2000). Ascorbate also reacts with the singlet oxygen generated by Type II reactions to produce hydrogen peroxide. Hydrogen peroxide has a considerably longer lifetime than singlet oxygen and consequently a greater diffusion distance; therefore, enhanced production of hydrogen peroxide via reactions with ascorbate may enhance the efficacy of PDT (Kramarenko *et al.*, 2006a).

An investigation into the enhancement of the lethal photosensitisation of *S. aureus* and the photodynamic inactivation of selected staphylococcal virulence factors is described in this chapter. The effect of solvent and the addition of ferrous sulphate and ascorbic acid on the efficacy of photodynamic inactivation using methylene blue in conjunction with laser light of 665 nm were investigated.

5.2 Materials and methods

5.2.1 The effect of solvent on the lethal photosensitisation of *S. aureus*

S. aureus 8325-4 and EMRSA-16 were maintained and grown for experimental purposes as described in section 2.3.1. Lethal photosensitisation of *S. aureus* 8325-4 and EMRSA-16 was carried out as described in section 2.6, with the following modifications. Methylene blue was suspended in either sterile, distilled water or MIX at a final concentration of 5 µM. MIX was prepared as described by George and Kishen and consisted of glycerol, ethanol and distilled water in a ratio of 30:20:50 (George & Kishen, 2008).

5.2.2 The effect of ferrous sulphate and ascorbic acid on the lethal photosensitisation of *S. aureus*

S. aureus 8325-4 and EMRSA-16 were maintained and grown for experimental purposes as described in section 2.3.1. Lethal photosensitisation of *S. aureus* 8325-4 and EMRSA-16 was carried out as described in section 2.6, with the following modifications. Fifty microlitres of methylene blue at a final concentration of 5 µM (S+) or PBS (S-) was added to an equal volume of the inoculum in sextuplicate wells of a sterile, flat-bottomed, untreated 96-well plate, three of which were irradiated with 4.38 J of 665 nm laser light, with stirring (L+) whilst the remaining three wells were incubated in the dark (L-). In addition, 50 µL of a 1:1 mixture of methylene blue (final concentration of 5 µM) and ascorbic acid/ferrous sulphate solution (final concentrations of 50 µM ferrous sulphate and 250 µM ascorbic acid or 100 µM ferrous sulphate and 500 µM ascorbic acid) were added to an equal volume of the inoculum in a further six wells and either irradiated as above or kept in the dark.

In addition, the optical density at 661 nm of the solution was measured in order to assess whether the addition of ferrous sulphate and ascorbic acid had an effect on light absorbance by methylene blue.

5.2.3 The effect of ferrous sulphate and ascorbic acid on the inactivation of *S. aureus* V8 protease by methylene blue and 665 nm laser light

Fifty microlitres of methylene blue at a final concentration of 5 μM was added to an equal volume of V8 protease (final concentration of 5 μg/mL) in sextuplicate wells of a sterile, flat-bottomed, untreated 96-well plate and either irradiated with 4.38 J of 665 nm laser light (L+), with stirring or kept in the dark (L-). Fifty microlitres of a 1:1 mixture of methylene blue (final concentration of 5 μM) and ascorbic acid/ferrous sulphate solution (final concentrations of 50 μM ferrous sulphate and 250 μM ascorbic acid) was added to an equal volume of V8 protease in a further six wells and irradiated or incubated in the dark as above. Fifty microlitres of PBS was added to an equal amount of V8 protease in a further six wells, which were treated as above (S-). After irradiation, the azocasein hydrolysis assay was performed as described in section 2.7.1.

5.2.4 The effect of ferrous sulphate and ascorbic acid on the inactivation of *S. aureus* sphingomyelinase by methylene blue and 665 nm laser light

Sphingomyelinase from *S. aureus* was diluted to a final concentration of 0.5 Units/mL in 250 mM Tris-HCl buffer with 10 mM magnesium chloride, pH 7.4 at 37°C. Twenty five microlitres of sphingomyelinase was added to an equal volume of either 5 μM methylene blue (S+) or 25 μL PBS (S-) and irradiation of the enzyme suspension was carried out as described previously with the appropriate controls (L-S-, L-S+, L+S-). Twenty five μL of a 1:1 mixture of methylene blue (final concentration of 5 μM) and ascorbic acid/ferrous sulphate solution (final concentrations of 50 μM ferrous sulphate and 250 μM ascorbic acid) was added to an equal volume of

sphingomyelinase in a further three wells and irradiated for one minute. As additional controls, three wells containing a 1:1 mixture of ascorbic acid/ferrous sulphate solution (final concentrations of 50 µM ferrous sulphate and 250 µM ascorbic acid) and PBS were added to an equal volume of sphingomyelinase and kept in the dark (L-). After irradiation/dark incubation, the spectrophotometric assay for sphingomyelinase assay was carried out as described in section 4.2.11.

5.3 Results

5.3.1 The effect of solvent on the lethal photosensitisation of *S. aureus*

5.3.1.1 *S. aureus* 8325-4

Figure 5.1 shows that no enhancement of photodynamic killing was observed using MIX as a solvent for methylene blue compared with distilled water; rather a significant protective effect was observed ($P < 0.05$; ANOVA).

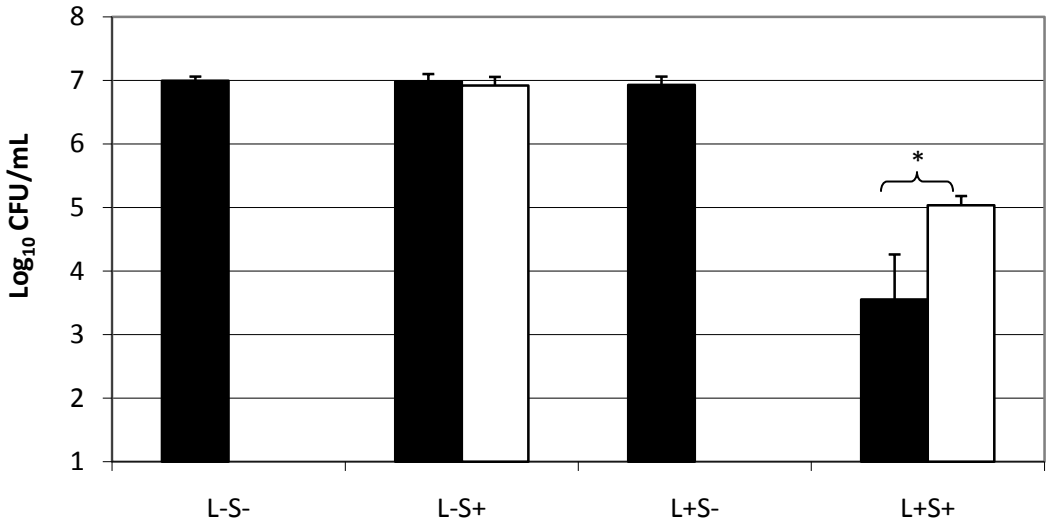

Figure 5.1 The effect of solvent on the lethal photosensitisation of *S. aureus* 8325-4 using methylene blue and 665 nm laser light.

An equal volume of either PBS (S-), methylene blue in distilled water (■) or MIX (□) (S+) was added to 50 µL of the bacterial suspension and either kept in the dark (L-) or exposed to 4.38 J of 665 nm laser light (L+). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. * $P < 0.05$ (ANOVA). Experiments were performed twice in triplicate and the combined data are shown.

5.3.1.2 EMRSA-16

It can be seen from Figure 5.2 that using MIX as a solvent also did not result in increased photodynamic killing of EMRSA-16 using methylene blue and 665 nm laser light. A similar protective effect was observed for *S. aureus* 8325-4; however, unlike *S. aureus* 8325-4 the protective effect of MIX was not significant for EMRSA-16 ($P > 0.05$; ANOVA).

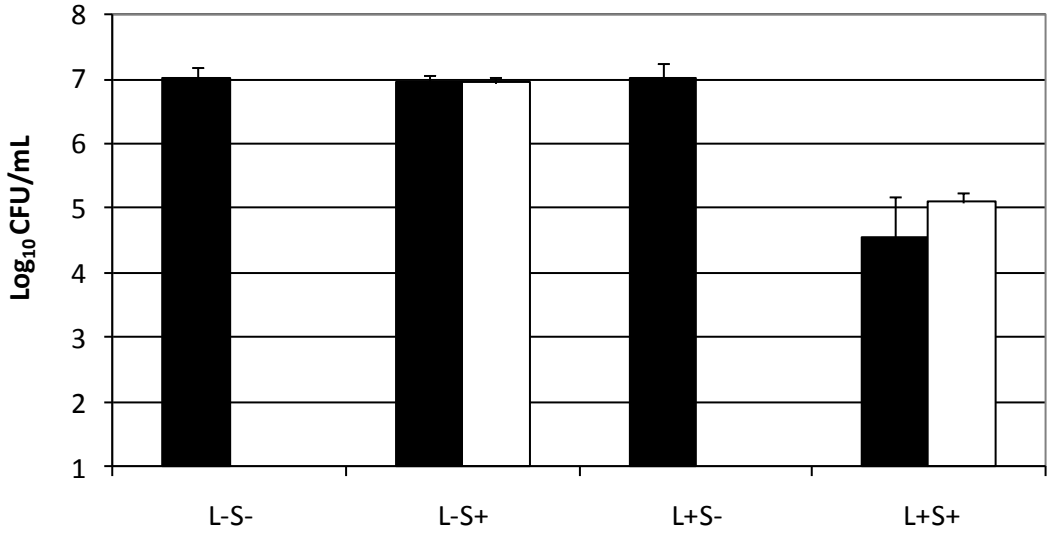

Figure 5.2 The effect of solvent on the lethal photosensitisation of EMRSA-16 using methylene blue and 665 nm laser light.

An equal volume of either PBS (S-), methylene blue in distilled water (■) or MIX (□) (S+) was added to 50 µL of the bacterial suspension and either kept in the dark (L-) or exposed to 4.38 J of 665 nm laser light (L+). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. Experiments were performed twice in triplicate and the combined data are shown.

5.3.2 The effect of solvent on the inactivation of V8 protease

Using MIX as a solvent for methylene blue caused a reduction of 80 % ± 19 % in the proteolytic activity of *S. aureus* V8 protease, compared with 68 % ± 17 % when H_2O was used as a solvent. This difference was not found to be statistically significant ($P > 0.05$; ANOVA).

5.3.3 The effect of ferrous sulphate and ascorbic acid on the lethal photosensitisation of *S. aureus*

5.3.3.1 *S. aureus* 8325-4

The addition of 50 µM ferrous sulphate and 250 µM ascorbic acid to 5 µM methylene blue resulted in approximately one-$\log_{10}$ increased kill compared with 5 µM methylene blue alone when irradiated with laser light of 665 nm (see Figure 5.3). This equated to a 97.14% kill versus 71.95% kill for methylene blue with and without the addition of ascorbic acid/ferrous sulphate respectively. This difference was found to be highly significant ($P < 0.001$; ANOVA). Whilst not as effective as the enhancement achieved with the addition of 50 µM ferrous sulphate and 250 µM ascorbic acid, the addition of 100 µM ferrous sulphate and 500 µM ascorbic acid resulted in an increased kill of 92.60% compared with methylene blue alone; however this difference was not statistically significant ($P > 0.05$; ANOVA).

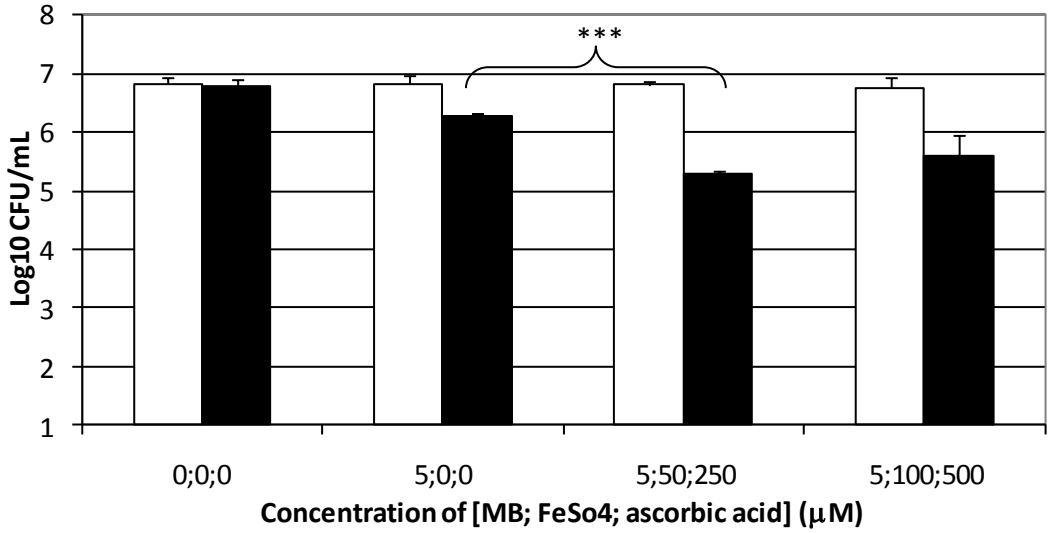

Figure 5.3 The effect of $FeSO_4$ and ascorbic acid on the lethal photosensitisation of *S. aureus* 8325-4 using methylene blue and 665 nm laser light.

An equal volume of either PBS, 5 μM methylene blue (final concentration) or 5 μM methylene blue with the addition of $FeSO_4$ and ascorbic acid (final concentrations of 50 μM and 100 μM or 250 μM and 500 μM respectively) was added to 50 μL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed twice in triplicate and the combined data are shown.

5.3.3.2 EMRSA-16

As shown by Figure 5.4, the addition of 50 µM $FeSO_4$ and 250 µM ascorbic acid to methylene blue did not result in enhanced killing of EMRSA-16 compared with methylene blue alone. Treatment of EMRSA-16 with laser light and 5 µM methylene blue resulted in a 36% kill, compared with a 38% kill when treated with 5 µM methylene blue plus 50 µM $FeSO_4$ and 250 µM ascorbic acid ($P > 0.05$; ANOVA).

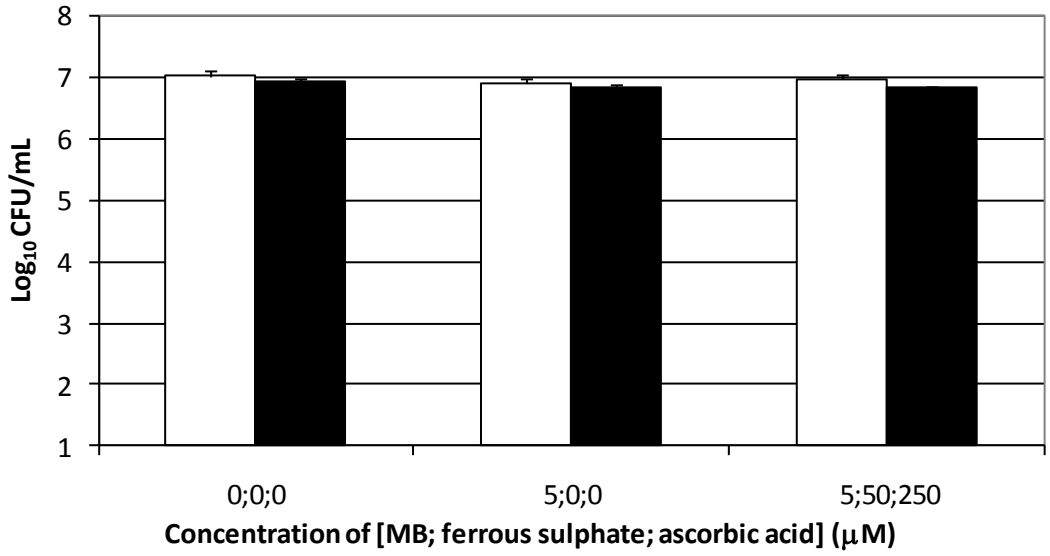

Figure 5.4 The effect of $FeSO_4$ and ascorbic acid on the lethal photosensitisation of EMRSA-16 using methylene blue and 665 nm laser light.

An equal volume of either PBS, 5 µM methylene blue (final concentration) or 5 µM methylene blue with the addition of $FeSO_4$ and ascorbic acid (final concentrations of 50 µM and 250 µM, respectively) was added to 50 µL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. Experiments were performed twice in triplicate and the combined data are shown.

5.3.4 The effect of ferrous sulphate and ascorbic acid on the inactivation of staphylococcal virulence factors

5.3.4.1 V8 protease

Figure 5.5 shows the effect of $FeSO_4$ and ascorbic acid on the photodynamic inactivation of *S. aureus* V8 protease by methylene blue and 665 nm laser light. The addition of $FeSO_4$ and ascorbic acid did not result in increased photodynamic inactivation of V8 protease; on the contrary, it appeared to have a significant protective effect against the effect of photosensitisation ($P > 0.05$; ANOVA).

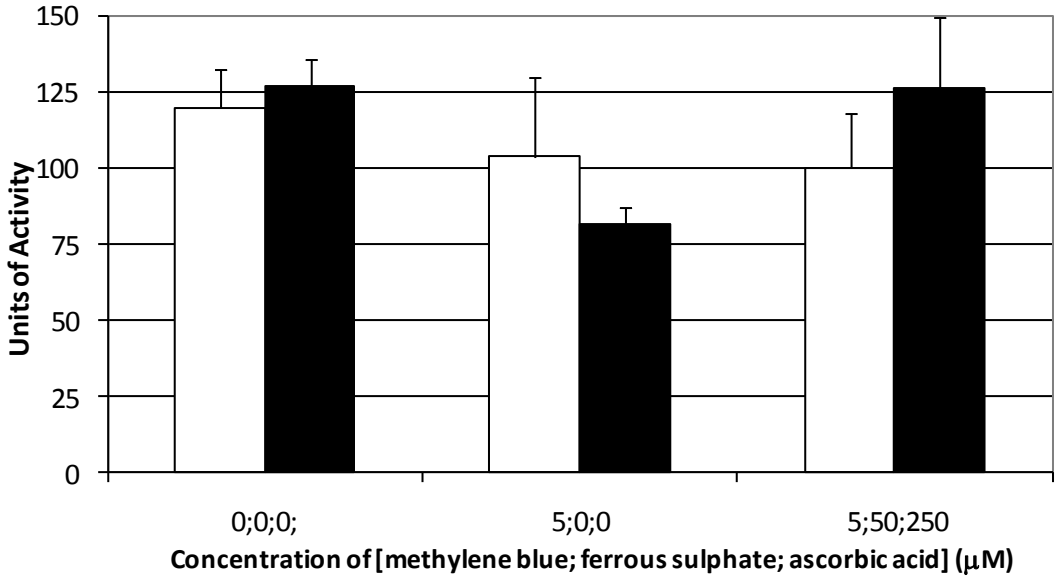

Figure 5.5 The effect of $FeSO_4$ and ascorbic acid on the photodynamic inactivation of *S. aureus* V8 protease using methylene blue and 665 nm laser light.

An equal volume of either PBS, 5 µM methylene blue (final concentration) or 5 µM methylene blue with the addition of $FeSO_4$ and ascorbic acid (final concentrations of 50 µM and 250 µM respectively) was added to V8 protease and samples were either exposed to 4.38 J of laser light (■) or kept in the dark (□). The activity of the V8 protease was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. Experiments were performed twice in triplicate and the combined data are shown.

5.3.4.2 Sphingomyelinase

As previously observed with the V8 protease, the addition of 50 μM ferrous sulphate and 250 μM ascorbic acid to methylene blue had a protective effect against the inactivation of sphingomyelinase by methylene blue and 665 nm laser light. Lethal photosensitisation of the sphingomyelinase in the presence of 5 μM methylene blue, 50 μM ferrous sulphate and 250 μM ascorbic acid resulted in a 34% ± 5% reduction in activity compared with a 56% ± 10% reduction in activity when the enzyme was treated with 4.38 J of laser light and 5 μM methylene blue.

There was no difference in the optical density at 661 nm between methylene blue alone and with the addition of either 50 μM ferrous sulphate/250 μM ascorbic acid or 100 μM ferrous sulphate/500 μM ascorbic acid.

5.4 Discussion

It has been suggested that using MIX as a solvent for methylene blue may result in a better photooxidation potential, attributed to the prevention of photosensitiser aggregation and a proposed increased half-life of singlet oxygen due to the presence of ethanol in the formulation (George & Kishen, 2007). It has also been demonstrated that methylene blue dissolved in MIX resulted in a significantly greater killing of *E. faecalis* compared with methylene blue dissolved in water alone (George & Kishen, 2008). Therefore, it was hypothesised that the MIX solvent may have the potential to enhance the photodynamic killing of *S. aureus* and the photoinactivation of staphylococcal virulence factors. However, photosensitisation of *S. aureus* using MIX did not result in increased killing of either the meticillin-sensitive strain 8325-4 or the meticillin-resistant strain EMRSA-16. On the contrary, a significant protective effect was observed in the case of *S. aureus* 8325-4.

