

Killing of organisms responsible for wound infections using a light-activated antimicrobial agent

Submitted by

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Declaration

I, Ghada Omar certify that the work presented in this thesis is the result of my own investigations. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Ghada Omar

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Abstract

Infected wounds are a major cause of hospital-acquired infections and these are difficult to treat due to the emergence of antibiotic-resistant bacteria. This project is concerned with evaluating a novel antimicrobial approach involving the photosensitizer indocyanine green (ICG) which generates reactive oxygen species when irradiated with near-infrared (NIR) light which enables good tissue penetration. The photo-susceptibility of common wound-infecting organisms to ICG coupled with NIR-light was investigated. All species were susceptible to killing. ICG at a concentration of 25 µg/mL enabled the killing of the Gram-positive species (*Staphylococcus aureus* and *Streptococcus pyogenes*), higher concentrations (100-200µg/mL) were necessary to achieve substantial kills of the Gram-negative species (*Pseudomonas aeruginosa* and *Escherichia coli*). Both high and low fluences were able to kill 99.999% of the Gram-positive bacteria. High fluence irradiation was necessary to kill 99.99% of the Gram-negative bacteria. The pulsed-mode of irradiation was as effective as the continuous-mode for killing the Gram-positive species. Yet only the continuous-mode of irradiation was able to kill *P. aeruginosa*. Biofilms of *Staph. aureus* and *P. aeruginosa* were susceptible to disruption and killing by ICG-photosensitization. A significant enhancement of lethal photosensitization of *Staph. aureus* was achievable using gold-nanoparticles and antioxidants. Significant kills (>99%) were achieved in the presence of serum and 100 µg/mL ICG. A low oxygen concentration reduced the kills to 96.77% and 71.62% for *Staph. aureus* and *Strep. pyogenes* respectively. Mechanistic studies revealed that killing was mediated mainly by reactive-oxygen species.

In vivo studies in mice showed that ICG and continuous-NIR light could achieve kills of 96%, 93% and 78-91% for *P. aeruginosa*, *Strep. pyogenes* and *Staph. aureus* respectively.

The results of these *in vitro* and *in vivo* studies imply that ICG-PDT could be an effective means of decreasing the microbial burden in wounds.

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Abbreviations

°C	Degree Celsius
¹ O ₂	Singlet oxygen
ACP	Antioxidant carrier photosensitizer
ALA	Aminolaevulinic acid
AuNPs	Gold nanoparticles
BHI	Brain Heart Infusion
CFU	Colony-forming units
CLSM	Confocal laser scanning microscopy
cm	Centimetre
CNS	Coagulase-negative staphylococci
CSE	Control standard endotoxin
CVLU	Chronic venous leg ulcer
D ₂ O	Deuterium oxide
DM	Diabetes mellitus
DMMB	Dimethylmethylene blue
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
E-MRSA	Epidemic methicillin-resistant <i>Staphylococcus aureus</i>
EPR	Electron Paramagnetic Resonance
EPS	Extracellular polymeric substance
Er : YAG	Erbium-doped Yttrium aluminium garnet
Eusol [®]	Edinburgh University Solution of Lime
FeSO ₄	Ferrous Sulfate
GaAlAs	Gallium aluminium arsenide
h	Hour
HAIs	Hospital-acquired infections
Hb	Haemoglobin
HbO ₂	oxyhaemoglobin
He-Ne	Helium/neon
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HS	Horse serum
HSA	Human serum albumin
ICG	Indocyanine green
LAL	Limulus amoebocyte lysate
LB	Luria broth
LED	Light emitting diode
LP	Lethal photosensitization
LPS	Lipopolysaccharide
LRW	LAL reagent water
MAL-PDT	Methyl aminolevulinate photodynamic therapy
MB	Methylene blue
MDR	Multidrug-resistant
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSA	Mannitol salt agar
msec	Millisecond

MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
NaSCN	Sodium thiocyanate
NB	Nutrient broth
Nd : YAG	Neodymium-doped Yttrium aluminium garnet
NIR	Near-Infrared
nm	Nanometer
NMB	New methylene blue
OSCN	Hypothiocyanate
PBS	Phosphate buffered saline
PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PFF	Proximal femoral fracture
PIT	Pre-irradiation time
PMNL	Polymorphonuclear leukocytes
Ppm	Parts per million
PS	Photosensitizer
PTMPP	Meso-mono-phenyl-tri(N-methyl-4-pyridyl)-porphyrin
QS	Quorum sensing
RB	Rose Bengal
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	sodium dodecyl sulfate
sec	Second
SLDs	Super Luminous Diodes
SnCe6	tin (IV) chlorin e6
SOD	Superoxide dismutase
SOSGR	Singlet oxygen sensor green reagent
Spp.	Species
SPR	Surface plasmon resonance
SSD	Silver sulfadiazine
SSI	Surgical site infection
TBO	Toluidine blue O
TEM	Transmission electron microscopy
TNF- α	Tumour necrosis factor
TSB	Tryptic soy broth
UV	Ultraviolet
Vit	Vitamin
VSV	Vesicular stomatitis virus
W	Watt
μ g	Microgram
μ L	Microlitre
μ M	Micro-molar

Chapter 1

Introduction

1.1 Wounds

The skin is the outer covering of the body, which protects it from any external harm. A disruption of the normal integrity of the anatomic structure and function of the skin is defined as a wound (Lazarus *et al*, 1994). This includes injury of underlying tissues/ organs caused by surgery, a blow, a cut, chemicals, heat/cold, friction/shear force, pressure or as a result of disease (Bale, 2000).

1.1.1 Classification and types of wounds

There is no definite method of classifying wounds. Wounds can be referred to by their anatomical site, e.g. abdominal or axillary wound (Bale, 2000). Wounds are popularly categorized by their level of chronicity as either an acute or a chronic wound. Acute wounds usually follow trauma or inflammation and are caused by external damage to intact skin and usually heal within six weeks. Surgical wounds, bites, burns, minor cuts and abrasions, and more severe traumatic wounds such as lacerations and crush or gunshot injuries are examples of acute wounds. In contrast, chronic wounds, in addition to failing to heal after six weeks, have characteristic pathological associations due to underlying endogenous mechanisms associated with a predisposing condition that ultimately compromises the integrity of dermal and epidermal tissue that inhibit or delay healing (Bowler *et al.*, 2001; Kumar & Leaper, 2008). Pressure ulcers, venous leg ulcers, and diabetic foot ulcers are examples of chronic wounds (De la Torre & Chambers, 2008). These wounds are visible evidence of an underlying condition such as extended pressure on the tissues, compromised tissue perfusion as a consequence of impaired arterial supply (peripheral vascular disease) or impaired venous drainage (venous hypertension) and metabolic diseases such as diabetes mellitus (DM), or even poor nutrition (Bowler *et al.*, 2001).

In addition, wounds can be classified by their aetiology, for example bite, stab, or by the type of their closure (e.g. primary or secondary intention) or by the depth of tissue involvement and complexity of the injury (Kumar & Leaper, 2005). Wound depth is classified by the initial level of tissue destruction evident in the wound: superficial, partial-thickness, or full-thickness (Barnard & Allison, 2009). The USA National Research Council categorizes wounds according to the degree of contamination, which has implications for wound

management and predicts infection risk (Table 1-1. Leaper, 2006; Kumar & Leaper, 2008).

Table 1-1: The USA National Research Council wound classification

Classification	Criteria	Infective Risk (%)	Examples
Clean (Class I)	Uninfected operative wound No acute inflammation Primarily closure No entrance to respiratory, gastrointestinal, biliary, and urinary tracts No break in aseptic technique Closed drainage used if necessary	<2	Non-implant Mastectomy Herniorrhaphy Implant Hip replacement Hernioplasty
Clean-contaminated (Class II)	Elective entry into respiratory, biliary, gastrointestinal, urinary tracts with minimal spillage No evidence of infection or major break in aseptic technique	<10	Appendectomy Cholecystectomy Elective lung resection
Contaminated (Class III)	Absence of purulent inflammation Gross spillage from gastrointestinal tract Penetrating traumatic wounds <4 hours old Major break in aseptic technique	About 20	Stab wound Non-perforated appendicitis A perforated bowel.
Dirty-infected (Class IV)	Purulent inflammation present Preoperative perforation of viscera Penetrating traumatic wounds >4 hours old		debridement of a pressure ulcer

1.1.2 Wound healing and factors adversely affecting it

Wound healing, following trauma or elective surgery, is a complex sequence of biological processes that include inflammation, migration, and remodelling. Healing takes place in an attempt to not only repair but also to recompense for the loss of function which has occurred as a result of tissue damage (Jones *et al.*, 2004). Healing failure leads to a prolonged recovery period and extensive limitations to function. A number of factors can disturb normal wound healing processes, slowing them down or completely impairing them. These factors can be intrinsic or extrinsic (Table 1-2. Bale, 2000; Percival, 2002; Halloran & Slavin, 2002). Infection is the most critical factor, which has detrimental effects on wound healing (Bale, 2000).