Using MIX as a solvent resulted in a slight enhancement of the photodynamic inactivation of V8 protease; however this was not found to be statistically significant. The results shown in this chapter do not support the hypothesis that using MIX as a solvent for methylene blue results in more efficacious photodynamic killing or enhanced photodynamic inactivation of staphylococcal virulence factors. Since previous experiments have shown that *S. aureus* is killed more effectively when irradiated in the presence of an enhancer of singlet oxygen lifetime (D_20) (Section 3.2.1.1.2), it is possible that MIX does not prolong the half-life of singlet oxygen enough for it to have a significant effect in this system. In addition, the system used by George and Kishen used a higher concentration of methylene blue, a different final concentration of MIX components (bacterial cells were resuspended in MIX, whereas resuspended cells were added 1:1 to MIX in the test system described in this chapter), a different bacterium, a higher bacterial cell

concentration and a higher light dose than those used in this study, all of which may contribute to the observed lack of enhancement (George & Kishen, 2008).

Ferrous sulphate and ascorbic acid were added to methylene blue following reports that these additives may enhance the photodynamic killing of oral squamous cell carcinoma cells (Kelley *et al.*, 1997). It was proposed that cytotoxicity is enhanced in the presence of ferrous sulphate and ascorbic acid due to increased lipid radical production. The singlet oxygen produced by a triplet state photosensitiser reacting with molecular oxygen can then react with polyunsaturated fatty acids such as those in biological membranes to produce lipid peroxides. In the presence of ascorbic acid, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}), which can then catalyse the production of free radicals from the lipid peroxides formed by the reaction of singlet oxygen with fatty acids, thus increasing cytotoxicity.

The addition of ascorbate alone has also been reported to enhance the efficacy of photodynamic killing of breast cancer and leukaemia cells due to the ability of ascorbate to react with singlet oxygen ($^1O_2$) to produce hydrogen peroxide (H_2O_2), which has a longer half-life and greater diffusion distance than singlet oxygen (Kramarenko *et al.*, 2006a; Kramarenko *et al.*, 2006b; Rozanova Torshina *et al.*, 2007). The production of hydrogen peroxide in this manner is shown by the equation below:

Ascorbate + $^1O_2 \rightarrow H_2O_2$ + dehydroascorbate

Hydrogen peroxide can in turn react with biological structures resulting in the production of hydroxyl radicals, thus initiating a cytotoxicity cascade (Kramarenko *et al.*, 2006b). The addition of ferrous sulphate and ascorbate to a photosensitiser formulation could therefore present a simple method of enhancing the efficacy of PDT.

The photodynamic killing of *S. aureus* 8325-4 was shown to be enhanced by approximately one $\log_{10}$ when methylene blue was irradiated with laser light in the presence of ferrous sulphate and ascorbic acid compared with methylene blue alone, suggesting that increased free radical production and consequently increased oxidative damage to the cell occurs in these conditions. This enhancement of photodynamic killing was statistically significant ($P < 0.001$;ANOVA).

In contrast, the killing of EMRSA-16 was not found to be enhanced under these conditions. Meticillin-resistant *S. aureus* has previously been shown to be less susceptible to photodynamic killing than meticillin-sensitive strains (Embleton *et al.*, 2002; Wainwright *et al.*, 1998). The reduced susceptibility of EMRSA-16 may be due to the presence of capsular polysaccharides that may affect penetration of the photosensitiser, and therefore limit the damage of reactive oxygen species. Thicker cell walls have been observed in MRSA compared with MSSA (Hiramatsu *et al.*, 1997); consequently this difference in the cell wall between MSSA and MRSA may play a role in the differing susceptibilities.

In Gram-positive microorganisms, for singlet oxygen to react with fatty acids present in the bacterial cell membrane, it must first penetrate the cell wall. In the case of decreased uptake or binding, less damage to the cell wall would result in a lower level of oxidative damage from these secondary mechanisms of radical generation. It is possible that Gram-negative bacteria may be more susceptible than Gram-positive microorganisms to this method of enhanced cytotoxicity due to the reaction of singlet oxygen with components of the outer membrane (Dahl *et al.*, 1989).

Photosensitisation of the V8 protease and sphingomyelinase using methylene blue and laser light in the presence of ascorbic acid and ferrous sulphate did not result in increased photodynamic inactivation of these enzymes compared with methylene

blue alone. As the lipid peroxides key to the increased cytotoxicity of PDT in the presence of ferrous sulphate and ascorbate are formed by the reaction of reactive oxygen species with biomolecules (e.g. the bacterial cytoplasmic membrane), the lack of increased photodynamic inactivation of the V8 protease and sphingomyelinase may be due to the absence of lipid-containing biomolecules in the test system, which involved the purified enzymes only (Wainwright, 2000). It is therefore possible that in a whole cell system, increased photodynamic inactivation of staphylococcal enzymes may be achieved.

However, Fenton-like reactions may also be initiated by the reaction of transition metals with hydrogen peroxide (as opposed to a lipid peroxides), and damage to proteins in this manner has been documented in cell-free systems (Dean *et al.*, 1997; Stadtman, 1990). Hydrogen peroxide may be generated as a result of the reaction of hydroxyl radicals produced by Type I reactions (Wainwright, 2000); therefore it is possible that initiation of Fenton-like reactions did occur in this system, but maybe not at high enough frequency to cause damage to the protein. As the enhancing effect of antioxidants on PDT is concentration dependent (Jakus & Farkas, 2005), enhancement of photodynamic inactivation may be achieved by optimisation of the concentration of ascorbate in the system.

As previously stated, ascorbate alone can react with singlet oxygen to produce the longer-lived hydrogen peroxide; however it did not enhance the photodynamic inactivation of the V8 protease or sphingomyelinase in this system. It is possible that photodynamic inactivation of these enzymes is not mediated by hydrogen peroxide, rather by other reactive oxygen species. In addition, the protective effect against photodynamic inactivation observed when ferrous sulphate and ascorbic acid were added to the methylene blue solution may be due to the antioxidant properties of ascorbic acid. Ascorbic acid is a powerful antioxidant, and reacts with reactive

oxygen species to produce a terminal, poorly-reactive semidihydroascorbate radical (Valko *et al.*, 2006). As previously discussed, the enhancing effect of antioxidants is concentration-dependent; therefore there may be a threshold concentration where a switch from protection to enhancement is observed. Further research would be necessary to investigate this further.

Other approaches to the enhancement of the activity of photosensitisation may also be investigated. It has been demonstrated that gold nanoparticles can enhance the TBO-mediated photodynamic killing of *S. aureus* (Gil-Tomas *et al.*, 2007; Narband *et al.*, 2008). It was proposed that the gold nanoparticles may enhance the light capture by TBO and direct the photodynamic reaction towards the generation of reactive oxygen species such as hydroxyl radicals rather than singlet oxygen. It is therefore possible that enhancement of the photodynamic inactivation of staphylococcal virulence factors may be achieved using such a system.

5.5 Summary

Treatment of *S. aureus* 8325-4 with methylene blue in the presence of 50 μM ferrous sulphate and 250 μM ascorbic acid resulted in approximately one-$\log_{10}$ increased kill compared with 5 μM methylene blue alone when irradiated with laser light of 665 nm. This enhancement of photodynamic inactivation was not observed for EMRSA-16, V8 protease or sphingomyelinase. MIX was found to be an ineffective solvent for methylene blue when used for the photodynamic killing of *S. aureus* species, as it provided a protective effect against lethal photosensitisation compared with methylene blue suspended in water alone. These results suggest that additives such as ferrous sulphate and ascorbic acid to the photosensitiser formulation may result in enhanced antibacterial activity for certain strains of *S. aureus*; however, enhanced photodynamic inactivation of *S. aureus* protease or sphingomyelinase may not occur at concentrations of the additives required for enhancement of bacterial killing. Further optimisation of the concentrations of these additives may result in more favourable results. There is also scope for further investigations using novel methods of enhancement such as the addition of gold nanoparticles to the photosensitiser formulation.

6 The susceptibility of *Staphylococcus aureus* small colony variants to lethal photosensitisation

6.1 Introduction

Small colony variants (SCVs) of *S. aureus* were first isolated from infected lesions in the 1950s (Goudie & Goudie, 1955; Hale, 1951; Sherris, 1952). SCVs of *S. aureus* are a naturally-occurring subpopulation found in certain infections and are characterised by a slow growth rate and small colony size relative to the parent strain (von Eiff, 2008). Chronic antibiotic exposure is also believed to result in the generation of SCVs, as they have been isolated from patient groups receiving long-term antibiotic therapy, such as cystic fibrosis and osteomyelitis patients (Proctor *et al.*, 1998). The most commonly encountered SCVs from clinical samples have deficiencies in either the electron transport chain or thymidine biosynthesis, although other types of SCVs such as auxotrophs for carbon dioxide have also been observed (Proctor *et al.*, 2006).

SCVs typically have a doubling time of approximately 180 minutes, compared with 20 minutes for typical strains of *S. aureus* (Proctor *et al.*, 1998). SCVs also have different colony morphology and biochemical profiles compared with the classic *S. aureus* phenotype; colonies are generally non-pigmented and non-haemolytic, and strains show slow coagulase activity and decreased sugar fermentation (Proctor, 2000).

S. aureus SCVs have been isolated from abscesses, soft tissue and joint infections, blood, and the pulmonary tract, and have been associated with infections that are persistent, recurrent and antibiotic-resistant (Goring *et al.*, 2001; Proctor, 2000; Seifert *et al.*, 1999; von Eiff *et al.*, 2001). The slow growth rate of these organisms can cause difficulties in laboratory identification as they are easily overgrown by

other species in a mixed culture and therefore may be easily missed in patient samples, a problem compounded by their fastidious growth requirements and atypical colony morphology (Proctor *et al.*, 1998; Proctor, 2000).

The ability to form SCVs may be considered a virulence mechanism in itself, as SCVs are relatively unstable and may revert to a more virulent phenotype on completion of antibiotic therapy and/or once the host immune response has subsided, therefore acting as a defence mechanism against elimination (Proctor & Peters, 1998). This extraordinary ability of SCVs is exemplified by relapses of infection following extended disease-free periods and intensive parenteral antibiotic therapy (Proctor *et al.*, 1995).

SCVs often display lower susceptibility to antimicrobial agents such as aminoglycosides, due to the interruption of the electron transport chain as a result of auxotrophy for menadione, hemin or thymidine (von Eiff, 2008). Defects in the electron transport chain result in reduced uptake of cationic antibiotic compounds (Proctor & Peters, 1998). Resistance to β-lactams has also been noted due to the slow growth rate of SCVs, and the consequent reduced cell wall division of small colony variants (Proctor & von Humboldt, 1998).

Menadione and hemin are the most common auxotrophies observed in clinical SCVs (Jonsson *et al.*, 2003). Although SCVs that are auxotrophs for hemin and menadione are phenotypically similar, differences in metabolism between the two types of SCV have been documented. Von Eiff *et al.* demonstrated that a mutant with an insertion in the *menD* gene had a more severe defect in carbon metabolism than a mutant with an insertion in the *hemB* gene, due to the increased number of metabolic pathways that utilise menadione compared with hemin (von Eiff *et al.*, 2006). In addition to differences in carbon metabolism, differences in gene expression have been observed between these two types of SCV, with the most

significant differences in expression observed for genes involved in anaerobic respiration and fermentation (Kohler *et al.*, 2008). SCVs with mutations in *menD* and *hemB* also differ in their ability to survive in host tissues; *hemB* SCVs have been primarily recovered from sites such as bone and sputum, whereas *menD* SCVs have been isolated from a wider range of tissues (Bates *et al.*, 2003).

SCVs have been associated with higher virulence than the parent strain. In a murine septic arthritis model, a *hemB* small colony variant was found to produce approximately 20 times more protease than the parent strain (Jonsson *et al.*, 2003). Whilst mice inoculated with the *hemB* mutant had a significantly lower bacterial burden than those inoculated with the parent strain, the small colony variant was associated with a significantly higher severity of arthritis. It was proposed that the small colony variant was more virulence on a per organism basis than the parent strain. Increased production of other virulence factors has also been noted. Vaudaux *et al*. described increased expression of fibrinogen and fibronectin-binding proteins in a *hemB* small colony variant of *S. aureus*; adhesion to fibronectin and fibrinogen-coated surfaces was significantly enhanced compared with the parent strain (Vaudaux *et al.*, 2002).

Due to the persistent nature of these organisms, often despite prolonged antibiotic treatment, novel therapeutic options are required for the effective elimination of SCVs. SCVs have been isolated from persistent, antibiotic-resistant skin infections, which represent one of the areas in which PDT is particularly relevant (Coman *et al.*, 2008; Seifert *et al.*, 1999; von Eiff *et al.*, 2001). In the following chapter, the susceptibility of two *S. aureus* SCVs to lethal photosensitisation using methylene blue and tin chlorin e6 in combination with laser light of the appropriate wavelength is described. SCVs with mutations in the *hemB* and *menD* genes were used for these experiments, as strains with these mutations are the most commonly observed isolates obtained from clinical samples (Jonsson *et al.*, 2003).

6.2 Materials and methods

6.2.1 The effect of photosensitiser dose on the lethal photosensitisation of *Staphylococcus aureus* small colony variants

The strains used in this study were *S. aureus* LS-1 and its isogenic mutant with a deletion of the *hemB* gene (LS-1ΔhemB constructed by Dr John Wright, UCL), *S. aureus* 8325-4 and its isogenic mutant with a disruption in the *menD* gene *S. aureus* D1324 (a gift from Professor Richard Proctor) (Bates *et al.*, 2003). Bacteria were maintained as described in section 2.3.1 and lethal photosensitisation was performed as described in sections 2.5.2 and 2.6.

6.2.2 The effect of laser light dose on the lethal photosensitisation of *Staphylococcus aureus* small colony variants

The small colony variants and their parent strains were maintained as described in section 2.3.1. Experiments to determine the effect of laser light dose on lethal photosensitisation were performed as described in sections 2.5.1 and 2.6.

6.3 Results

6.3.1 *Staphylococcus aureus hemB* small colony variant

6.3.1.1 The effect of photosensitiser dose on the lethal photosensitisation of a *S. aureus hemB* small colony variant

6.3.1.1.1 Methylene blue

The effect of methylene blue dose on the lethal photosensitisation of *S. aureus* LS-1 (parent strain) and *S. aureus* LS-1ΔhemB when irradiated with laser light from a 665nm diode laser can be seen in Figure 6.1 and Figure 6.2, respectively. Although photosensitisation with 20 μM methylene blue and 4.38 J of 665 nm laser light resulted in highly significant killing of both the parent strain and the small colony variant ($P < 0.001$; ANOVA), significantly less killing was observed for the small colony variant compared with the parent strain ($P < 0.05$; ANOVA). Lethal photosensitisation with this regimen resulted in an approximate 3.5 log kill for *S. aureus* LS-1, compared with a 2.5 log kill for *S. aureus* LS-1ΔhemB. *S. aureus* LS-1 also appeared more susceptible to photodynamic killing at lower concentrations of photosensitiser (5 μM) compared with the *hemB* small colony variant; however there was no significant difference between the two strains ($P > 0.05$; ANOVA).

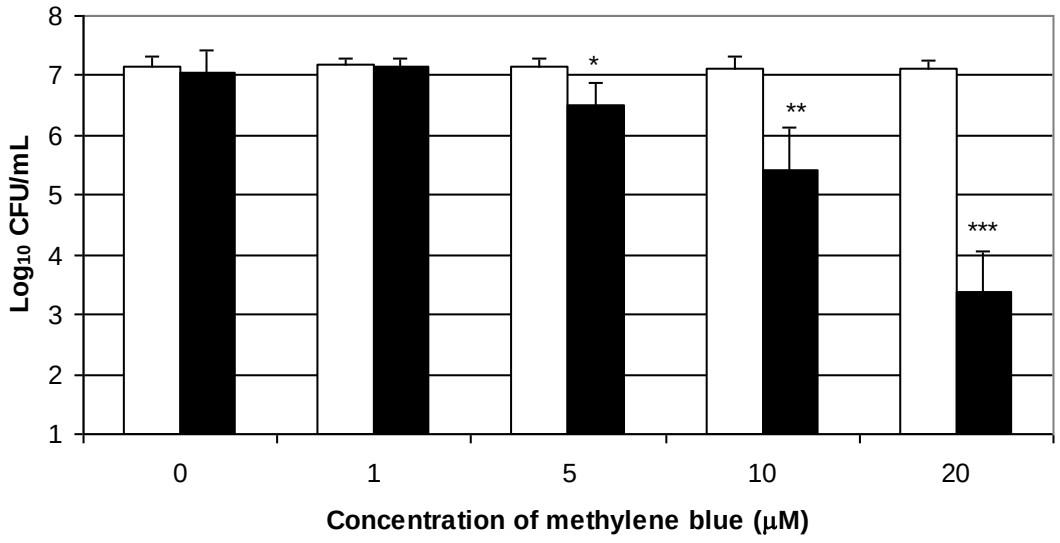

Figure 6.1 The effect of methylene blue dose on the lethal photosensitisation of *S. aureus* LS-1

An equal volume of either PBS or methylene blue (concentrations ranging from 1-20 μM) was added to 50 μL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. * P <0.05, ** P < 0.01, *** P < 0.001 (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

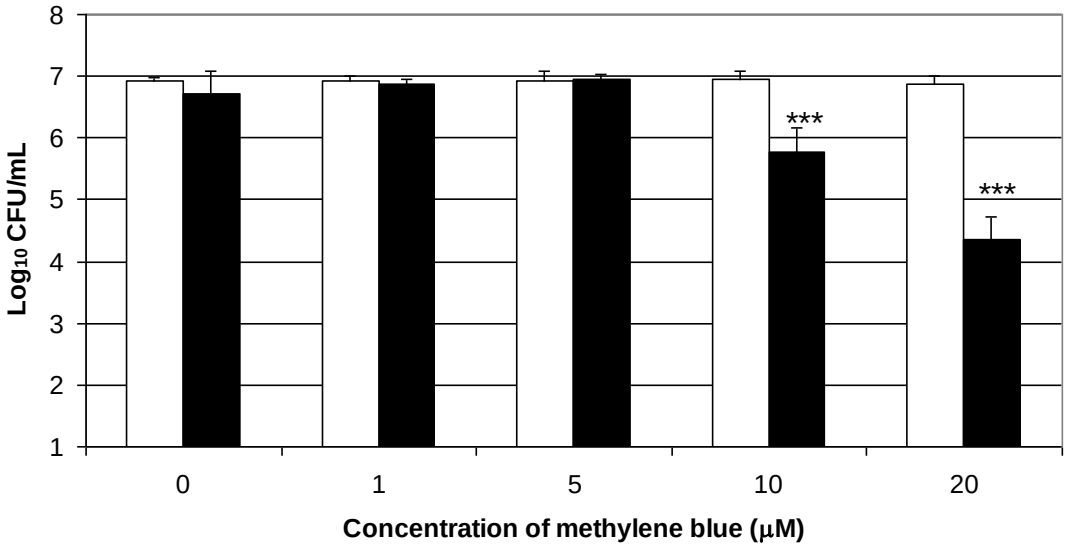

Figure 6.2 The effect of methylene blue dose on the lethal photosensitisation of *S. aureus* LS-1ΔhemB

An equal volume of either PBS or methylene blue (concentrations ranging from 1-20 μM) was added to 50 μL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

6.3.1.1.2 Tin chlorin e6

The effect of tin chlorin e6 dose on the lethal photosensitisation of LS-1 and the *hemB* mutant is shown in Figure 6.3 and Figure 6.4, respectively. As previously observed for methylene blue, highly significant kills were achieved for both LS-1 and *S. aureus* LS-1ΔhemB when irradiated with laser light from a 633 nm laser in the presence of tin chlorin e6; however, a significant difference between the kills achieved using 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light for *S. aureus* LS-1 and LS-1ΔhemB was also observed ($P < 0.05$; ANOVA). However, the small colony variant appeared to be more sensitive to tin chlorin e6 at lower concentrations compared with the parental strain, with significant kills being observed at 1 and 5 µM tin chlorin e6 for the *hemB* small colony variant and not for the parental strain. The difference in kill between the two strains was significant for 1 µM tin chlorin e6, with a 28.67% kill being achieved with the *hemB* SCV compared with 19.39% for the parent strain ($P < 0.05$; ANOVA). Whilst a lower reduction in viable CFU/mL was observed for the *hemB* strain compared with the parent strain for 5 or 10 µM photosensitiser, this difference in susceptibility was not found to be significant ($P > 0.05$; ANOVA).

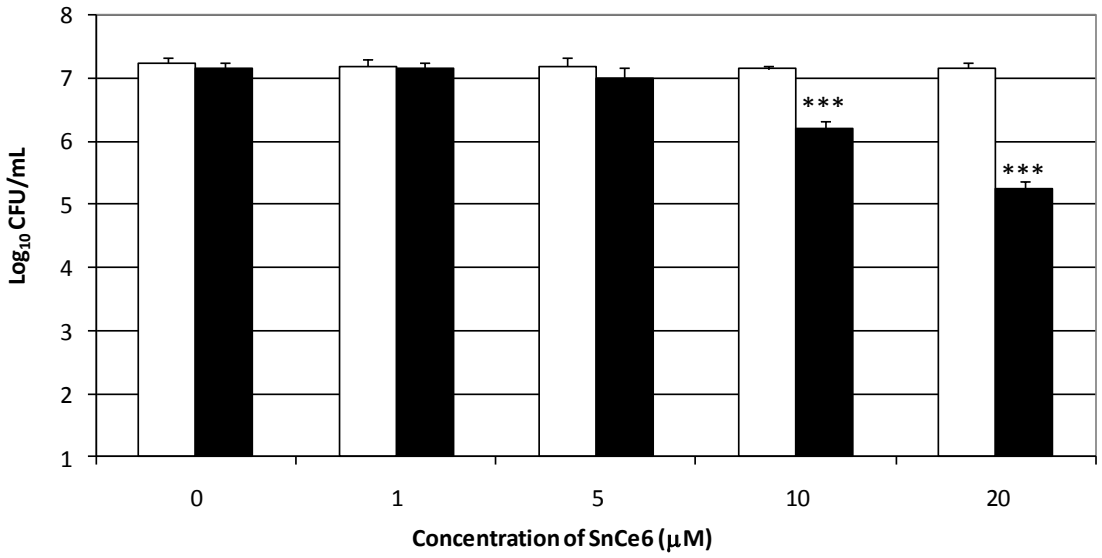

Figure 6.3 The effect of tin chlorin e6 dose on the lethal photosensitisation of *S. aureus* LS-1

An equal volume of either PBS or tin chlorin e6 (concentrations ranging from 1-20 μM) was added to 50 μL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 633 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

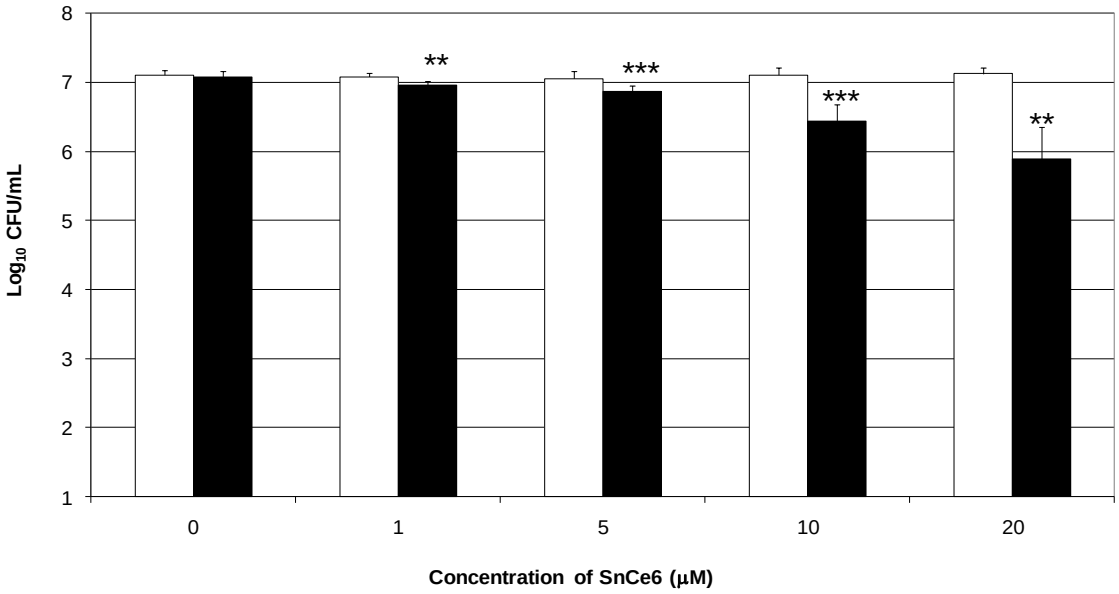

Figure 6.4 The effect of tin chlorin e6 dose on the lethal photosensitisation of *S. aureus* LS-1ΔhemB

An equal volume of either PBS or tin chlorin e6 (concentrations ranging from 1-20 μM) was added to 50 μL of the bacterial suspension and either kept in the dark (□) or exposed to 4.38 J of 633 nm laser light (■). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. ** $P < 0.01$, *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

6.3.1.2 The effect of laser light dose on the lethal photosensitisation of a *S. aureus hemB* small colony variant

6.3.1.2.1 Laser light of 665 nm

Figure 6.5 and Figure 6.6 show the effect of 665 nm laser light dose on the lethal photosensitisation of *S. aureus* LS-1 and *S. aureus* LS-1ΔhemB, in the presence or absence of 20 µM methylene blue, respectively. Although highly significant kills were observed for both strains at all light doses, there was a highly significant difference in susceptibility between the strains for 4.38 J and 8.76 J of 665 nm laser light. There was no significant difference between the kills observed at 21.9 J of laser light in the presence of 20 µM methylene blue; however, a borderline significant kill was observed for the hemB small colony variant when it was treated with 21.9 J of 665 nm laser light in the absence of photosensitiser, which was not observed for the parent strain (P = 0.049; ANOVA).

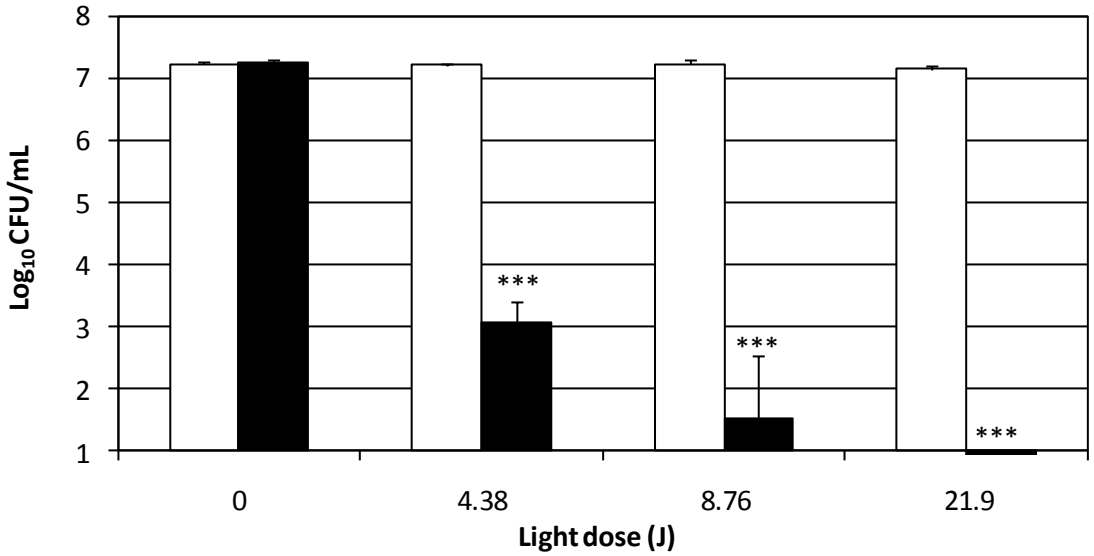

Figure 6.5 The effect of 665 nm laser light dose on the lethal photosensitisation of *S. aureus* LS-1

An equal volume of either PBS (□) or methylene blue (■) (20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 4.38 J of 665 nm laser light. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

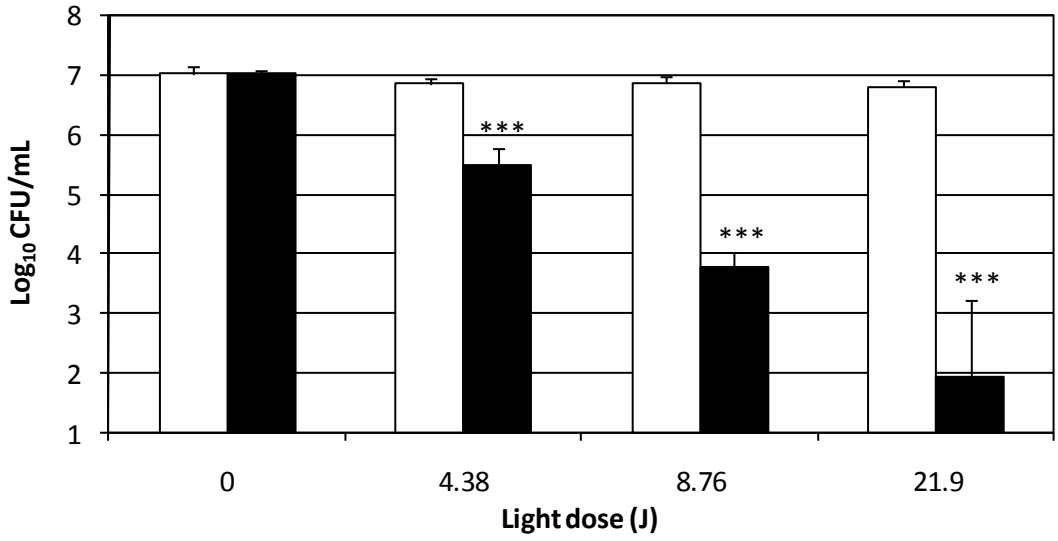

Figure 6.6 The effect of 665 nm laser light dose on the lethal photosensitisation of *S. aureus* LS-1ΔhemB

An equal volume of either PBS (□) or methylene blue (■) (20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 4.38 J of 665 nm laser light. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

6.3.1.2.2 Laser light of 633 nm

The effect of 633 nm laser light dose on the lethal photosensitisation of *S. aureus* LS-1 and the hemB SCV mutant is shown in Figure 6.7 and Figure 6.8. Following irradiation with 4.38 J of 633 nm laser light in the presence of 20 µM tin chlorin e6, a significant difference in the reduction of viable CFU/mL was observed between *S. aureus* LS-1 and *S. aureus* LS-1ΔhemB ($P < 0.05$; ANOVA). No significant difference

was observed between the kills obtained for the parent strain and the *hemB* small colony variant at higher light doses in the presence of photosensitiser. However, a highly significant kill of *S. aureus* LS-1 was observed for 21.9 J of 633 nm laser light alone (no photosensitiser), which was not observed for the *hemB* small colony variant.

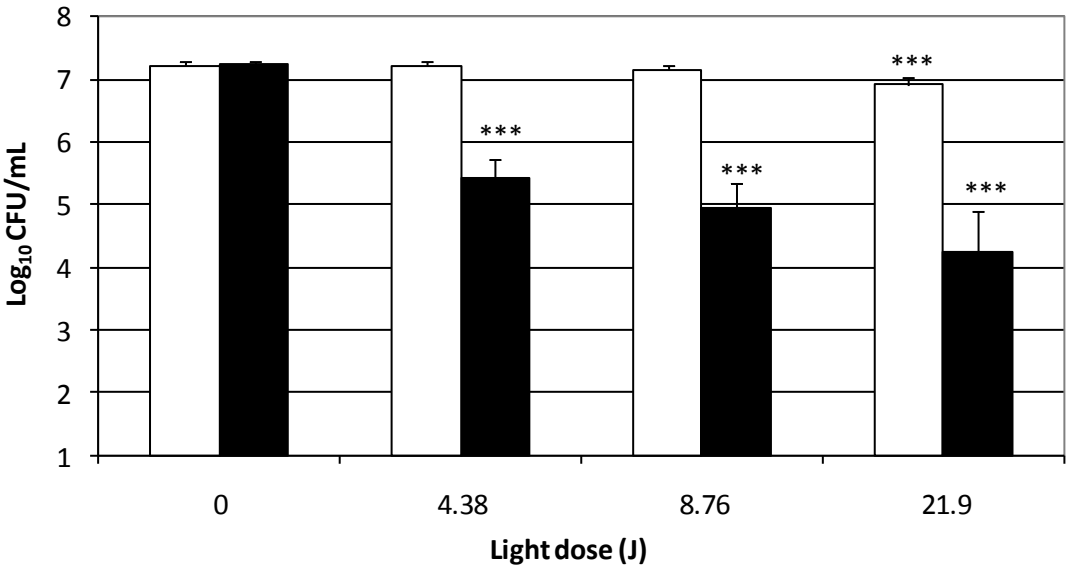

Figure 6.7 The effect of 633 nm laser light dose on the lethal photosensitisation of *S. aureus* LS-1

An equal volume of either PBS (□) or tin chlorin e6 (■) (20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 4.38 J of 633 nm laser light. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

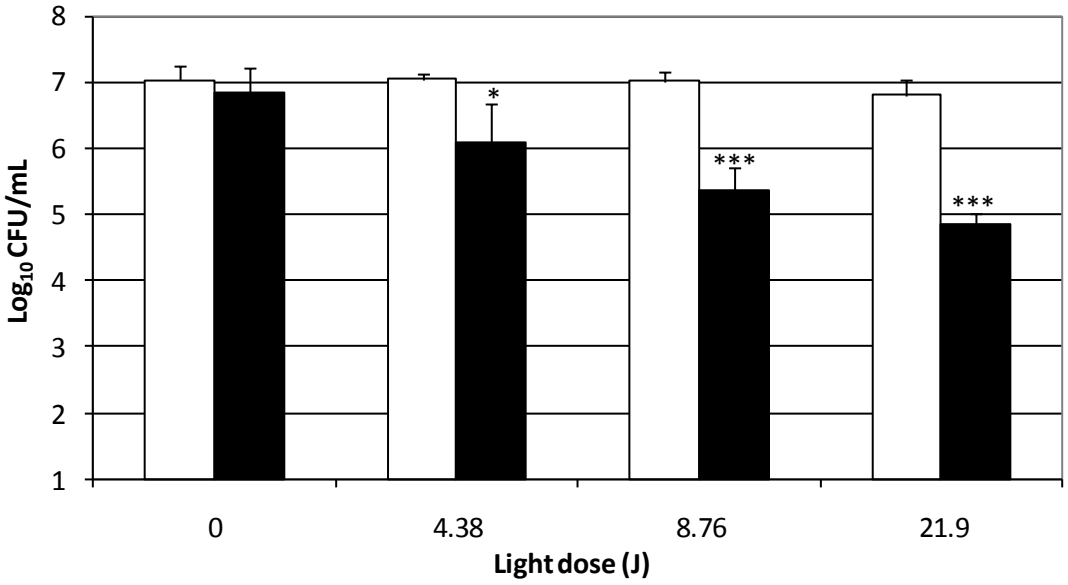

Figure 6.8 The effect of 633 nm laser light dose on the lethal photosensitisation of *S. aureus* LS-1ΔhemB

An equal volume of either PBS (□) or tin chlorin e6 (■) (20 µM) was added to 50 µL of the bacterial suspension and either kept in the dark or exposed to 4.38 J of 633 nm laser light. After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. * $P < 0.05$, *** $P < 0.001$ (ANOVA). Experiments were performed three times in triplicate and the combined data shown.

6.3.2 *Staphylococcus aureus menD* small colony variant

6.3.2.1 The effect of methylene blue and 665 nm laser light on the lethal photosensitisation of a *S. aureus menD* small colony variant

The effect of 20 µM methylene blue and 4.38 J of 665 nm laser light on *S. aureus* 8325-4 and its isogenic *menD* mutant is shown in Figure 6.9. No significant difference was observed between the kills achieved for the parent strain and the *menD* SCV ($P > 0.05$; ANOVA).

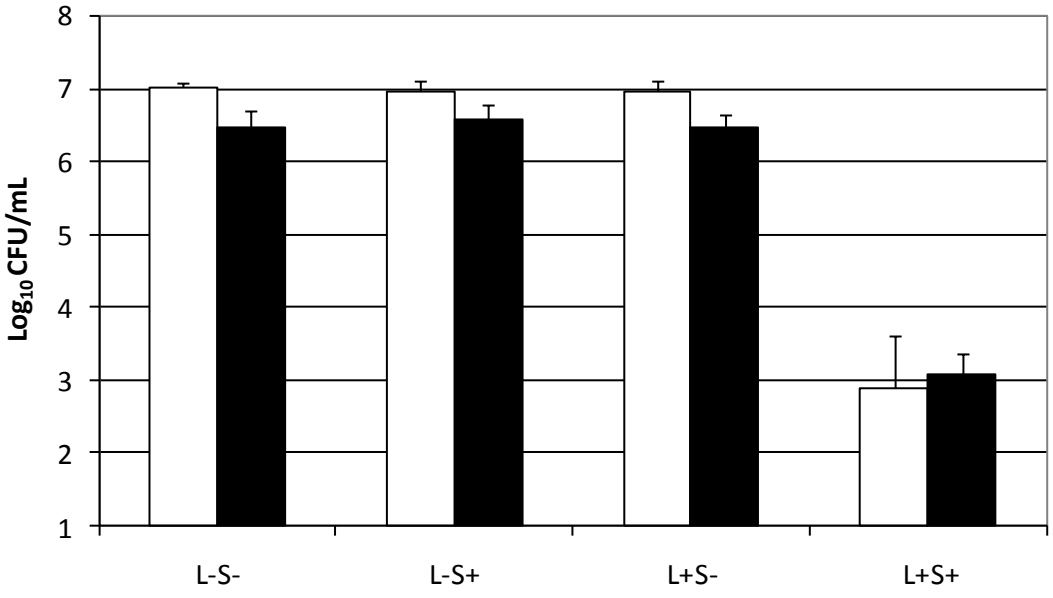

Figure 6.9 The effect of methylene blue and 665 nm laser light on the lethal photosensitisation of *S. aureus* 8325-4 and the *menD* small colony variant

An equal volume of either PBS (S-) or20 µM methylene blue (S+) was added to 50 µL of *S. aureus* 8325-4 (□) or the *menD* SCV (■). The bacterial suspensions were either kept in the dark (L-) or exposed to 4.38 J of 665 nm laser light (L+). After irradiation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. Experiments were performed three times in triplicate and the combined data shown.

6.3.2.2 The effect of tin chlorin e6 and 633 nm laser light on the lethal photosensitisation of a *S. aureus menD* small colony variant

The effect of lethal photosensitisation using 20 µM tin chlorin e6 and 4.38 J of 633 nm laser light is shown in Figure 6.10. As observed for methylene blue, there was no significant difference in the susceptibility of the parental strain or the *menD* small colony variant to photodynamic killing ($P > 0.05$; ANOVA).

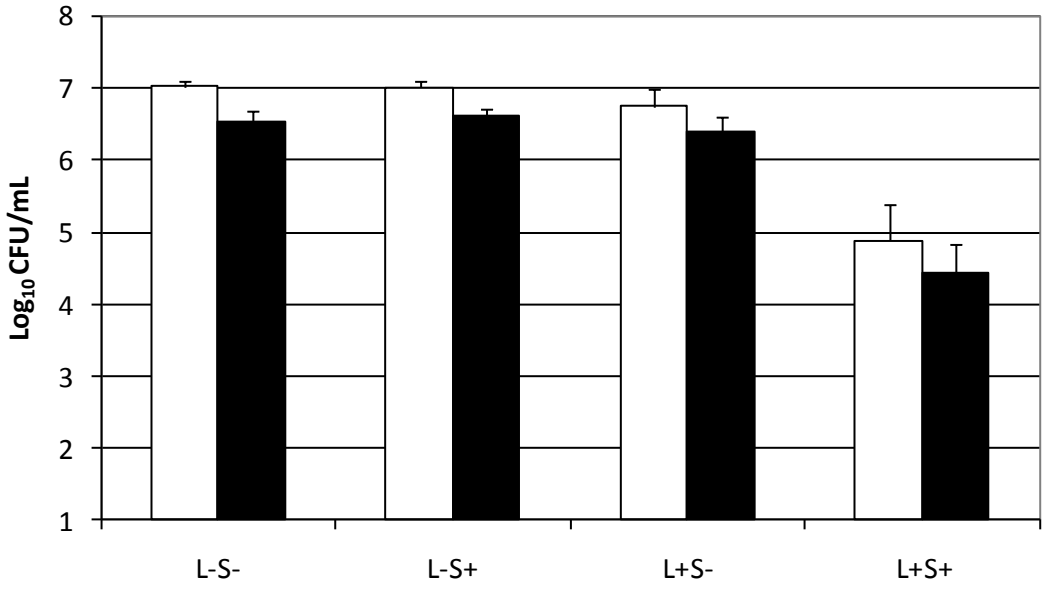

Figure 6.10 The effect of tin chlorin e6 and 633 nm laser light on the lethal photosensitisation of *S. aureus* 8325-4 and *S. aureus menD* small colony variant

An equal volume of either PBS (S-) or20 µM tin chlorin e6 (S+) was added to 50 µL of a suspension of either *S. aureus* 8325-4 (□) or the *menD* SCV (■). The bacterial suspensions were either kept in the dark (S-) or exposed to 4.38 J of 665 nm laser light (S+). After irradiation/dark incubation, samples were serially diluted and the surviving CFU/mL enumerated. Error bars represent the standard deviation from the mean. Experiments were performed three times in triplicate and the combined data are shown.