Table 1-2: Factors influencing tissue healing

Intrinsic factors	Extrinsic factors
Impaired nutrition status (e.g. protein, zinc & Vitamins A, B, C and E deficiency) Aging process Dehydration Underlying diseases (e.g. diabetes mellitus, cancer & HIV) Insufficient blood supply and oxygen (Smoking may produce hypoxia, Hypoxemia) Hypercatabolic states (neoplasia, uraemia & jaundice)	Poor surgical technique (i.e.Hematoma) Curative therapy (cytotoxic agents, steroids & radiation therapy) Inappropriate wound management (poor dressing technique) Excess Pressure (e.g. Pressure, shear and friction) Adverse psychosocial factors (stress & anxiety) Infection The presence of necrotic tissue and foreign materials

1.2 Wound Infections

1.2.1 Historical background of wound infections

Since time began, wound infections remain a significant dilemma that challenges physicians all over the world. Sumerian's cuneiform tablets that are believed to be older than 2,000 BC are considered to be the earliest written evidence in which wounds and their treatments were mentioned (Majno, 1975; Ovington, 2002). Thereafter, the Egyptians and the Greeks were two of the first civilizations to practice medicine in a systematic and well documented manner. The Egyptians recognized the cardinal signs of wound infection and inflammation, also they identified subsequent antiseptic treatments such as decoction of willow which contained salicin and sodium salts as well as honey, which nowadays has been shown to be beneficial in inhibiting the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) in infected leg ulcers (Sipos *et al.*, 2004). The Greeks were the first to classify wounds into acute and chronic (Ovington, 2002). Hippocrates used vinegar, wine or sea water to irrigate infected wounds to accelerate the healing process (Cope, 1958). In the first century, Celsus (ca 25 BC—ca 50 BC) described how tissue reacted to microbes and named the four major signs of inflammation: heat, pain, swelling, and redness (Cope, 1958). Throughout the Middle Ages and the Renaissance, there were limited advances in wound care.

The most profound advances, both technological and clinical, came with the development of microbiology and cellular pathology in the 19th century. Louis Pasteur (1822-1895), Joseph Lister (1827-1912) and Koch (1843-1910)

revolutionized the entire concept of wound infection. In 1864, Pasteur proved that fermentation was due to the presence of micro-organisms in the environment. In 1865, Lister adapted the germ theory of Pasteur to reduce mortality rates after amputation (Cope, 1958). Lister used antiseptics such as creosote to sterilize open wounds to prevent sepsis and amputation. Later on, Lister reduced cross-contamination by spraying or washing hands, instruments and bandages with carbolic acid (phenol) prior to performing surgery. After he introduced antiseptic techniques to surgery, mortality rates from gangrene in hospitals where he practiced dropped from almost 48% to only 15% (Newsom, 2008). In 1877 Koch followed Lister's ideas about sepsis. He used high-powered microscopy to demonstrate bacteria in wounds, and showed that different bacteria were involved. By 1881 Koch perfected the 'plate technique' that allowed identification of different bacteria from the appearance of their colonies. Alexander Ogston, was the first to name staphylococcus and show that *Staphylococcus aureus* caused abscesses in 1882. As time went by, antiseptics was replaced by aseptic techniques (Newsom, 2008).

Between 1870 and 1873 the mortality rate following amputations in the great centres of civilisation (England, France, Germany, and America) was 30 – 50% (Newsom, 2008). This was due to 'Hospitalism' a word which was introduced by Sir James Simpson. Hospitalism is now known as "hospital-acquired surgical-site infection". Shock, erysipelas (streptococcal infection) or pyaemia (staphylococcal infection) and hospital gangrene were the big post-operative killers (Newsom, 2008). The scope of wound infections was also dominant in times of war. In 1883, during the American Civil War, both necrotizing tissue infection and tetanus resulted in over 17,000 deaths (Singhal *et al.*, 2009). During the First World War (WWI), Antoine Depage (Belgian military surgeon, 1862-1925) reintroduced wound debridement and delayed wound closure together with the microbiological assessment of wounds. He used antiseptics especially Dakin's solution (hypochlorite of soda) as an adjunct to wound debridement and excision for treating infected wounds (Helling & Daon, 1998). Alexander Fleming (1881-1955) performed many of his bacteriological studies during WWI and is credited with the discovery of penicillin in 1929. Afterwards, a series of antibacterial drugs that exert a strong bactericidal effect against a number of micro-organisms, including the septic organisms were discovered

such as the sulphonamides and prontosil (Cope, 1958). The discovery and development of antibiotics opened a new era in the management of wound infections. Unfortunately, elimination of the sepsis affecting wounds has not come to an end due to the emergence of antibiotic-resistant bacterial strains and the nature of more challenging surgical intervention in immunocompromised, oncologic and organ transplant patients.