6.4 Discussion

Small colony variants of *S. aureus* may present a challenge for both clinicians and the diagnostic laboratory alike (Proctor & Peters, 1998). Due to the increased resistance to antibiotics typical of these strains, it would be advantageous to develop a therapeutic strategy with a differing mode of action to those antibiotics for which lower susceptibility is typically seen (Proctor *et al.*, 1998). As the mechanism of action of PDT is non-specific, it may therefore represent a novel treatment option for superficial infections caused by *S. aureus* SCVs (Wainwright, 2005). In this chapter, the ability of light-activated antimicrobial agents in combination with laser light to induce the photodynamic killing of two strains of *S. aureus* SCVs was assessed

In this study, both the hemin and menadione auxotrophic small colony variants of *S. aureus* were found to be susceptible to photodynamic killing with methylene blue and tin chlorin e6. The *S. aureus hemB* small colony variant was found to be less susceptible than its parent strain (*S. aureus* LS-1) to lethal photosensitisation using methylene blue and tin chlorin e6; however, highly significant kills were achieved using both photosensitisers. Significantly lower kills were observed for the *hemB* SCV following treatment with 20 µM methylene blue and 4.38 J of 665 nm diode laser light; kills of 99.98 and 99.73% were achieved for the parent strain and *hemB* SCV, respectively. With the highest light dose used (21.9 J), there was no significant difference between the kills observed for both strains, indicating that the decreased susceptibility of the SCV strain may be overcome by increasing the light dose.

For tin chlorin e6, treatment with 20 µM photosensitiser and 4.38 J of 633 nm HeNe laser light also resulted in significantly less killing of the *hemB* SCV strain compared with the parent strain. Kills of 98.98 and 94.01% were observed for the parent strain

and SCV, respectively. As observed with 665 nm laser light, at higher doses of 633 nm laser light there was no significant difference between the parent and the *hemB* SCV in their susceptibility to photodynamic killing. No significant difference between the viability of *S. aureus* LS-1 and the *hemB* SCV was observed following treatment with 8.76 J of 633 nm laser light and 20 μM tin chlorin e6, whereas the *hemB* SCV was significantly less susceptible than the parent strain to 8.76 J of 665 nm laser light and 20 μM methylene blue.

Iradiation with 21.9 J of 633 nm laser light in the absence of a photosensitiser caused significant killing of *S. aureus* LS-1, but not of the *hemB* small colony variant ($P < 0.001$; ANOVA). This may be due to the presence of endogenous photosensitisers in the parent strain that are not produced or produced at lower concentrations in the *hemB* SCV. *S. aureus* is known to produce several light-absorbing compounds, such as the carotenoid compound staphyloxanthin, that gives *S. aureus* colonies their distinctive golden colour (Pelz et al., 2005). A reduction in pigmentation is a well documented phenotype of *S. aureus* SCVs (Proctor, 2000); therefore this decrease may account for the different susceptibilities to laser light alone observed between the parent and SCV strains.

The *menD* small colony variant was found to be equally susceptible to photodynamic killing using methylene blue and tin chlorin e6 as its parental strain (*S. aureus* 8325-4). Treatment with 20 μM methylene blue and 4.38 J of 665 nm laser light resulted in kills of 99.99 and 99.96% for the parent strain and small colony variant, respectively. Following treatment with tin chlorin e6 and 633 nm laser light, kills of 99.28 and 99.20% were achieved for the parent strain and small colony variant, respectively.

Although primarily associated with exposure to aminoglycosides, *in vitro* exposure to triclosan, a commonly-used biocide, has also been shown to result in the

formation of SCVs, raising issues over the use of triclosan as a topical skin decolonisation agent (Proctor, 2000). It has been proposed that use of triclosan for this purpose could lead to a selective pressure in favour of small colony variants on the skin surface, which could potentially be transferred between patients, and also come into contact with wounds and thus initiate infection (Seaman *et al.*, 2007b). Triclosan-impregnated polymers such as sutures may also provide a selective pressure for small colony variants, as well as an entrance route into the body where infection may be initiated (Seaman *et al.*, 2007a). Bayston *et al*. found that triclosan-containing silicone could induce the formation of small colony variants of meticillin-resistant *S. aureus*, and warned of implications for the long-term use of triclosan-containing polymers (Bayston *et al.*, 2007). Given the findings reported in this chapter, PDT could potentially be used as an alternative method of skin decontamination in cases of SCV carriage.

PDT has previously been proposed for the decontamination of the anterior nares in cases of MRSA carriage (Embleton *et al.*, 2002; Street *et al.*, 2009; Zolfaghari *et al.*, 2009); and cases of infections associated with concomitant colonisation of the anterior nares by *S. aureus* SCVs have been reported in the literature (Hale, 1951; Proctor *et al.*, 1995; von Eiff *et al.*, 2001). Decontamination of the nares may therefore be useful in such cases where the nares represent a reservoir of infection, and PDT may well be applicable in such cases.

PDT could also be used to treat other skin areas and wounds infected with *S. aureus* SCVs, as SCVs have been implicated in numerous cases of skin and skin structure infections (Abele-Horn *et al.*, 2000; Coman *et al.*, 2008; Gomez-Gonzalez *et al.*, 2010; Rahman, 1977; Sherris, 1952; von Eiff *et al.*, 2001). The potential of PDT as a treatment modality for skin and skin structure infections is well recognised, and therefore could present an attractive alternative to antibiotics in skin infections caused by SCVs (Wainwright, 2010; Zeina *et al.*, 2001).

The optimum treatment regime for infections caused by small colony variants of *S. aureus* has not yet been determined (von Eiff, 2008). Due the increased resistance of SCV strains to some conventional antibiotics, a therapy that has a non-specific mode of action rather than one that is dependent on growth of the organism would be desirable. As PDT is generally believed to exert its killing effect via non-specific membrane damage and thus resistance is unlikely to develop, PDT could represent a novel method of treating superficial infections (Hamblin & Hasan, 2004; Wainwright, 2005). Consequently, PDT may be a potential treatment option for superficial infections caused by SCV strains of *S. aureus*, particularly in cases of antibiotic-resistant strains.

Meticillin-resistant SCVs of *S. aureus* have been observed and implicated in diverse types of infections (Bulger & Bulger, 1967; Coman *et al.*, 2008; Gomez-Gonzalez *et al.*, 2010; Seifert *et al.*, 1999). It would be useful to assess the efficacy of photodynamic killing against meticillin-resistant SCVs, as PDT could be applicable in the eradication of these organisms. It has been shown in Chapter 3 that both photosensitisers used in this chapter are effective against MRSA; therefore it is possible that PDT could also be effective against meticillin-resistant SCV strains.

6.5 Summary

Small colony variants of *S. aureus* are susceptible to photodynamic killing using the photosensitisers methylene blue and tin chlorin e6, in combination with laser light of the appropriate wavelength. Although the SCVs were less susceptible than their parent strains, highly significant kills were achieved using both methylene blue and tin chlorin e6, suggesting that PDT may be of use in the treatment of superficial infections caused by these organisms, especially considering their lower susceptibility to some antibiotics. Increasing the light dose delivered by the 665 nm and 633 nm lasers eliminated the difference in susceptibility between the SCVs and parent strains, demonstrating that it is possible to overcome the decreased sensitivity of the SCVs to photodynamic killing. In addition, PDT may also have a role in the decolonisation of the anterior nares, which have been found to be a reservoir of infection in cases of recurrent skin infections caused by SCVs (von Eiff *et al.*, 2001).

7 Inactivation of key virulence factors of *Porphyromonas gingivalis* using light-activated antimicrobial agents

7.1 Introduction

Porphyromonas gingivalis is one of the major aetiological agents of periodontitis, an inflammatory disease of the periodontium that involves progressive bone, and eventually tooth, loss (Hajishengallis, 2009). *P. gingivalis* produces a number of virulence factors that are believed to play significant roles in the pathology of periodontitis, and may therefore represent targets for antimicrobial therapy (Yilmaz, 2008).

Lipopolysaccharide is considered a key virulence factor of periodontopathogens due to its ability to initiate the host inflammatory response (Wilson, 2004). LPS-mediated induction of cytokine production has been implicated in bone resorption, inhibition of collagen synthesis and the induction of destructive host metalloproteinases, which contribute to bone loss and inhibition of tissue repair (Lamont & Jenkinson, 1998). *P. gingivalis* LPS is released in large quantities in outer membrane vesicles, which are able to penetrate periodontal tissue and elicit an immune response (Darveau *et al.*, 2004); consequently LPS can continue to exert its destructive effects after antibiotic therapy has successfully eradicated the microorganism (Wilson, 2004).

The synthesis of large amounts of proteolytic enzymes is characteristic of *P. gingivalis*, and is one of the features that distinguishes it from other members of the genus *Porphyromonas*; consequently, the proteolytic activity of *P. gingivalis* has been identified as a potential target for novel therapeutic strategies (Cutler *et al.*, 1995). *P. gingivalis* produces a number of multifunctional enzymes with proteolytic functions, which are involved in host colonisation, microbial nutrition, host tissue

destruction and resistance to host defences (Potempa *et al.*, 2000; Travis & Potempa, 2000).

The haemagglutinins of *P. gingivalis* have been presented as putative virulence factors due to their role in hemin acquisition and colonisation of host tissues (Holt *et al.*, 1999). Haemagglutinating proteins are also thought have immunostimulatory properties, and thus contribute to the pathology of periodontal disease via their induction of proinflammatory cytokine production (Zhang *et al.*, 2005). The importance of *P. gingivalis* haemagglutinin in the pathology of bone loss has been demonstrated by the observation of lower bone loss in rats immunised with recombinant haemagglutinin B (Katz *et al.*, 1999).

PDT has several applications as a therapeutic option in periodontology, including disinfection of roots and the management and treatment of periodontitis (Jose *et al.*, 2010). As PDT systems for periodontitis are commercially available, the effect of photosensitisation on virulence factors produced by the causative microorganisms of this disease represents an important area of interest. The photosensitisation of selected *P. gingivalis* virulence factors with the light-activated antimicrobial agents methylene blue and tin chlorin e6 in the presence of laser light of 665 nm and 633 nm respectively is described in the following chapter. The effect of concentrations of photosensitiser ranging from 1 to 20 μM and laser light of the appropriate wavelength on a selection of secreted enzymes and surface proteins was investigated. In addition, preliminary investigations into the mechanism of photodynamic inactivation were performed.

7.2 Materials and methods

7.2.1 Limulus Amebocyte Lysate assay for lipopolysaccharide activity

Photosensitisation experiments were performed using 50 µl of 20 ng/mL *P. gingivalis* 1690 LPS (a gift from Professor Richard Darveau) for a final concentration of 10 ng/mL and either an equal volume of photosensitiser (S+) or pyrogen-free water (PFW) (S-). For methylene blue experiments, a final concentration of 67 µM was used and for tin chlorin e6, a final concentration of 59 µM was used. Samples were either exposed to 21.9 J of 665 nm laser light for methylene blue-treated samples, or 633 nm laser light for tin chlorin e6-treated samples. Experiments were performed in triplicate. For tin chlorin e6, only preliminary experiments were performed to determine if photosensitisation had any effect on the activity of LPS and therefore only L-S- and L+S+ samples were processed.

Following irradiation/dark incubation, the Limulus Amebocyte Lysate (LAL) test was performed as follows. An aqueous extract of amebocytes from *Limulus polyphemus* (Pyrotell® reagent; Associates of Cape Cod Inc) was reconstituted with PFW. Control Standard Endotoxin (CSE; Associates of Cape Cod Inc) was used as a positive control for the LAL assay. CSE was reconstituted with 1 mL PFW to give a concentration of 10 ng/mL and placed in a sonicating water bath for 15 minutes. After sonication, the CSE stock solution was vortexed for 30 seconds before making doubling dilutions ranging from 10 ng/mL to 0.156 ng/mL. The test solution of *P. gingivalis* LPS was diluted in a similar manner. Aliquots of 10 µL Pyrotell were spotted onto the lid of a pyrogen-free 96-well plate and 10 µL of each dilution of the CSE and *P. gingivalis* LPS were then added to the Pyrotell, starting from the most dilute. The lid was covered with another 96-well plate lid, placed in a moisture chamber and incubated at 37°C for 60 minutes. Following incubation, 1 µL of 0.2% methylene blue in 70% ethanol was added to the top of each spot. A star-like formation by the methylene blue on top of the spot indicated a positive result. The assay endpoint was defined as the

lowest concentration of endotoxin required for a positive result, and the fold change in the concentration of *P. gingivalis* LPS required to cause a positive result compared with the negative control (L-S-) was calculated.

7.2.2 Azocasein assay for protease activity

P. gingivalis W50 culture supernatant was prepared according to section 2.4. Photosensitisation experiments and the azocasein hydrolysis assay for measuring total *P. gingivalis* W50 protease activity were performed as described in sections 2.5 and 2.7.2, respectively. For photosensitiser-dose experiments, 21.9 J of laser light was used to irradiate the samples.

7.2.3 The effect of deuterium oxide and L-tryptophan on the photodynamic inactivation of *P. gingivalis* W50 proteolytic activity

Experiments to assess the effect of an enhancer of singlet oxygen lifetime (deuterium oxide) and singlet oxygen scavenger (L-tryptophan) on the proteolytic activity of *P. gingivalis* W50 were carried out according to section 2.8.3.

7.2.4 Haemagglutination assay

Photosensitisation experiments were performed according to section 2.5, using 50 µL of *P. gingivalis* W50 culture supernatant (prepared according to section 2.4) and an equal volume of photosensitiser. Following irradiation/dark incubation, the haemagglutinating activity was assessed using the haemagglutination assay as described below.

Rabbit blood (E&O laboratories) was centrifuged for 10 minutes at 5590 x *g*. Cells were washed with PBS and resuspended to a concentration of 2%. Following irradiation/dark incubation, samples were removed and placed into column 1 of a v-

bottomed 96-well plate and diluted using doubling dilutions in PBS so that the final volume in each well was 50 µL. PBS was used as a negative control for haemagglutinating activity. Aliquots of 50 µL of the rabbit erythrocyte solution were added to each well and the plates incubated at 37°C for 10 minutes with gentle shaking, in the dark. Plates were then incubated overnight at room temperature in the dark. Following overnight incubation, the haemagglutinating titre was defined as the highest dilution giving rise to haemagglutination. Haemagglutination appeared as a pink carpet of cells on the bottom of the plate. A negative result (no haemagglutination) appeared as a pellet of cells in the bottom of the well.

7.3 Results

7.3.1 The effect of photosensitisation on the activity of *P. gingivalis* lipopolysaccharide

The effect of treatment of *P. gingivalis* 1690 LPS with methylene blue or tin chlorin e6, irradiated with laser light of the appropriate wavelength, is shown in Table 7.1. Irradiation of *P. gingivalis* LPS with diode laser light of 665 nm in the presence of 67 µM methylene blue significantly inhibited the LPS activity by 4-fold compared with the untreated control ($P < 0.001$; ANOVA). Treatment of *P. gingivalis* 1690 LPS with 59 µM tin chlorin e6 and 21.9 J of 633 nm HeNe laser light had no effect on the concentration of endotoxin required for a positive result compared with the control.

	Median fold decrease	
	Methylene blue	**Tin chlorin e6**
L-S+	0	Not tested
L+S-	0	Not tested
L+S+	4 ***	0

Table 7.1 The effect of methylene blue or tin chlorin e6 and 21.9 J of laser light on the activity of *P. gingivalis* 1690 LPS

An equal volume of either methylene blue (final concentration of 67 µM), tin chlorin e6 (final concentration of 59 µM) (S+) or PFW (S-) was added to *P. gingivalis* 1690 LPS and samples were either exposed to 21.9 J of 665 nm laser light (for methylene blue-treated samples) or 633 nm laser light (for tin chlorin e6-treated samples) (L+) or kept in the dark (L-). The LPS activity was assessed using the Pyrotell® LAL assay. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

Experiments performed using the standard doses of the methylene blue and tin chlorin e6 described in previous chapters (maximum final concentration of 20 μM) demonstrated that these concentrations of photosensitiser did not affect the activity of the LPS as assessed by the Pyrotell® LAL assay (data not shown).

7.3.2 The effect of photosensitiser dose on the photodynamic inactivation of *P. gingivalis* proteases

7.3.2.1 Methylene blue

The effect of photosensitisation with methylene blue and laser light of 665 nm on the protease activity of *P. gingivalis* W50 culture supernatant is shown in Figure 7.1. It can be seen that when irradiated with 21.9 J laser light of 665 nm, all concentrations of methylene blue tested (final concentrations of 1 to 20 μM) caused a highly significant reduction in the proteolytic activity compared with untreated samples ($P < 0.001$; ANOVA). The highest reduction in activity observed was 65% compared with the untreated control.

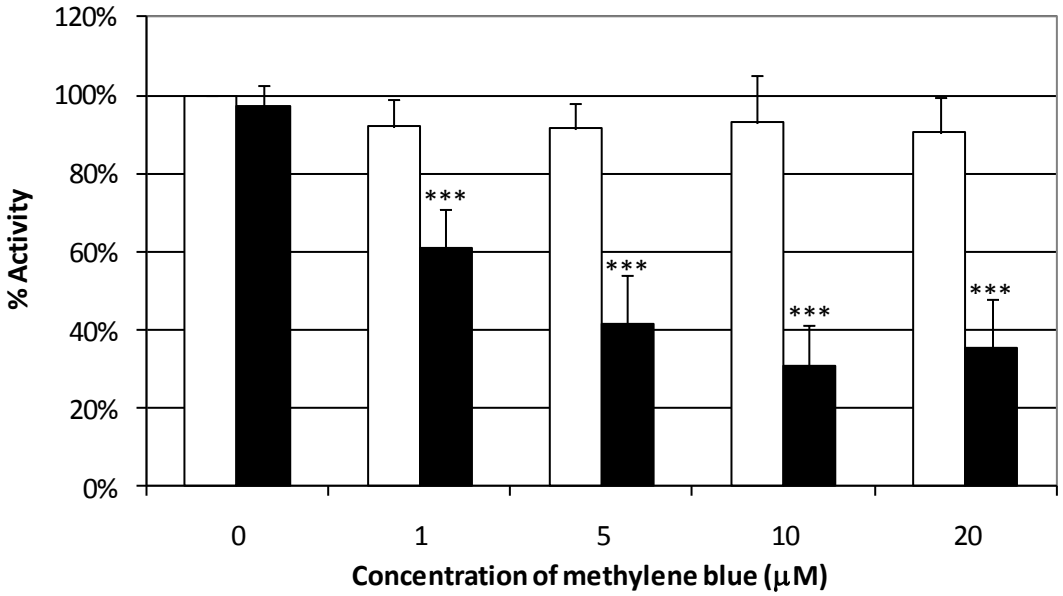

Figure 7.1 The effect of methylene blue dose and 21.9 J of 665 nm laser light on the proteolytic activity of *P. gingivalis* W50 supernatants.

An equal volume of *P. gingivalis* W50 culture supernatant was added to either 300 μL PBS or 20 μM methylene blue and either kept in the dark (□) or exposed to 4.38 J of 665 nm laser light (■). Following irradiation, the protease activity was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

7.3.2.2 Tin chlorin e6

The effect of photosensitisation with tin chlorin e6 and 633 nm laser light on the protease activity of *P. gingivalis* W50 culture supernatant is shown in Figure 7.2. Irradiation of the culture supernatant in the presence of all concentrations of tin chlorin e6 (1 to 20 μM) resulted in a highly significant reduction in the proteolytic activity compared with the untreated control. The maximum inhibition of activity observed using these conditions was 88%; this was considerably higher than the

inactivation seen using the methylene blue and the 665 nm laser light regime. The 633 nm laser light alone also appeared to have an inhibitory effect on the proteolytic activity of the culture supernatant, although this was not consistently observed.

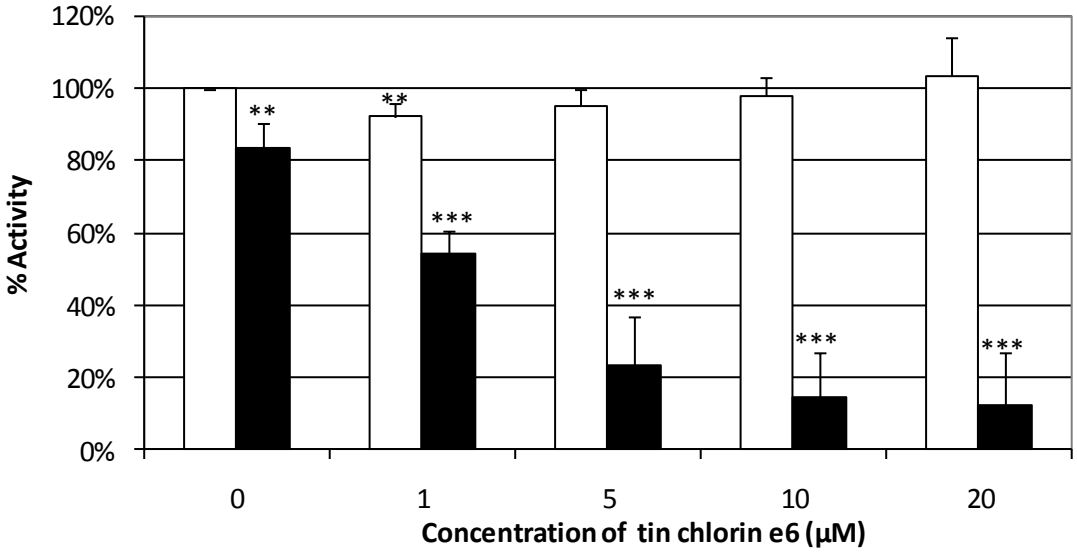

Figure 7.2 The effect of tin chlorin e6 dose and 21.9 J of 633 nm laser light on the proteolytic activity of *P. gingivalis* W50 supernatant.

An equal volume of *P. gingivalis* W50 culture supernatant was added to either 300 μL PBS or 20 μM tin chlorin e6 and either kept in the dark (□) or exposed to 4.38 J of 633 nm laser light (■). Following irradiation, the protease activity was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. ** P <0.01, *** P < 0.001 (ANOVA). Experiments were performed three times and the combined data are shown.

7.3.3 The effect of laser light dose on the photodynamic inactivation of *P. gingivalis* proteases

7.3.3.1 Laser light of 665 nm

The effect of 665 nm laser light dose on the proteolytic activity of *P. gingivalis* W50 culture supernatant is shown in Figure 7.3. A significant reduction in proteolytic activity was observed for all light doses tested when the culture supernatant was irradiated in the presence of 20 μM methylene blue, with highly significant reductions observed for light doses ≥ 8.76 J ($P < 0.001$; ANOVA). Irradiation with 21.9 J of 665 nm laser light in the presence of 20 μM methylene blue resulted in a 66% reduction in the proteolytic activity compared with the untreated control.

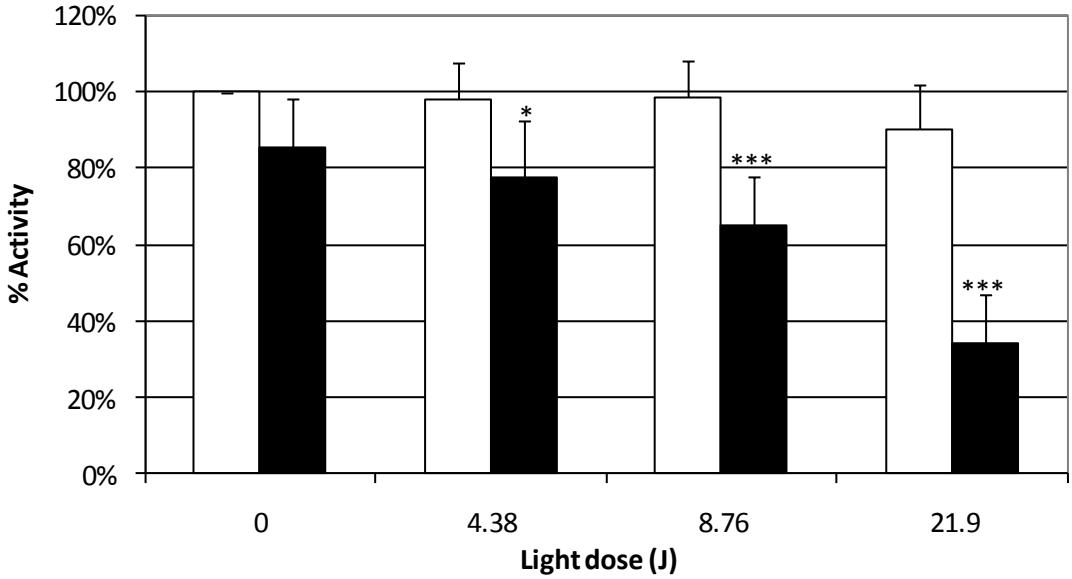

Figure 7.3 The effect of 20 µM methylene blue and 665 nm laser light dose on the proteolytic activity of *P. gingivalis* W50 culture supernatant.

An equal volume of *P. gingivalis* W50 culture supernatant was added to either 300 µL PBS (□) or 20 µM methylene blue (■) and either kept in the dark or exposed to 665 nm laser light. Following irradiation, the protease activity was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. * $P < 0.05$, *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

7.3.3.2 Laser light of 633 nm

Figure 7.4 shows the effect of 633 nm laser light dose on the proteolytic activity of the culture supernatant. A highly significant reduction in proteolytic activity was observed following treatment of the culture supernatant with 20 µM tin chlorin e6 and all light doses tested ($P < 0.001$; ANOVA). Irradiation with 21.9 J of 633 nm laser light in the presence of 20 µM tin chlorin e6 resulted in a 74% reduction in the

proteolytic activity, compared with the 66% reduction in activity observed using 20 µM methylene blue and 665 nm laser light. In addition, a highly significant inactivation of proteolytic activity was observed following treatment with 20 µM tin chlorin e6 and 4.38 J of laser light, which was not achieved using the same concentration of methylene blue.

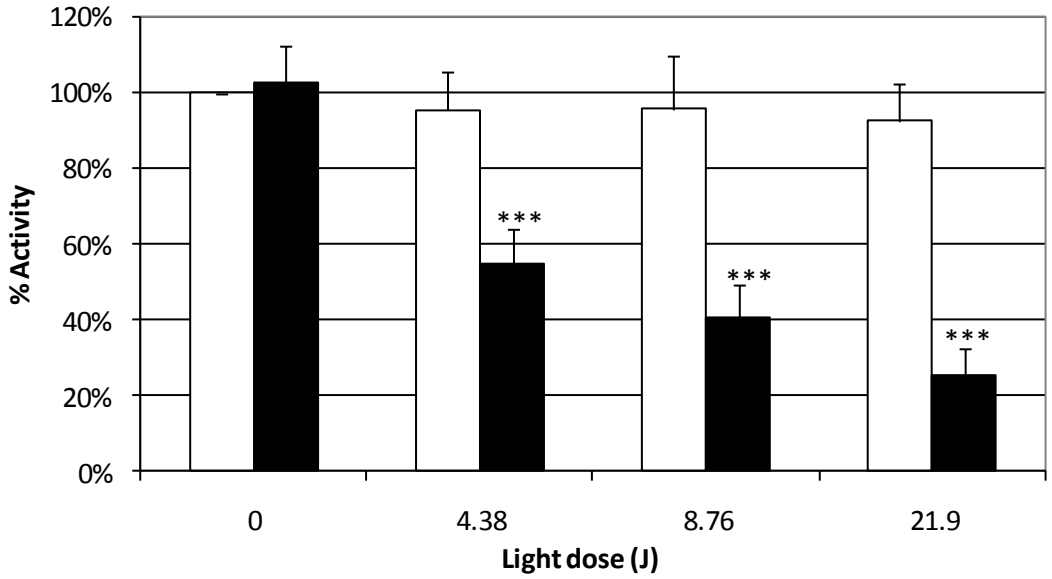

Figure 7.4 The effect of 633 nm laser light dose on the proteolytic activity of *P. gingivalis* W50 culture supernatant.

An equal volume of *P. gingivalis* W50 culture supernatant was added to either 300 µL PBS (□) or 20 µM tin chlorin e6 (■) and either kept in the dark or exposed to 633 nm laser light. Following irradiation, the protease activity was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. *** $P < 0.001$ (ANOVA). Experiments were performed three times and the combined data are shown.

7.3.4 The effect of a singlet oxygen enhancer and scavenger on the photodynamic inactivation of *P. gingivalis* proteases

Figure 7.5 shows the effect of the singlet oxygen lifetime enhancer D_2O and the singlet oxygen scavenger L-tryptophan on the photodynamic inactivation of *P. gingivalis* W50 proteases using 5 μM methylene blue and 21.9 J of 665 nm laser light. Whilst the addition of D_2O resulted in a small reduction in proteolytic activity after treatment with methylene blue and laser light of 665 nm, it did not significantly enhance the inactivation of proteolytic activity ($P > 0.05$; ANOVA). In contrast, L-tryptophan significantly reduced the effectiveness of photodynamic inactivation by methylene blue and laser light of 665 nm ($P < 0.01$; ANOVA).

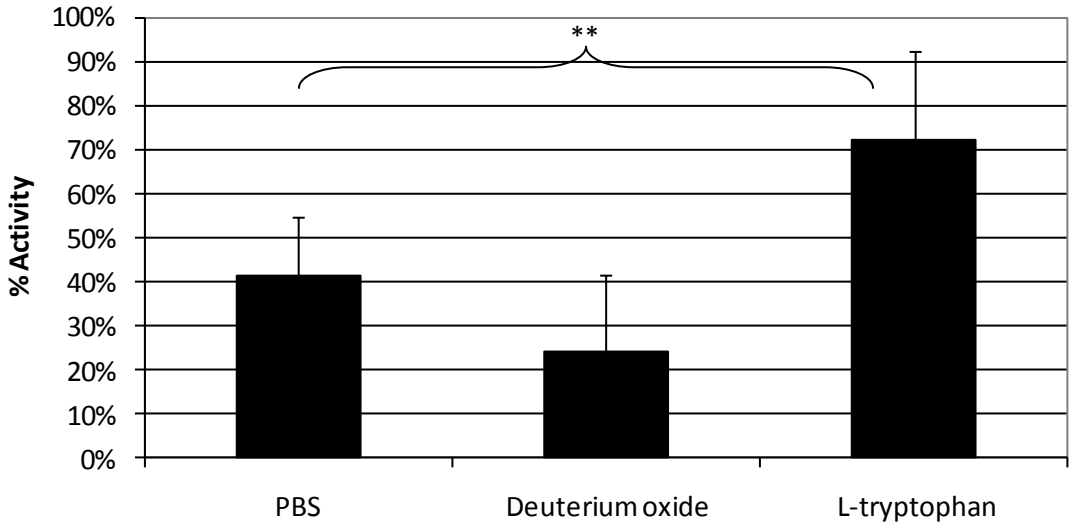

Figure 7.5 The effect of a singlet oxygen enhancer and scavenger on the photodynamic inactivation of *P. gingivalis* W50 proteases.

P. gingivalis W50 culture supernatant was irradiated with 21.9 J of 665 nm laser light in the presence of 5 μM methylene blue dissolved in PBS, D_2O or 10 mM L-tryptophan. Following irradiation, protease activity was assessed using the azocasein hydrolysis assay. Error bars represent the standard deviation from the mean. ** $P < 0.01$ (ANOVA). Experiments were performed three times and the combined data are shown.

7.3.5 The effect of photosensitiser dose on the photodynamic inactivation of *P. gingivalis* haemagglutinin

7.3.5.1 Methylene blue

Table 7.2 shows the effect of methylene blue dose on the haemagglutinating activity of *P. gingivalis* W50 culture supernatant. When irradiated with 21.9 J of 665 nm laser light, a methylene blue dose-dependent decrease in haemagglutinating activity was observed. Significant reductions in activity were observed following

photosensitisation using 10 and 20 µM methylene blue, which resulted in median 6- and 8-fold reductions in the haemagglutinating titre of the supernatant compared with the untreated control, respectively ($P < 0.001$; ANOVA). Neither laser light nor methylene blue alone had an effect on the activity of the supernatant.

Photosensitiser	**Median haemagglutinating titre**	
concentration (µM)	**L-**	**L+**
0	1/1024	1/1024
1	1/1024	1/512
5	1/1024	1/256
10	1/1024	1/128
20	1/1024	1/64

Table 7.2 The effect of methylene blue dose on the activity of *P. gingivalis* W50 haemagglutinin activity.

An equal volume of either PBS or 20 µM methylene blue was added to 50 µL *P. gingivalis* W50 culture supernatant and samples were either exposed to 21.9 J 665 nm laser light (L+) or kept in the dark (L-). Following irradiation/dark incubation, the haemagglutinating activity was assessed using the haemagglutination assay. The haemagglutinating titre was calculated as the reciprocal of the lowest concentration of culture supernatant causing haemagglutination. Experiments were performed three times and the combined data are shown.

7.3.5.2 Tin chlorin e6

The effect of tin chlorin e6 dose on the haemagglutinating activity of *P. gingivalis* W50 culture supernatant is shown in Table 7.3. Tin chlorin e6 had a dose-dependent inhibitory effect on haemagglutinating activity when irradiated with 21.9 J of 633 nm laser light; all doses of tin chlorin e6 resulted in a highly significant reduction in activity compared with the control ($P < 0.001$; ANOVA). Photosensitisation using 20

μM tin chlorin e6 and 21.9 J of 633 nm laser light was significantly more efficacious than the equivalent doses of methylene blue and 665 nm laser light, which resulted in 14- and 8-fold reductions in activity, respectively ($P < 0.001$; Mann-Whitney *U* test). There was no effect of photosensitiser or laser light alone on the haemagglutinating activity of the culture supernatant.

Photosensitiser	**Median haemagglutinating titre**	
concentration (μM)	**L-**	**L+**
0	1/512	1/512
1	1/512	1/128
5	1/512	1/16
10	1/512	1/8
20	1/1024	1/4

Table 7.3 The effect of tin chlorin e6 dose on the activity of *P. gingivalis* W50 haemagglutinin activity.

An equal volume of either PBS or 20 μM tin chlorin e6 was added to 50 μL *P. gingivalis* W50 culture supernatant and samples were either exposed to 21.9 J of 633 nm laser light (L+) or kept in the dark (L-). Following irradiation/dark incubation, the haemagglutinating activity was assessed using the haemagglutination assay. The haemagglutinating titre was calculated as the reciprocal of the lowest concentration of culture supernatant causing haemagglutination. Experiments were performed three times and the combined data are shown.

7.3.6 The effect of laser light dose on the photodynamic inactivation of *P. gingivalis* haemagglutinin

7.3.6.1 Laser light of 665 nm laser light

The effect of 665 nm laser light dose on the haemagglutinating activity of *P. gingivalis* W50 culture supernatant is shown in Table 7.4. Irradiation of culture supernatant in the presence of 20 μM methylene blue resulted in a light dose-dependent inactivation of activity, with a 10-fold reduction in activity observed with the highest light dose tested (21.9 J). All laser light doses tested resulted in a highly significant reduction in haemagglutininating titre compared with the untreated control when the culture supernatant was irradiated in the presence of methylene blue ($P \leq 0.001$; ANOVA). A laser light dose of 21.9 J also had a small inhibitory effect in the absence of photosensitiser, causing a median 2-fold reduction in activity; however, this was not significant ($P > 0.05$; ANOVA). There was no inhibitory effect of methylene blue in the absence of laser light.

Light dose (J)	Median haemagglutinating titre	
	S-	S+
0	1/256	1/256
4.38	1/256	1/128
8.76	1/256	1/64
21.9	1/128	1/8

Table 7.4 The effect of 665 nm laser light dose on the activity of *P. gingivalis* W50 haemagglutinin activity.

An equal volume of either PBS (S-) or 20 µM methylene blue (S+) was added to 50 µL *P. gingivalis* W50 culture supernatant and samples were either exposed to 4.38, 8.76 or 21.9 J of 665 nm laser light or kept in the dark. Following irradiation/dark incubation, the haemagglutinating activity was assessed using the haemagglutination assay. The haemagglutinating titre was calculated as the reciprocal of the lowest concentration of culture supernatant causing haemagglutination. Experiments were performed three times and the combined data are shown.

7.3.6.2 Laser light of 633 nm

Table 7.5 shows that treatment of *P. gingivalis* W50 culture supernatant with 20 µM tin chlorin e6 and 633 nm laser light resulted in a significant, laser light-dose-dependent reduction in haemagglutinating activity ($P < 0.001$; ANOVA). Photosensitisation with 21.9 J 633 nm laser light and 20 µM tin chlorin e6 resulted in a significantly greater reduction in activity (14-fold) than that observed for equivalent doses of methylene blue and 633 nm laser light (10-fold) ($P < 0.01$; ANOVA). A 2-fold reduction in activity was observed when the supernatant was treated with 21.9 J of laser light in the absence of photosensitiser (S-) ($P > 0.05$; ANOVA). There was no inhibitory effect of tin chlorin e6 in the absence of light.

Light dose (J)	Median haemagglutinating titre	
	S-	S+
0	1/512	1/512
4.38	1/512	1/128
8.76	1/512	1/32
21.9	1/256	1/4

Table 7.5 The effect of 633 nm laser light dose on the activity of *P. gingivalis* W50 haemagglutinin activity.

An equal volume of either PBS (S-) or 20 µM tin chlorin e6 (S+) was added to 50 µL *P. gingivalis* W50 culture supernatant and samples were either exposed to 4.38, 8.76 or 21.9 J of 633 nm laser light or kept in the dark. Following irradiation/dark incubation, the haemagglutinating activity was assessed using the haemagglutination assay. The haemagglutinating titre was calculated as the reciprocal of the lowest concentration of culture supernatant causing haemagglutination. Experiments were performed three times and the combined data are shown.

7.4 Discussion

Secreted bacterial products and/or surface-associated components are believed to be the primary cause of tissue destruction in chronic periodontal disease. Surface-associated material of *P. gingivalis* has been shown to stimulate bone resorption, inhibit bone formation, and inhibit the proliferation of osteoblasts, keratinocytes and monocytes (Wilson *et al.*, 1993b).

Photodynamic therapy is well-suited to the treatment of periodontal diseases, as conventional treatments such as antibiotics are difficult to maintain at a suitable concentration in the periodontal pocket (Jori *et al.*, 2006). A photosensitiser can be applied to the periodontal pocket, and laser light delivered via a fibreoptic can be placed directly into the periodontal pocket (Packer *et al.*, 2000). In addition, antibiotics may not penetrate bacterial biofilms (Maisch, 2007); however, it has been well documented that light-activated antimicrobial agents can achieve killing of bacteria within plaque biofilms (Konopka & Goslinski, 2007).

The killing of *P. gingivalis* by both methylene blue and tin chlorin e6, in combination with laser light of the appropriate wavelength, has been described in Chapter 3. As photodynamic killing of the organism was found to be highly successful, the ability of the photosensitisers to inactivate virulence mechanisms of *P. gingivalis* would be advantageous.

LPS is considered to be one of the most important virulence factors of periodontopathogens (Wilson, 2004). *P. gingivalis* LPS can stimulate the release of a number of effector molecules such as the proinflammatory cytokines TNF-α, IL-6 and IL-1β, and prostaglandin E_2. These molecules contribute to the inflammation and bone resorption seen in chronic periodontitis both directly and indirectly

(Wilson, 1995). LPS plays a direct role in bone resorption by the activation of osteoclasts and inhibition of osteoblast differentiation (Lamont & Jenkinson, 1998; Xing *et al.*, 2010). LPS from *P. gingivalis* has also been shown to inhibit bone collagen synthesis, and consequently bone formation (Millar *et al.*, 1986). In addition, LPS can stimulate an IgG response, which has been associated with persistent alveolar bone resorption and prolonged periodontal destruction (Sakai *et al.*, 2001).

Outer membrane vesicles containing LPS are released by *P. gingivalis*. These vesicles are able to penetrate periodontal tissue where they contribute to the innate immune response against *P. gingivalis*, which is believed to be partly responsible for the destruction associated with periodontal disease (Darveau *et al.*, 2004). Therefore, these vesicles also represent a target for PDT as the LPS does not have to be associated with the bacterium to cause deleterious effects.

P. gingivalis 1690 LPS was found to be susceptible to photodynamic inactivation by methylene blue and laser light of 665 nm; however treatment with tin chlorin e6 and HeNe laser light had no effect on its activity. A median 4-fold reduction in activity was observed following treatment of the LPS with a relatively high concentration of methylene blue (67 µM) and 21.9 J of 665 nm laser light. It is thought that photosensitisation induces protein-protein and protein-lipid crosslinking in the bacterial membrane (Bhatti *et al.*, 2002); such cross-linking may therefore occur when the LPS is irradiated with methylene blue and laser light and consequently inhibit its biological activity. Lipid peroxidation may also occur as a result of singlet oxygen generation (Wolnicka-Glubisz *et al.*, 2009).

P. gingivalis LPS activity was inactivated to a lesser degree than that seen for other virulence factors. Lipopolysaccharides are large, comparatively complex molecules; the lower susceptibility of LPS to photodynamic inactivation could be due to a lower availability of oxidisable amino acids compared with the other proteinaceous

virulence factors investigated, for example the cysteine proteases, which have oxidisable amino acids at the active site.

Komerik *et al*. demonstrated that photosensitisation of *Escherichia coli* LPS using toluidine blue O (TBO) and 633 nm HeNe laser light decreased the bioactivity of LPS in a photosensitiser and light dose-dependent manner. It was proposed that the reduction in activity may be accompanied by structural changes to the LPS that may also affect the barrier function of the Gram-negative outer membrane (Komerik *et al.*, 2000). Although a dose-dependent inactivation of *P. gingivalis* was not observed in the experiments documented in this chapter, it is possible that this may be achieved using higher concentrations of photosensitiser and/or laser light. The LPS of *E. coli* and *P. gingivalis* have been shown to differ in their amino acid composition, which may account for the difference in their susceptibilities to photodynamic inactivation (Koga *et al.*, 1985).