1.2.2 Contamination, colonization and infection

Within any wound, there is an interaction between the host and the micro-organisms adherent to the wound. The interaction may vary from simple contamination through colonization on to local infection, and finally to disseminated infection which is characterized by the presence of fever, cellulitis, and/or septicaemia (Figure 1-1). It is important to have a clear understanding of the interactions involved in the process of infection. Contamination can be defined as the presence of micro-organisms within the wound with no evident multiplication (e.g. contamination by soil organisms in an open wound). Once the micro-organisms start to multiply with no apparent host reaction or clinical signs, this is termed colonization. Skin commensals include *Staphylococcus epidermidis* and *Corynebacterium* species, which in most circumstances increase the rate of wound healing (Schultz *et al.*, 2003). A transitional stage of local infection or “critical colonization” will follow, if the micro-organisms continue to multiply and impede the rate of healing (Collier, 2004; Edwards & Harding, 2004; Scanlon, 2005).

The critical load of bacteria plays a key role in delaying wound healing since bacteria stimulate the release of pro-inflammatory mediators (e.g. matrix metalloproteinases MMPs), which impair healing. A non-healing wound can be identified clinically once the wound margins fail to contract. An increase of serous exudates within fragile bright red granulation tissue may be evident. This appearance is due to the stimulation of angiogenesis and the production of an imperfect brittle matrix that leads to bleeding at the wound surface (Schultz *et al.*, 2003).

In the case of critical colonization, it is believed that delayed healing may be attributed to microbial factors. This concept was based on the improvement of

the healing once the wound was treated with antimicrobial agents (Fleck, 2006). There are three hypotheses explaining how bacteria delay wound healing without any apparent host response (e.g. inflammatory or immunological response). These include immuno-evasion (Allen *et al.*, 2005), the formation of biofilms (Serralta *et al.*, 2001), and the inhibition of cellular responses during wound healing (Stephens *et al.*, 2003). These modes can occur when the wound is colonized by a certain number of invasive bacteria (White & Cutting, 2006a).

The wound colonization can deteriorate to local infection or critical colonization with increasing bacterial multiplication and pathogenicity. The overt clinical signs associated with local wound infection can be summarized as follows: discharge of pus with swelling, pain, erythema and local warmth, evidence of surrounding tissue involvement or wound breakdown; wound appears unhealthy or deteriorating, and probing infection to the bone (cellulitis, lymphangitis, osteomyelitis or gangrene). However if the infection becomes systemic a general fever, rigours, chills, hypotension, and multiple organ failure may proceed (Sibbald *et al.*, 2003).