Although little research has been done on the direct inactivation of the biological activity of *P. gingivalis* LPS, there has been much interest in novel periodontitis therapies that reduce the negative effects of LPS, such as the release of proinflammatory cytokines by LPS-stimulated cells, especially using “non-traditional” approaches. For example, turmeric, elderflower, cranberry and Japanese apricot extracts have all been shown to have anti-inflammatory effects on cells treated with LPS (Bodet *et al.*, 2006; Chen *et al.*, 2008; Harokopakis *et al.*, 2006; Morimoto *et al.*, 2009). The anti-inflammatory effect of photodynamic treatment is discussed further in chapter 8.

P. gingivalis proteases are multifunctional enzymes that have been proposed as virulence factors due to their role in the degradation of host periodontal tissue, activation of host degradative enzymes, and the deregulation of host inflammatory processes (Kuramitsu *et al.*, 1995). The extracellular cysteine proteases of *P.*

gingivalis, such as the gingipains, are considered key virulence factors of the bacterium (Potempa *et al.*, 2003); *P. gingivalis* also produces several other enzymes with proteolytic activity such as periodontain, collagenase, protease-haemagglutinin, peptidase and an endothelin converting-like enzyme (Curtis *et al.*, 2001).

The proteases of *P. gingivalis* are able to degrade host proteins such as collagen, extracellular matrix components such as fibronectin, immunoglobulins, cytokines, complement factors and coagulation factors (Kadowaki *et al.*, 2000). Specifically, the arg- and lys-gingipains have been shown to confer resistance to complement, and are essential for the growth of *P. gingivalis* in the presence of human serum (Grenier *et al.*, 2003).

Extracellular Arginine-specific proteases are considered important virulence factors in periodontal disease; three forms of arg-specific proteases have been identified in *P. gingivalis* W50 culture supernatants (Rangarajan *et al.*, 1997). Arg-gingipains have been shown to be responsible for major damage caused to human gingival fibroblasts (Grenier *et al.*, 2003). A role for arg-gingipains in the colonisation of host tissues has also been suggested as a protease-deficient mutant was showed to have significantly lower attachment to human epithelial cells and less attachment to host matrix proteins (Tokuda *et al.*, 1996).

Gingipains also have haemagglutinating, adhesion and haemoglobin-binding activity (Potempa *et al.*, 2000). In addition, gingipains play a role in the processing and/or maturation of other virulence factors such as fimbriae, haemagglutinins and the haemoglobin receptor protein (Kadowaki *et al.*, 2000).

Several studies have shown the importance of gingipains in the virulence of *P. gingivalis*. Inhibition of *P. gingivalis* W50 protease activity in a murine virulence

model significantly reduced the ability of the bacterium to produce lesions and mortality (Kesavalu *et al.*, 1996). Kesavalu *et al*. also demonstrated that a protease-depleted mutant was significantly less virulent than the parent strain. Immuninisation against gingipains has been shown to provide protection against lesion development and mortality in murine lesion models (Genco *et al.*, 1998; Yonezawa *et al.*, 2001).

The proteases of *P. gingivalis* have been suggested as a potential target for periodontitis therapy due to their contribution to the pathology associated with the disease (Imamura, 2003). Kadowaki *et al*. suggested the attenuation of *P. gingivalis* virulence may be achieved by the use of proteinase inhibitors or gingipain-specific antibodies (Kadowaki *et al.*, 2000).

The proteolytic activity of culture supernatants of *P. gingivalis* W50 was shown to be inhibited by both methylene blue and tin chlorin e6 when irradiated with laser light of the appropriate wavelength. Photodynamic treatment with both photosensitisers significantly reduced the proteolytic activity of the supernatant compared with untreated samples. With the highest combination of photosensitiser and light dose tested (20 μM photosensitiser and 21.9 J laser light), methylene blue and tin chlorin e6 caused reductions in proteolytic activity of 66% and 74%, respectively. These data show that significant inhibition of proteolytic activity can be achieved at photosensitiser and light doses that also effectively kill *P. gingivalis*; at these doses, both methylene blue and tin chlorin e6 killed > 99.99% of bacteria. Photosensitiser concentrations as low as 1 μM were also shown to have a highly significant inhibitory effect on the proteolytic activity of the organism, demonstrating that inactivation of proteolytic enzymes may occur even at photosensitiser concentrations at which killing of *P. gingivalis* is not achieved.

The addition of an enhancer of singlet oxygen lifetime did not significantly increase photodynamic inactivation, suggesting that either singlet oxygen may not be the major reactive oxygen species involved in inactivation, or that the inactivation is not dependent on the lifetime of the singlet oxygen. L-tryptophan was found to significantly reduce the efficacy of the photodynamic inactivation. Although L-tryptophan is not specifically a singlet oxygen scavenger, the results demonstrate the importance of reactive oxygen species in the photodynamic inactivation of these enzymes. These results are similar to those observed for the *S. aureus* V8 protease, as described in sections 4.3.2.3 and 4.3.2.4.

Braham *et al*. have recently demonstrated that photosensitisation of *P. gingivalis* with a commercial formulation of methylene blue and laser light of 670 nm can inactivate proteolytic activity as well as effectively killing the organism (Braham *et al.*, 2009). In addition, Packer *et al*. have shown that proteolytic enzymes of *P. gingivalis* W50 are susceptible to photodynamic inactivation by TBO and HeNe laser light, observing a light dose-dependent inactivation of activity. It was proposed that photodynamic inactivation of proteolytic enzymes was due to oxidisation of active site thiol groups, as the majority of the proteolytic activity of *P. gingivalis* is attributed to arginine- and lysine-specific cysteine proteases (gingipains) (Packer *et al.*, 2000). The lys- and arg-gingipains have been identified as the major photolabile proteins in *P. gingivalis* (Bhatti *et al.*, 2001). It has been shown that photodynamic treatment can cause cysteine residues to cross-link via the formation of covalent bonds (Shen *et al.*, 1996); therefore proteolytic enzymes may be inactivated by the photodynamic oxidisation of active site cysteine residues.

Photodynamic inactivation has also been shown to be effective for the proteolytic enzymes of other microorganisms. It has been shown that a highly significant reduction in the proteolytic activity of *Pseudomonas aeruginosa* can be achieved using TBO and 633 nm laser light (Komerik *et al.*, 2000). Methylene blue and laser

light of 665 nm has also been shown to significantly reduce the activity of the V8 protease of *S. aureus* (Tubby *et al.*, 2009); tin chlorin e6 has also been shown to have similar activity when irradiated with 633 nm laser light (section 4.3.2).

Other methods of inhibiting the proteolytic activity of *P. gingivalis* proteases have also been investigated. Derivatives of catechins, components of green tea, have been shown to have an inhibitory effect on arginine-specific gingipains, and to a lesser extent, the lysine-specific gingipain (Okamoto *et al.*, 2004). Kadowaki *et al*. have also investigated synthetic compounds and substances derived from *Streptomyces* spp for their ability to inhibit arg-gingipains (Kadowaki *et al.*, 2003; Kadowaki & Yamamoto, 2003; Kadowaki *et al.*, 2004).

P. gingivalis is known to produce a number of haemagglutinins; at least eight molecules with haemagglutinating activity have been identified (Lamont & Jenkinson, 2000). Although the precise number and specific function of these proteins is unknown, they have been proposed as virulence factors due to their roles in hemin acquisition and modulation of the immune response (Katz *et al.*, 1999; Zhang *et al.*, 2005).

Haemagglutinating activity is associated with virulence as protoheme is a necessary requirement for *P. gingivalis*, and thought to be derived from erythrocytes in the periodontal pocket; therefore the ability to agglutinate and lyse erythrocytes is important for survival of the bacterium (Kadowaki *et al.*, 2000). Haemagglutinins from *P. gingivalis* are thought to lyse erythrocytes via the formation of small pores (Shah *et al.*, 1992). A further virulence mechanism has been proposed for haemagglutinin B, which has been shown to induce production of the proinflammatory cytokines IL-12, interferon-γ and TNF-α (Zhang *et al.*, 2005). Haemagglutinin B is a nonfimbrial adhesin that is expressed on the surface of *P. gingivalis.* Immunisation with haemagglutinin B has been shown to significantly

reduce periodontal bone loss in a rat oral infection model (Katz *et al.*, 1999). Immunization against haemagglutinins has also been shown to prevent recolonisation in human periodontitis patients, emphasising the potential importance of haemagglutinins as a virulence factor (Booth *et al.*, 1996).

Both photosensitisation regimens were shown to inactivate *P. gingivalis* W50 haemagglutinating activity, and inactivation was dependent on both photosensitiser concentration and laser light dose. The haemagglutinating activity was not effected by treatment with either photosensitiser or laser light alone, except for the highest light doses tested for 633 nm and 665 nm laser light, which both resulted in a small, 2-fold reduction in activity, although this was not found to be statistically significant ($P > 0.05$; ANOVA). Tin chlorin e6 was found to be the most effective photosensitiser when irradiated with 633 nm laser light; a maximum reduction in activity of 14-fold was observed, compared with the 10-fold reduction observed following treatment with methylene blue and laser light.

The photodynamic inhibition of haemagglutination observed may be partly due to inactivation of proteases with haemagglutinating activity. Cysteine proteases from *P. gingivalis* have been associated with haemagglutinating activity, and gingipains have been found in complexes with haemagglutinins (Curtis *et al.*, 2001; Pike *et al.*, 1994). Photosensitisation has been shown to destroy the haemagglutinin domain of the PrpRI protease of *P. gingivalis* (Bhatti *et al.*, 2001); therefore, it is possible that the activities of multiple proteins with haemagglutinating activity may be inhibited by the action of light-activated antimicrobial agents.

Although the specific inactivation of haemagglutinins has not been extensively studied, Tezuka *et al*. have shown that the haemagglutinating activity of *P. gingivalis* can be inhibited by immunisation against haemagglutinin A (Tezuka *et al.*, 2006). Similar results using antibodies have also been published (Abiko *et al.*, 2001; Tagawa

et al., 2004). Another approach using synthetic peptides to inhibit *P. gingivalis* haemagglutinating activity has also been demonstrated, and proposed as a novel therapeutic strategy against *P. gingivalis*-induced periodontal disease (Chang *et al.*, 2004).

7.5 Summary

Treatment with methylene blue and 665 nm laser light resulted in inhibition of the activity of *P. gingivalis* LPS, proteases and haemagglutinins. Both methylene blue and tin chlorin e6 significantly inhibited the proteolytic and haemagglutinating activities of *P. gingivalis* W50 culture supernatants when irradiated with laser light; however, only methylene blue and laser light of 665 nm was able to reduce the biological activity of *P. gingivalis* LPS. In addition, a higher concentration of methylene blue was required for inactivation of the biological activity of LPS compared with *P. gingivalis* protease and haemagglutinating activity. As the virulence factors of *P. gingivalis* are strongly implicated in the pathogenesis of periodontal disease, the ability of photodynamic treatment to inactivate these virulence determinants is a highly desirable property and would represent an advantage over conventional periodontitis therapies.

8 Assessment of the ability of light-activated antimicrobials to inactivate key modulators of the immune response

8.1 Introduction

The proinflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) play key roles in the pathology associated with inflammation (Watkin *et al.*, 2007). Whilst proinflammatory cytokines are part of the normal host immune response to infection, excessive production and exposure to these cytokines can cause damage to host tissues and have deleterious effects on the healing process. TNF-α is predominantly produced by macrophages, and is involved in a number of inflammatory processes such as the recruitment of neutrophils and monocytes, stimulation of chemokine production, and apoptosis of target cells, whilst IL-6 has proinflammatory functions such as the induction of acute phase protein production, T cell activation, and B cell proliferation and antibody secretion (Elgert, 2009). IL-6 is produced by a number of cell types, including monocytes, fibroblasts and keratinocytes (Kishimoto, 1989).

Proinflammatory cytokines are believed to play an important role in the pathology associated with periodontitis due to their multimodal destruction of host tissue, which ultimately results in alveolar bone loss (Graves & Cochran, 2003). TNF-α, and IL-6 have been detected in periodontitis lesions (Takashiba *et al.*, 2003); therefore, the local inactivation of these cytokines may be beneficial in the treatment of periodontitis and may contribute to the healing of the disease.

P. gingivalis is an important periodontal pathogen and produces a number of virulence factors that are believed to contribute to periodontal destruction via induction of proinflammatory cytokine production (Wang & Ohura, 2002). *P. gingivalis* LPS can stimulate a number of cell types to produce proinflammatory

cytokines, including macrophages and gingival fibroblasts. Gingival fibroblasts are responsive to stimulation with *P. gingivalis* LPS and are believed to play a major role in the pathogenesis of periodontitis due, to production of IL-1, IL-6 and interleukin-8 (IL-8) (Tabeta *et al.*, 2000). Gingival fibroblasts from inflamed tissues have been shown to produce significantly higher levels of interleukin-1β, IL-6 and TNF-α compared with gingival fibroblasts from healthy tissues (Wang *et al.*, 2003). Macrophages are also strongly associated with the progression of periodontal disease and are found in high numbers in gingival tissues; these cells have been shown to produce a variety of cytokines and chemokines upon stimulation with *P. gingivalis* LPS or whole bacterial cells (Zhou *et al.*, 2005). Oral epithelial cells may also produce proinflammatory cytokines, and have been shown to produce IL-6 upon stimulation with the arg-protease of *P. gingivalis* (Lourbakos *et al.*, 2001).

P. gingivalis haemagglutinins are also implicated in the overproduction of cytokines that is observed in periodontal disease. Haemagglutinin B has been shown to induce the production of cytokines, in particular TNF-α, from macrophages *in vitro* (Zhang *et al.*, 2005). Significantly higher levels of interferon-γ have also been observed following stimulation of lymphoid cells with haemagglutinin B (Katz *et al.*, 1999).

Steffen *et al.* observed that heat-killed cells of *P. gingivalis* induced the production of significantly higher levels of IL-6 and IL-8 compared with viable bacteria, demonstrating that proinflammatory cytokine production can occur even after the bacterium has been killed (Steffen *et al.*, 2000). LPS and bacterial proteases have both been found to exert effects on the host after eradication of the infecting bacterium, which may account for this phenomenon (Wilson, 1995). Consequently, a treatment that had the ability to inactivate locally-produced cytokines, as well as kill pathogenic bacteria and inactivate bacterial virulence factors may be of use in the treatment of periodontitis.

Proinflammatory cytokines have also been proposed as mediators of pathology in inflammatory skin disorders such as psoriasis and atopic eczema, and anti-cytokine therapy has consequently been explored as a potential treatment for these diseases (Belloni *et al.*, 2008; Ettehadi *et al.*, 1994; Pereira *et al.*, 2006). These inflammatory skin diseases are commonly associated with colonisation of the skin by *S. aureus*, which is believed to exacerbate the conditions (Tomi *et al.*, 2005). Consequently, a treatment which not only had anti-inflammatory properties but also antimicrobial action may also show promise for the treatment of such skin disorders.

S. aureus superantigens, α-haemolysin and Protein A are all thought to interact with keratinocytes and cause the production of proinflammatory cytokines (Portugal *et al.*, 2007). It has been demonstrated that *S. aureus* α-haemolysin can induce the production of IL-6 and to a lesser extent, TNF-α; sphingomyelinase (β-haemolysin) has also been found to induce inflammation by lysing cells containing inflammatory mediators (Fournier & Philpott, 2005). The α-haemolysin of *S. aureus* can cause inflammation via the formation of pores in host cell membranes and subsequent host cell lysis, and by the stimulation of cellular signalling cascades that result in the release of proinflammatory cytokines (Haslinger *et al.*, 2003).

Other staphylococcal products such as peptidoglycan and teichoic acids have also been shown to induce TNF-α and IL-6 production *in vitro* (Wang *et al.*, 2000). In addition, *S. aureus* invasion of osteoblasts results in the release of IL-6 and consequent exacerbation of bone destruction (Liang & Ji, 2007). The induction of proinflammatory cytokine release can therefore be considered one of the virulence mechanisms of *S. aureus*. Inactivation of these cytokines may consequently be beneficial in the treatment of staphylococcal infections for which PDT is indicated, such as superficial skin infections.

Due to the deleterious effect proinflammatory cytokines may have on host tissues in infections such as periodontitis and staphylococcal skin infections, it would be desirable to reduce their local activity as well as killing the infecting microorganism(s). Such a reduction in inflammatory activity may result in less damage to host tissues and aid the healing process. This chapter describes the effect of light-activated antimicrobial agents on the biological activities of TNF-α and IL-6.

8.2 Materials and methods

8.2.1 Maintenance of cell lines

8.2.1.1 L929 fibroblast cell line

The mouse L929 fibroblast cell line was purchased from the European Collection of Cell Cultures and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10 % foetal calf serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin. Cells were seeded at a density of approximately $4x10^5$ cells/mL and incubated at 37°C in an atmosphere of 95% air and 5% CO_2 using a humidified incubator.

8.2.1.2 B9 hybridoma cell line

A B9 mouse B cell hybridoma cell line was purchased from the European Collection of Cell Cultures. The cell line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) supplemented with 5 % foetal calf serum, 50 µM 2-mercaptoethanol (Invitrogen) and 50 pg/mL IL-6 (Invitrogen) at a density of $1x10^3$ – $9x10^5$ cells/mL and incubated at 37°C in an atmosphere of 95% air and 5% CO_2 using a humidified incubator.

8.2.2 L929 fibroblast bioassay to determine the effect of photosensitisation on the activity of TNF-α

50 µL of photosensitiser was added to an equal volume of TNF-α (final concentration of 1 ng/mL in DMEM, Biosource) and photosensitisation studies were performed as described in section 2.5, with the following modification: DMEM was used instead of PBS for S- tests. Following irradiation/dark incubation, TNF-α activity was assessed using the L929 fibroblast bioassay as described below. Experiments were performed three times in triplicate.

L929 cells were maintained according to section 8.2.1.1. A 100 µL aliquot of a fibroblast suspension containing 4×10^5 cells/mL was added to each well of a 96-well flat-bottom microtitre plate and incubated overnight at 37°C in an atmosphere of 95% air and 5% CO_2 using a humidified incubator. Following photosensitization experiments, the growth medium was then aspirated and replaced with 50 µL fresh medium. A 50 µL test sample was added to each row and serially diluted in supplemented DMEM (as described in section 8.2.1.1) using doubling dilutions. Following dilution, 50 µl of 8 µg/mL actinomycin D-mannitol (Sigma-Aldrich, UK) solution was added to each well to sensitise the cells to TNF-induced lysis. The plates were then incubated for 18 hours in a 37°C, 95% air and 5% CO_2 humidified incubator.

After incubation, the supernatant was aspirated from each well and the L929 cells washed once with 200 µL of tissue culture grade PBS. A 50 µL aliquot of 0.05% crystal violet in 20% ethanol was added to each well and the plates incubated for 10 minutes at room temperature. The plates were rinsed with water to remove the crystal violet and allowed to dry overnight, following which, 100 µl of 100% methanol was added to each well to elute the stain. The absorbance at 590 nm (OD_{590}) was then measured using a Dynex microtitre plate reader. Control wells containing DMEM only (no TNF-α) were used as a measure of maximal crystal violet

uptake, i.e. 0% cell lysis, and wells in which 100% cell lysis was observed were used as blanks. The concentration of TNF-α required to cause 50% lysis of L929 cells (LD_{50}) was calculated. Wells that exhibited an OD_{590} closest to 50% of the arithmetic mean were considered to represent 50% lysis of the L929 cells.

8.2.3 B9 cell proliferation assay to determine the effect of photosensitisation on the activity of IL-6

Aliquots of 100 µL tin chlorin e6 were added to an equal volume of IL-6 (final concentration of 0.25 ng/mL in B9 medium without IL-6) and photosensitisation studies were performed as described in section 2.5, using B9 medium instead of PBS for S- tests. Following irradiation/dark incubation, IL-6 activity was assessed using the B9 cell proliferation assay as described below. Experiments were performed three times in triplicate.

B9 cells were maintained according to section 8.2.1.2. B9 cells were plated in triplicate in flat-bottomed 96-well microtitre plates at a density of 1×10^4 cells per well. Test solutions of IL-6 were serially diluted in B9 medium (without IL-6) using doubling dilutions to a final concentration of 2 pg/mL, following which, 100 µL of each dilution was added to 100 µL B9 cell suspension. The cells were then incubated in the dark for 48 hours at 37°C in an atmosphere of 95% air and 5% CO_2 using a humidified incubator. Cell proliferation was assessed using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, UK). Microtitre plates were centrifuged at 145 x *g* for 10 minutes and the supernatant removed and discarded. Cells were resuspended in 200 µL fresh B9 medium (without IL-6) and 22 µL 5 mg/mL MTT in PBS was added to each well. Plates were incubated at 37°C for 2 hours in the dark, after which they were centrifuged at 145 x *g* for 10 minutes. Eighty microlitres of the supernatant was then removed and discarded, and 100 µL dimethylsulfoxide (Sigma-Aldrich, UK) was added to dissolve the tetrazolium product. The optical density at

590 nm was read using a Dynex microplate reader using 650 nm as the background control. Wells containing medium only were used as blanks and untreated IL-6 wells were used as a measure of maximum proliferation. The concentration of IL-6 required to cause 50% proliferation of B9 cells (ED_{50}) was calculated.

8.2.4 Photosensitisation experiments for SDS PAGE analysis

Photosensitisation experiments were performed using 25 µL of TNF-α (final concentration of 40 µg/mL) or IL-6 (final concentration of 20 µg/mL), which was added to an equal volume of methylene blue (final concentration of 20 µM) (S+) or PBS (S-). Laser light dose experiments were then performed according to section 2.5.1. After photosensitisation or dark incubation, samples were analysed by SDS PAGE, performed according to section 2.8.

8.3 Results

8.3.1 The effect of photosensitiser dose on the photodynamic inactivation of TNF-α

8.3.1.1 Methylene blue

The effect of methylene blue and diode laser light of 665 nm on the biological activity of TNF-α is shown in Table 8.1. Neither photosensitiser nor laser light alone had a significant inhibitory effect on the activity of TNF-α ($P > 0.05$; Mann-Whitney U Test); however, treatment with methylene blue and 665 nm laser light resulted in a reduction in biological activity that increased with methylene blue concentration. Irradiation of TNF-α in the presence of concentrations of methylene blue ≥ 5 μM resulted in a significant increase in the LD_{50} ($P < 0.01$; Mann-Whitney *U* Test). Treatment of the TNF-α with 20 μM methylene blue and 21.9 J laser light resulted in more than a 10-fold increase in the concentration of TNF-α required to achieve the LD_{50} compared with the untreated control.

Concentration of methylene blue (μM)	Median LD_{50} (ng/mL)	
	L-	L+
0	0.08	0.09
1	0.12	0.14
5	0.08	0.19 **
10	0.11	0.41 **
20	0.10	> 1 **

Table 8.1 The effect of photosensitiser dose on the biological activity of TNF-α when treated with methylene blue and laser light

An equal volume of either methylene blue (1 to 20 μM) or L929 medium was added to 50 μl TNF-α and samples either exposed to 21.9 J 665 nm laser light (L+) or incubated in the dark (L-). Following irradiation/dark incubation, TNF-α activity was assessed using the L929 bioassay. ** $P < 0.01$, Mann-Whitney *U* Test. Experiments were performed three times in triplicate and the combined data are shown.

8.3.1.2 Tin chlorin e6

The effect of tin chlorin e6 dose on the biological activity of TNF-α is shown in Table 8.2. Neither photosensitiser nor laser light alone had a significant inhibitory effect on the activity of the TNF-α ($P > 0.05$; Mann-Whitney *U* Test). Treatment of the cytokine with ≥ 5 μM tin chlorin e6 and laser light of 633 nm caused a highly significant increase in the LD50 compared with the untreated control ($P < 0.001$; Mann-Whitney *U* Test). Treatment with ≥ 10 μM tin chlorin e6 and 633 nm laser light resulted in the complete inactivation of activity as detectable by this assay, whilst 5 μM tin chlorin e6 in the presence of laser light caused a 15-fold increase in

the concentration of TNF-α required to achieve the LD_{50} compared with untreated samples, demonstrating that TNF-α was highly susceptible to photodynamic inactivation with this photosensitiser.

Concentration of tin chlorin e6 (μM)	Median LD_{50} (ng/mL)	
	L-	L+
0	0.04	0.05
1	0.04	0.04
5	0.04	0.60 ***
10	0.04	>1.0 ***
20	0.05	>1.0 ***

Table 8.2 The effect of photosensitiser dose on the biological activity of TNF-α when treated with methylene blue and laser light

An equal volume of either tin chlorin e6 (1-20 μM) or L929 medium was added to 50 μl TNF-α and samples either exposed to 21.9 J 633 nm laser light (L+) or incubated in the dark (L-). Following irradiation/dark incubation, TNF-α activity was assessed using the L929 bioassay assay. *** $P < 0.001$, Mann-Whitney *U* Test. Experiments were performed three times in triplicate and the combined data are shown.

8.3.2 The effect of laser light dose on the photodynamic inactivation of TNF-α

8.3.2.1 Laser light of 665 nm

The effect of 665 nm laser light dose on the biological activity of TNF-α is shown in Table 8.3. Only a laser light dose of 21.9 J in combination with methylene blue had a significant inhibitory effect on the biological activity of the TNF-α ($P < 0.001$; Mann-

Whitney *U* Test). Laser light in the absence of photosensitiser did not significantly affect the biological activity of the cytokine ($P > 0.05$; Mann-Whitney *U* Test). Despite the highly significant reduction in activity observed following photosensitisation with 21.9 J 665 nm laser light and 20 µM methylene blue, a lower level of inactivation was observed for this set of experiments compared with the methylene blue dose response experiments, suggesting some variability in the test system.

Light dose (J)	**Median LD_{50} (ng/mL)**	
	S-	**S+**
0	0.05	0.07
4.38	0.06	0.10
8.76	0.04	0.10
21.9	0.05	0.18 ***

Table 8.3 The effect of laser light dose on the biological activity of TNF-α when treated with methylene blue and 665 nm laser light

An equal volume of either 20 µM methylene blue (S+) or L929 medium (S-) was added to 50 µl TNF-α and samples either exposed to 4.38 J, 8.76 J or 21.9 J 665 nm laser light or incubated in the dark. Following irradiation/dark incubation, TNF-α activity was assessed using the L929 bioassay assay. *** $P < 0.001$, Mann-Whitney *U* Test. Experiments were performed three times in triplicate and the combined data are shown.

8.3.2.2 Laser light of 633 nm

Table 8.4 shows the effect of 633 nm laser light dose on the photodynamic inactivation of TNF-α. Laser light doses ≥ 4.38 J completely inhibited the biological activity of the TNF-α as detectable by this assay when the cytokine was irradiated in the presence of 20 μM tin chlorin e6. Neither laser light nor photosensitiser alone had a significant inhibitory effect on the biological activity of TNF-α ($P > 0.05$; Mann-Whitney *U* Test).

Light dose (J)	**Median LD_{50} (ng/mL)**	
	S-	**S+**
0	0.04	0.04
4.38	0.04	>1.0 ***
8.76	0.04	>1.0 ***
21.9	0.04	>1.0 ***

Table 8.4 The effect of laser light dose on the biological activity of TNF-α when treated with tin chlorin e6 and 633 nm laser light

An equal volume of either 20 μM tin chlorin e6 (S+) or L929 medium (S-)was added to 50 μl TNF-α and samples either exposed to 4.38 J, 8.76 J or 21.9 J 633 nm laser light or incubated in the dark. Following irradiation/dark incubation, TNF-α activity was assessed using the L929 bioassay assay. *** $P < 0.001$, Mann-Whitney *U* Test. Experiments were performed three times in triplicate and the combined data are shown.

8.3.3 SDS PAGE analysis of TNF-α

SDS PAGE analysis (Figure 8.1) showed that after photosensitisation with 20 μM methylene blue and 665 nm laser light, more bands derived from TNF-α were apparent on the gel. The higher molecular weight bands present after photosensitisation were approximately 2- and 3-times the weight of the untreated TNF-α (approximately 30 and 45 kDa respectively, compared with 15 kDa for the untreated protein), suggesting that photosensitisation may cause cross-linking of the cytokine.

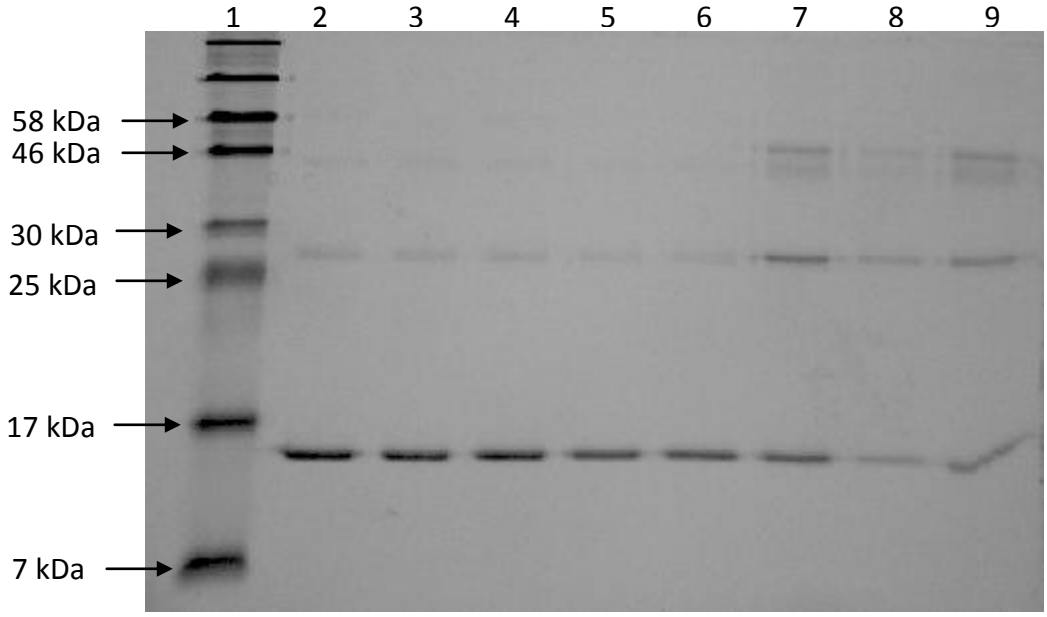

Figure 8.1 SDS PAGE analysis of TNF-α irradiated with 20 µM methylene blue and 665 nm laser light doses of 4.38 J, 8.76 J or 21.9 J.

Tumour necrosis factor-α was either kept in the dark (L-) or irradiated with laser light doses of 4.38 J, 8.76 J or 21.9 J (L+) in the presence of an equal volume of either PBS (S-) or 20 µM methylene blue (S+). Following irradiation, samples were analysed by SDS PAGE using a 5% stacking gel and 15% resolving gel under denaturing conditions. Lane 1: molecular weight marker, lane 2: L-S-, lane 3: L-S+, lane 4: L+S- (4.38 J), lane 5: L+S- (8.76 J), lane 6: L+S- (21.9 J), lane 7: L+S+ (4.38 J), lane 8: L+S+ (8.76 J), lane 9: L+S+ (21.9 J). The apparent molecular mass of TNF-α was approximately 15 kDa. The molecular mass of TNF-α was 17.5 kDa according to the source (Invitrogen).

8.3.4 The effect of photosensitiser dose on the photodynamic inactivation of IL-6

8.3.4.1 Methylene blue

Preliminary studies showed that methylene blue interfered with both the MTT assay and B9 cell proliferation; consequently the investigation of the effect of photosensitisation on IL-6 bioactivity using this photosensitiser could not be performed.

8.3.4.2 Tin chlorin e6

The effect of photosensitiser dose on the photodynamic inactivation of IL-6 is shown in Table 8.5. Tin chlorin e6 concentrations ≥ 5 μM had a significant inhibitory effect on the biological activity of IL-6 when irradiated with 21.9 J 633 nm laser light ($P < 0.05$; Mann-Whitney *U* Test). Treatment of IL-6 with tin chlorin e6 in the absence of laser light did not have a significant effect on the activity of the cytokine ($P > 0.05$; Mann-Whitney *U* Test).

Concentration of tin chlorin e6 (μM)	Median ED_{50} (ng/mL)	
	L-	L+
0	0.01	0.01
1	0.01	0.03
5	0.01	0.04 *
10	0.01	> 0.06 ***
20	0.01	0.06 **

Table 8.5 The effect of photosensitiser dose on the biological activity of IL-6 when treated with tin chlorin e6 and 4.38 J 633 nm laser light.

An equal volume of either tin chlorin e6 (1 to 20 μM) or B9 medium (without IL-6) was added to 100 μL IL-6 and samples either exposed to 4.38 J 633 nm laser light (L+) or incubated in the dark (L-). Following irradiation/dark incubation, IL-6 activity was assessed using the B9 cell proliferation assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney *U* Test. Experiments were performed three times in triplicate and the combined data are shown.

8.3.5 The effect of laser light dose on the photodynamic inactivation of IL-6

Treatment of IL-6 with 20 µM tin chlorin e6 and laser light doses ≥ 8.76 J resulted in the complete inhibition of bioactivity as detectable by the B9 cell proliferation assay, as can be seen in Table 8.6. The increase in the ED_{50} was highly significant compared with the untreated control ($P < 0.001$; Mann Whitney *U* Test).

Light dose (J)	Median ED_{50} (ng/mL)	
	S-	S+
0	0.02	0.03
4.38	0.02	> 0.06 ***
8.76	0.03	> 0.06 ***
21.9	0.02	> 0.06 ***

Table 8.6 The effect of laser light dose on the biological activity of IL-6 when treated with 20 µM tin chlorin e6 and 633 nm laser light.

An equal volume of either 20 µM tin chlorin e6 (S+) or B9 medium (without IL-6) (S-) was added to 100 µL IL-6 and samples either exposed to 4.38 J, 8.76 J or 21.9 J 633 nm laser light or incubated in the dark. Following irradiation/dark incubation, IL-6 activity was assessed using the B9 cell proliferation assay. *** $P < 0.001$, Mann-Whitney *U* Test. Experiments were performed three times in triplicate and the combined data are shown.

8.3.6 SDS PAGE analysis of IL-6

SDS PAGE analysis (Figure 8.1) showed that bands derived from IL-6 after photosensitisation with 20 µM tin chlorin e6 and 633 nm laser light became less well-defined, and more smeared with increasing irradiation time compared with untreated samples. After treatment with 20 µM tin chlorin e6 and 21.9 J laser light, the band derived from the IL-6 was almost undetectable, suggesting a major effect of photosensitisation on the protein. IL-6 bands also appeared slightly less defined after treatment with laser light in the absence of photosensitiser, suggesting some effect of laser light alone on the cytokine, although there was no significant loss in the biological activity (see Table 8.6).

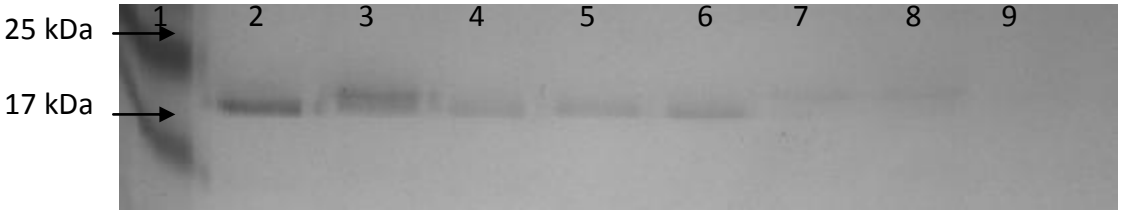

Figure 8.2 SDS PAGE analysis of IL-6 irradiated with 20 µM tin chlorin e6 and 633 nm laser light doses of 4.38 J, 8.76 J or 21.9 J.

Interleukin-6 was either kept in the dark (L-) or irradiated with 633 nm laser light doses of 4.38 J, 8.76 J or 21.9 J (L+) in the presence of an equal volume of either PBS (S-) or 20 µM tin chlorin e6 (S+). Following irradiation, samples were analysed by SDS PAGE using a 5% stacking gel and 15% resolving gel under denaturing conditions. Lane 1: molecular weight marker, lane 2: L-S-, lane 3: L-S+, lane 4: L+S- (4.38 J), lane 5: L+S- (8.76 J), lane 6: L+S- (21.9 J), lane 7: L+S+ (4.38 J), lane 8: L+S+ (8.76 J), lane 9: L+S+ (21.9 J). The apparent molecular mass of IL-6 was approximately 20 kDa. The molecular mass of IL-6 was 21.3 kDa, according to the source (Invitrogen).

8.4 Discussion

Methylene blue and tin chlorin e6 were both shown to have an inhibitory effect on the biological activity of TNF-α when irradiated with laser light of the appropriate wavelength. Photodynamic inactivation was more effective using tin chlorin e6 and 633 nm laser light, with an >25-fold increase in concentration of cytokine required to produce a LD_{50} being observed after photosensitisation with 20 μM tin chlorin e6 and a low laser light dose (4.38 J). This observed difference between the two treatments may be due to the difference in charge between the two photosensitisers and consequent differences in binding of the photosensitisers to the cytokines. IL-6 was also shown to be susceptible to photodynamic inactivation using tin chlorin e6, with high levels of inactivation being achieved following treatment with photosensitiser concentrations ≥ 10 μM.

TNF-α is one of the cytokines implicated in the pathology of psoriasis (Ettehadi *et al.*, 1994; Trent & Kerdel, 2004), and IL-6 levels have also been shown to be increased in psoriasis plaques (Grossman *et al.*, 1989). As psoriasis patients have also been shown to be frequently colonised by toxigenic *S. aureus*, (Tomi *et al.*, 2005), PDT may be well suited for the decontamination of psoriatic lesions as it may be able to reduce inflammation, as well as killing *S. aureus* (Chapter 3) and inactivating its toxins (Chapter 4). PDT has already been used with some success for the treatment of psoriasis; however the use of topical photosensitisers has not yet been fully explored (Fritsch *et al.*, 1998).

S. aureus colonisation has also been proposed to play a role in promoting inflammation in atopic dermatitis, a chronic inflammatory skin disorder, and atopic skin has been shown to be highly colonised compared with healthy subjects (Birnie *et al.*, 2008); therefore decolonisation of atopic skin may be beneficial to the treatment of this disorder. As corticosteroids have been shown to improve eczema and decrease staphylococcal colonisation of atopic skin, inflammation is thought to

be involved in the pathology of this condition (Nilsson *et al.*, 1992). However, topical corticosteroids have been frequently associated with a number of side effects, and drug tolerance can occur, particularly when used to treat chronic conditions such as psoriasis (Fisher, 1995). It would therefore be desirable to reduce both inflammation and skin colonisation with a therapy that is not associated with such side effects. PDT is not associated with tolerance due to its mechanism of action, and would therefore seem a potential candidate for the treatment of this disease, particularly in cases where skin colonisation by *S. aureus* is problematic for the patient. In addition, staphylococcal proteases have been implicated in the pathogenesis of atopic dermatitis (Miedzobrodzki *et al.*, 2002); as PDT has been shown to inactivate the V8 protease of *S. aureus* (Chapter 4), this therapy could be extremely beneficial as it has the potential to target multiple factors involved in the pathology of this disease. Skin disorders are well-suited for treatment with PDT as they are easily accessible for topical photosensitiser application and laser light delivery (Wainwright, 2010).

Anti-TNF-α therapies such as anti-TNF-α antibodies and soluble TNF-α receptor agonists have been shown to be successful *in vivo* for the treatment of psoriasis (Ogilvie *et al.*, 2001; Ryan *et al.*, 2009; Trent & Kerdel, 2004), and inhibition of TNF-α (simultaneously with interleukin-1) has been shown to significantly reduce tissue inflammation and destruction in experimental periodontitis (Assuma *et al.*, 1998; Delima *et al.*, 2001). Despite these therapies showing efficacy, the use of anti-TNF-α therapies have been associated with severe side effects such as increased risk of opportunistic infections, lymphoproliferative diseases and reactivation of diseases such as multiple sclerosis and tuberculosis due to the systemic suppression of TNF-α (Nash & Florin, 2005). TNF-α plays an important role in the host defence against infection as well as mediating the inflammation typical of chronic inflammatory diseases such as psoriasis (Saraceno & Chimenti, 2008); therefore the inhibition of TNF-α activity at a specific site would be highly desirable in order to avoid therapy-

induced immunodeficiency. The localised nature of PDT would be well-suited for this purpose as it would not affect TNF-α activity at sites distal to the treatment site.

P. gingivalis LPS mediates inflammation by the induction of IL-6 and TNF-α production (Zhang *et al.*, 2008). TNF-α has been implicated in the tissue destruction and bone loss that occurs in periodontal disease (Graves & Cochran, 2003), and raised TNF-α levels have been shown in patients with aggressive periodontitis (Bastos *et al.*, 2009). Production of TNF-α by gingival T cells in periodontitis patients has been demonstrated and is induced by *P. gingivalis* LPS, in addition to other *P. gingivalis* virulence factors and periodontal pathogens (Baker, 2000). One of the major destructive effects of TNF-α in periodontitis is its ability to stimulate fibroblasts to produce proteinases that degrade extracellular matrix components (Takashiba *et al.*, 2003). As photodynamic treatment has been shown to inactivate proteolytic enzymes from both *S. aureus* (Chapter 4) and *P. gingivalis* (Chapter 6), it is possible that photosensitisation may also be able to inactivate host proteinases. PDT may therefore be able to limit tissue destruction due to the inactivation of both TNF-α and host proteinase activity. Further experiments would be useful to determine the effect of photodynamic treatment on host proteinases.

IL-6 levels in gingival crevicular fluid have been shown to be significantly elevated in periodontitis patients compared with healthy subjects (Mogi *et al.*, 1999) and this cytokine is associated with bone resorption and consequent tooth loss (Baker, 2000). *P. gingivalis* LPS has been shown to induce the production of IL-6 in human gingival fibroblasts and is thought to be responsible for sustaining the inflammatory response in periodontal disease (Ara *et al.*, 2009). Inhibition of IL-6 and TNF-α (along with other proinflammatory cytokines) has been suggested as a potential adjunctive therapy for periodontitis, and various attempts to inhibit production of these cytokines *in vitro* have been reported (Bodet *et al.*, 2008; Chen *et al.*, 2008; Zdarilova *et al.*, 2009); however these strategies rely on the direct treatment of

cytokine-producing cells. Photodynamic inactivation of the pre-formed cytokine *in situ* would result in less disturbance to host cells. Anti-IL-6 antibodies have been shown to inhibit cytokine-mediated bone resorption (Mundy, 1991), suggesting that inactivation of pre-formed cytokines is possible.

The mechanism of inactivation of these cytokines has not been fully determined; however SDS PAGE analysis suggests that photosensitisation may cross-link TNF-α molecules, resulting in the higher molecular weight bands that can be seen in Figure 8.1. In its active form, TNF-α exists as a homotrimer, and this homotrimeric structure is essential for the cytokine's biological activity (Tang *et al.*, 1996). The SDS PAGE technique used in this chapter disrupts quaternary protein structure; therefore the TNF-α homotrimer would be broken up into the 17.5 kDa monomers by the procedure. Figure 8.1 shows that TNF-α was present in L+S+ samples as dimers and trimers, which were observed in greater quantities compared with the control samples. It is therefore possible that photosensitisation caused further cross-linking of the monomers that was resistant to the denaturation process; however, it remains to be determined whether such an increase in cross-linking would affect the activity of the cytokine. TNF-α contains photooxidisable amino acids, which may cross-link via covalent bonds (Shen *et al.*, 1996). As the trimeric form of TNF-α is associated with biological activity, further analysis is necessary to elucidate the mechanism of inactivation.

IL-6 is a single chain glycoprotein, typically present in a heterogeneous mixture of forms with molecular weights ranging from 21 to 30 KDa as a result of post-translational modification, although higher molecular weight oligomeric complexes have also been observed (Simpson *et al.*, 1997). IL-6 bands appeared less defined after photosensitisation, indicating that changes to the protein had occurred. The four cysteine residues in IL-6 and the disulphide bonds formed between these residues are critical for its biological activity (Snouwaert *et al.*, 1991). As cysteine

residues have been shown to be photooxidisable, oxidisation of these amino acids may play a role in the photodynamic inactivation of this cytokine (Shen *et al.*, 1996).

Further investigations into the mechanism(s) of photodynamic inactivation of these cytokines are necessary to determine the effect of photosensitisation on the structure of the molecules. Native PAGE or the use of zymograms may be useful as these techniques are non-denaturing and the effect on photosensitisation on the native structure of the proteins may be observed.

8.5 Summary

Photosensitisation of the proinflammatory cytokines TNF-α and IL-6 resulted in a significant reduction in their biological activities. Both methylene blue and tin chlorin e6 were shown to significantly reduce the activity of TNF-α when irradiated with laser light of the appropriate wavelength. Treatment with tin chlorin e6 and 633 nm laser light was shown to be more effective than methylene blue for the photodynamic inactivation of TNF-α, with an over 25-fold decrease in activity being observed after treatment with 20 µM photosensitiser and a low laser light dose of 4.38 J. Although it was not possible to assess the effect of methylene blue on the activity of IL-6, tin chlorin e6 was shown to significantly reduce the biological activity of this cytokine when irradiated with 633 nm laser light. Neither laser light nor photosensitiser alone had a significant inhibitory effect on the activities of either cytokine. These results suggest that PDT may be able to reduce the biological activity of proinflammatory cytokines and therefore reduce the destructive inflammation associated with inflammatory disorders for which PDT is indicated as a potential treatment.

9 Conclusions

Light-activated antimicrobial agents are a promising novel antimicrobial strategy, in particular for the treatment of superficial, localised infections such as staphylococcal skin infections and periodontal disease due to the accessibility of these infections and the susceptibility of the causative microorganisms (Wainwright, 1998). The results presented in this thesis confirm that effective killing of *S. aureus* and *P. gingivalis* can be achieved using light-activated antimicrobial agents, and indicate that photodynamic treatment may also result in the inactivation of virulence factors implicated in the initiation and progression of disease. In addition, it has been demonstrated that light-activated antimicrobial agents may be able to reduce the activity of inflammatory mediators associated with disease pathology.

Highly significant reductions in the number of viable *S. aureus* and *P. gingivalis* were achieved using tin chlorin e6 or methylene blue in the presence of laser light. Methylene blue was found to be more efficacious than tin chlorin e6, with 20 µM methylene blue and 4.38 J of 665 nm laser light resulting in ≥ 99.99% kills of both microorganisms, compared with 92, 94 and 99.95% kills for MSSA, MRSA, and *P. gingivalis*, respectively when the same concentration of tin chlorin was used. The difference in efficacy observed between the two photosensitisers may be due to differences in their charge, as the cationic methylene blue may bind to the negatively-charged bacterial membrane more effectively than the anionic tin chlorin e6.

The treatment of periodontitis, in which *P. gingivalis* is considered a major aetiological agent, is well-suited to treatment using light-activated antimicrobials (Jori *et al.*, 2006). PDT has been shown to be non-inferior to scaling and root planing for the treatment of aggressive periodontitis in pilot clinical trials; however, the trials were limited by small size and were inadequately powered to detect significant

differences between the treatments (Azarpazhooh *et al.*, 2010). Large-scale trials are therefore required to further investigate the efficacy of PDT in the clinical setting. As antimicrobial resistance has been documented in *P. gingivalis*, it would also be useful to investigate the susceptibility of antibiotic-resistant strains of *P. gingivalis* to photodynamic inactivation (Ardila *et al.*, 2010).

The ability to effectively kill antibiotic-resistant bacteria would be highly advantageous, if not a requirement, for a novel antimicrobial strategy. The results presented in this thesis demonstrate that both meticillin-sensitive and meticillin-resistant strains of *S. aureus* are highly susceptible to photodynamic killing using methylene blue and tin chlorin e6. Irradiation with 21.9 J of 665 nm laser light in the presence of 20 μM methylene blue resulted in 99.999% kills for both MSSA and MRSA. Percentage kills of 99.93 and 99.83% were observed for MSSA and MRSA respectively, following photosensitisation using equivalent doses of tin chlorin e6 and 633 nm laser light. It was also shown that small colony variants of *S. aureus* were susceptible to lethal photosensitisation using methylene blue and tin chlorin e6. These variants often show reduced susceptibility to antibiotics, and so PDT may present a potential treatment option for infections caused by these strains (Proctor & von Humboldt, 1998; von Eiff, 2008).

The meticillin-resistant strain of *S. aureus* used for experimental purposes in this thesis was EMRSA-16, an important cause of hospital-acquired MRSA infections in the UK (Johnson *et al.*, 2001). A recent study by Zolfaghari *et al*. demonstrated that methylene blue and 670 nm laser light can successfully kill EMRSA-16 *in vivo* (Zolfaghari *et al.*, 2009); however, further *in vivo* studies would be useful to confirm these findings. In addition, with the current rise in CA-MRSA, further studies to determine the susceptibility of CA-MRSA strains to lethal photosensitisation would also be useful, especially as CA-MRSA infections typically present as abscesses and foliculitis, which are easily accessible for treatment with PDT (Elston, 2007).

Both methylene blue and tin chlorin e6 were also successful in inactivating or reducing the biological activity of key virulence factors of *S. aureus* and *P. gingivalis* at concentrations and light doses relevant for microbial killing. It was demonstrated that photosensitisation significantly reduced the ability of *S. aureus* to bind to human extracellular matrix components, although it was not effective at causing the detachment of *S. aureus* cells which were already bound to human extracellular matrix components.

Furthermore, photosensitisation with methylene blue and tin chlorin e6 reduced the activity of enzymes and toxins required for microbial nutrition and disease pathology such as the V8 protease. In addition, photosensitisation was also shown to be able to reduce the activity of *S. aureus* alpha- and beta-haemolysins, which are associated with evasion of the host immune response (Collins *et al.*, 2008; Jarry *et al.*, 2008; Tajima *et al.*, 2009). Therefore, photosensitisation may have the potential to reduce another aspect of the pathogen's virulence by increasing its susceptibility to killing by the host immune system. Inactivation of these virulence factors occurred in the presence of human serum, indicating that inactivation of virulence factors may be relevant *in vivo*.

Several novel approaches aimed at inhibiting the virulence of *S. aureus* are currently being explored. The disruption of quorum sensing has been proposed as a mechanism to reduce the virulence of *S. aureus*, as many of the secreted virulence factors of this organism are regulated by these systems, for example the accessory gene regulator system, which regulates expression of proteases, haemolysins and cell surface-associated binding proteins (Kaufmann *et al.*, 2008; Otto *et al.*, 1999; Park *et al.*, 2007). Disruption of *S. aureus* quorum sensing mechanisms has been shown to reduce pathogenicity in animal models of abscess infection (Wright *et al.*, 2005); however, this approach does not have direct antimicrobial action. In addition,

the inhibition of quorum sensing in *S. aureus* has been associated with an upregulation of protein A expression, which is also involved in virulence. In contrast, light-activated antimicrobial agents can reduce the activity of the virulence factors described in this thesis and also kill the microorganism(s) which produces them, thus giving photosensitisation the advantage of antimicrobial plus anti-virulence action.

Another interesting strategy aimed at reducing the virulence of staphylococci involves polyphenol compounds isolated from green tea and their derivatives. Such compounds have been found to reduce the activity of *S. aureus* coagulase and haemolytic activity, prevent biofilm formation in *Staphylococcus epidermidis*, and increase susceptibility of resistant strains to certain antibiotics (Bernal *et al.*, ; Blanco *et al.*, 2005; Shah *et al.*, 2008; Sudano Roccaro *et al.*, 2004). However, it has been proposed that these compounds exert their inhibitory effect on virulence by preventing secretion of the proteins rather than direct inhibition of activity. Although the prevention of secretion of virulence factors would be beneficial, the inactivation of preformed, secreted virulence factors would also be desirable as once secreted, such virulence factors may continue to cause deleterious effects after eradication of the bacterium.

The proteolytic enzymes of *P. gingivalis* are considered to play a major role in the virulence of the bacterium (Potempa *et al.*, 2003). A highly significant reduction in proteolytic activity was achieved following photosensitisation with methylene blue and tin chlorin e6, showing that PDT may be able to substantially reduce the tissue destruction associated with these enzymes. Photosensitisation with toluidine blue O and 633 nm laser light was previously shown by Packer *et al*. to be an effective inhibitor of *P. gingivalis* proteolytic activity (Packer *et al.*, 2000). The proteases of *P. gingivalis* have been a target for several other anti-virulence-based strategies. Curtis *et al*. observed reduced virulence of *P. gingivalis* in the presence of an inhibitor of lys-gingipain (Curtis *et al.*, 2002). As *P. gingivalis* produces several proteolytic

enzymes that are considered part of its virulence arsenal, the inhibition of activity of more than one class of proteolytic enzyme would be advantageous. Other protease inhibitors, including green tea derivatives, have also been shown to inhibit activity of the gingipains, although selectivity for a particular enzyme was also observed (Bania *et al.*, 2008; Okamoto *et al.*, 2004).

Although the LPS of *P. gingivalis* was found to be less susceptible to photodynamic inactivation compared with the other virulence factors described in this thesis, a small reduction was observed following treatment with higher doses of methylene blue. Komerik *et al*. previously demonstrated that a reduction in the biological activity of *P. gingivalis* LPS was achievable using toluidine blue O and 633 nm laser light (Komerik *et al.*, 2000); therefore, it is possible that higher levels of inactivation may be achieved with further optimisation of the photosensitiser and light dose. Other attempts to reduce the bioactivity of LPS from periodontal pathogens have been reported. There has been much interest in the investigation of the ability of extracts from foodstuffs and plants to reduce LPS-mediated inflammation (Bodet *et al.*, 2006; Bodet *et al.*, 2008; Chen *et al.*, 2008; Inaba *et al.*, 2005; La *et al.*, 2010; Zdarilova *et al.*, 2009). However, this approach focuses on the ability of the compounds to prevent production of proinflammatory cytokines rather than inactivation of the biological activity of the LPS. Since such extracts would exert their effect upon host cells, they may be associated with toxicity. The inactivation of the bioactivity of LPS would be more favourable because such compounds would be less likely to have toxic side effects.

While this thesis demonstrates that methylene blue and tin chlorin e6 show *in vitro* efficacy for the inactivation of important virulence factors from both *S. aureus* and *P. gingivalis*, further studies are required to assess the activity of these photosensitisers *in vivo*. Although the inactivation of *S. aureus* α- and β-haemolysins was demonstrated in the presence of human serum, a wound infection, for example,

represents a much more complex milieu. The ability of photodynamic treatment to reduce the activity of microbial virulence factors in animal and human tissue models would need to be investigated further; however, in such complex environments it may be more difficult to accurately evaluate the activities of virulence factors before and after treatment. It would also be useful to assess the *in vitro* ability of photosensitisation to inactivate virulence factors produced by clinical strains of *S. aureus* and *P. gingivalis* obtained from patient samples, as the activities may be different from those of purified enzymes and enzymes produced by laboratory strains. Differences in pathogenicity between laboratory reference strains and clinical isolates may arise due to loss of pathogenicity characteristics in strains adapted to the laboratory (Fux *et al.*, 2005).

As a consequence of the emergence of CA-MRSA, it would also be beneficial to assess the ability of PDT to inactivate virulence factors associated with these strains. CA-MRSA strains are characteristically associated with production of Panton-Valentine leukocidin (PVL), which is considered to be the major virulence factor of these isolates (Elston, 2007). Further studies to assess the ability of photosensitisation to reduce the activity of PVL would therefore be useful. Alpha-hemolysin is also believed to play an important role in CA-MRSA infections as virulence is substantially decreased in α-haemolysin-negative CA-MRSA in animal models (Deleo *et al.*, 2010). The results presented in this thesis demonstrate that the activity of α-haemolysin can be reduced by light-activated antimicrobial agents; consequently the ability of PDT to inactivate this virulence factor would be a favourable addition to the killing of CA-MRSA.

Proinflammatory cytokines, whilst an essential component of the immune system, may also have a deleterious effect on human tissue when dysregulated in the disease state. IL-6 and TNF-α are believed to play key roles in the pathology associated with inflammation, and have been shown to be induced by *S. aureus* and

P. gingivalis (Portugal *et al.*, 2007; Watkin *et al.*, 2007; Zhang *et al.*, 2008). The experiments described in this thesis demonstrate that photodynamic treatment is able to inactivate the biological activities of TNF-α and IL-6. Tin chlorin e6 was the most active photosensitiser, resulting in > 25-fold increase in the LD_{50} for TNF-α and complete inactivation of detectable IL-6 activity following irradiation with a low dose of 633 nm laser light. A local reduction in the activities of these cytokines may help to reduce the destruction associated with their overproduction. As previously discussed, there has been much interest in the anti-inflammatory activity of plant extracts, although these compounds have generally only been shown to inhibit the production of proinflammatory cytokines rather than reduce the biological activity of the cytokines. Methods of inactivating preformed cytokines have mostly focussed on monoclonal antibodies and soluble cytokine receptors, which are usually administered systemically and associated with serious side effects (Belloni *et al.*, 2008; Ellerin *et al.*, 2003; Nash & Florin, 2005). Consequently, the use of these therapies is only relevant to serious diseases that require systemic therapy.

Further studies are required to investigate the ability of photosensitisation to reduce the biological activity of proinflammatory cytokines *in vivo* and to assess any potential adverse effects resulting from such inactivation. The localised nature of PDT is advantageous, as treatment should not have a systemic effect on host cytokines at distant sites and should therefore not be associated with such side effects. The use of a topical light-activated antimicrobial agent that can also cause a local reduction in the activity of proinflammatory cytokines could therefore present a promising treatment option for superficial bacterial infections that are associated with inflammation-induced pathology. However, a successful reduction of biological activity *in vivo* would consequently also depend on close proximity of the cytokine to the photosensitiser molecules due to the short lifetime and diffusion of singlet oxygen.

The light-activated antimicrobial agents methylene blue and tin chlorin e6 were both shown to successfully kill *P. gingivalis* and *S. aureus* and reduce the activity of a selection of their virulence factors at concentrations and light-doses that may be clinically relevant. Methylene blue is approved for clinical staining procedures at 1% w/v (27 mM); the concentrations of photosensitiser used in the experiments described in this thesis were in the micromolar range, and therefore may be considered to have a safe toxicological profile (Wainwright, 2000). The Periowave™ system, which is licensed for oral disinfection in Canada and the European Union, utilises methylene blue at a concentration of 0.005% w/v in PBS plus a mucoadhesive, equivalent to a photosensitiser concentration of 135 µM (Cassidy *et al.*, 2009; Ondine Biomedical, 2010a). As methylene blue was shown to successfully kill *P. gingivalis* and reduce the activity of some of its virulence factors at lower concentrations, it is possible that a reduction in virulence may be observed with the commercial formulation.

Although the toxicity of tin chlorin e6 has not been fully determined, porphyrins are associated with low *in vivo* toxicity. Other metalloporphyrin photosensitisers have been used clinically and toxicity was generally only observed following relatively high doses (Stojiljkovic *et al.*, 2001). The major toxicity concern with chlorins is long-term skin photosensitivity (Detty *et al.*, 2004); however, as photosensitivity is observed following systemic administration, the topical use of the photosensitiser may avoid such systemic adverse events. Tin chlorin e6 is well suited for conjugation to targeting molecules due to reactive carboxy groups on side chains outside of its polycyclic core, and conjugation to a variety of moieties has been demonstrated (Embleton *et al.*, 2005; Narband, 2009; Rakestraw *et al.*, 1992). Consequently, targeting of the photosensitiser to bacterial cells may lower the toxicity to human cells.

The low irradiation times required for the successful killing of these microorganisms and reduction of their virulence potential are ideal for the clinical setting, as a short treatment duration would cause less discomfort for the patient and would minimise damage to host tissues. Although complete bacterial eradication was not observed with the photosensitiser concentrations and light doses investigated in this thesis, repeat treatment or further optimisation of the doses may achieve complete microbial killing. Methylene blue has been used successfully in clinical studies for the photodynamic decolonisation of MRSA from the anterior nares; indeed, the MRSAid™ system has also been approved in Canada and the European Union for this purpose and uses methylene blue and 670 nm diode laser light (Street *et al.*, 2009). The MRSAid™ system involves an irradiation time of 10 minutes (Ondine Biomedical, 2010b); as the results presented in this thesis demonstrate that staphylococcal virulence factors are susceptible to methylene blue-based photodynamic inactivation using shorter irradiation times, it is therefore likely that inactivation of these virulence factors would occur during such a treatment. The Periowave™ oral photodisinfection system utilises a shorter irradiation time of one minute per treatment site (Ondine Biomedical, 2010d); an equivalent irradiation time using the 665 nm laser described in this thesis (4.38 J) caused a significant reduction in the activity of *P. gingivalis* proteolytic and haemagglutinating enzymes, and therefore such reduction in activity may occur when the treatment is used *in vivo*.

The lack of specificity of PDT may cause damage to host tissues, although it has been proposed that the light doses required for microbial killing would not significantly affect adjacent host cells (Wilson, 2004). It has recently been demonstrated that treatment with 118 µM methylene blue and 360 J/cm^2 670 nm laser light did not cause any tissue necrosis in a murine model (Zolfaghari *et al.*, 2009). Nevertheless, the specificity may be increased by the conjugation of targeting molecules such as antibodies. This approach has been successfully investigated *in vitro* for antimicrobial PDT using bacteriophages and antibodies as targeting agents

conjugated to tin chlorin e6 (Embleton *et al.*, 2004; Embleton *et al.*, 2005). *In vivo* studies for anticancer PDT have also been performed using antibody-conjugated photosensitisers, demonstrating that this approach may be successfully employed in animal models (van Dongen *et al.*, 2004).

In summary, photosensitisation has the potential to not only reduce the bacterial burden in superficial skin and oral infections, but also to reduce the pathology associated with bacterial virulence factors and proinflammatory cytokines. Further research is necessary to evaluate this potential *in vivo*. Additional studies may also be required to determine strategies to enhance the activity and selectivity of light-activated antimicrobial agents in order to maximise the therapeutic potential of PDT.

Publications arising from this work

Tubby, S., Wilson, M. & Nair, S. P. (2009). Inactivation of Staphylococcal Virulence Factors Using a Light-Activated Antimicrobial Agent. *BMC Microbiol* **9**, 211.

This publication is included at the end of this volume for reference.

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