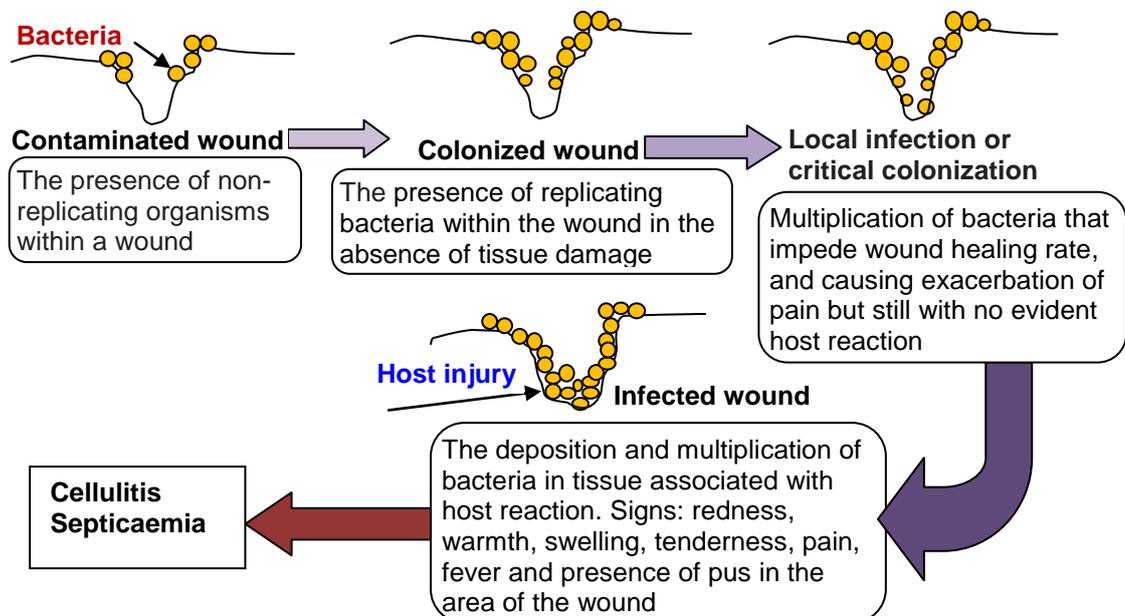


Figure 1-1: Diagram shows the different phases of bacterial interactions in a wound progressing from contamination to infection.

1.2.2.1 Definition of wound infection

A wound is a breach of the skin that can lead to infection and sepsis. Although all wounds are contaminated, most effectively resist invasive infection. Infection proceeds due to many factors including the number of bacteria per gram of tissue, the pathogenicity and virulence of the organism, and the ability of the host to counteract with an efficient immune system (Edwards & Harding, 2004). When the bacterial load exceeds 10^5 organisms per gram of tissue, or when the immune system becomes suppressed, infection develops (Robson *et al.*, 1999). However, beta-haemolytic streptococci cause infection at significantly lower levels and their presence in a wound biopsy can be indicative of infection and a delayed healing response regardless of the bacterial count (Edwards & Harding, 2004). Thus, the number of organisms in a wound cannot always be used as an indication of invasiveness (McGuckin *et al.*, 2003). Uncontrolled wound infection may result in not only delayed wound healing, but also may lead to fatal complications include cellulitis, septicaemia or even death (Scanlon, 2005; De la Torre & Chambers, 2008). The concept of infection, however is complex, it can be summarized in the following equation:

$$\text{Infection} = \frac{\text{Bacterial Load X virulence}}{\text{Host resistance}}$$

Although wound treatment and infection prevention strategies are advanced nowadays, some wounds are susceptible to infection. Several factors that predispose wounds to infection are well documented and influence the way colonized wounds may respond. These are local or systemic factors. Local factors include: wound criteria (mechanism of injury, location, area, depth and degree of chronicity), presence of dead tissue or foreign bodies, tissue oxygen levels (e.g. high risk of infection <25 mm Hg) (Kalani *et al.*, 1999), preoperative supplementation of oxygen. While systemic factors include general health status, extreme age (i.e. neonates and elderly), dietary imbalance (i.e. malnutrition), immunodeficiency secondary to inherited neutrophil defects, DM, alcoholism as well as long term drugs usage (such as corticosteroids, immunosuppressive agents used for organ transplants or chemotherapy) (Kingsley, 2001; Sibbald *et al.*, 2003; Cooper, 2005).

Acute wounds are more susceptible to infection than chronic wounds (Robson *et al.*, 1973). Wounds that have not been debrided of necrotic tissue can predispose the patient to systemic infection; therefore, measures to prevent wound infection must include debridement of eschar or necrotic tissue and particular wound cleansing, along with measures to prevent wound dehydration (Robson *et al.*, 1973; Hutchinson & McGuckin, 1990; McGuckin *et al.*, 2003). Nevertheless, sometimes these measures fail to prevent infections that consequently will have enormous consequences.

1.2.3 The impact of wound infections on both healthcare system and patients

Wound infection is a major cause of morbidity and mortality that is associated with subsequent high costs worldwide. Infected wounds affect many thousands of people in the United Kingdom. As a consequence, the management of these wounds is an ongoing problem which places a considerable drain on healthcare systems. Infections of surgical wounds are one of the most common causes of hospital acquired infection (HAI) in the United Kingdom. The cost of HAIs resulting from infected wounds is estimated to be £162 million per year (Plowman *et al.*, 1999). The Nosocomial Infection National Surveillance Service report (2002) showed that the incidence of HAIs linked to surgical wounds is as high as 10%. More to the point, 77% of the deaths of patients that underwent surgery are attributed to wound infection. Furthermore, it was estimated that surgical site infections are the most common infections among postoperative patients in the United States, accounting for approximately 25% of all nosocomial infections (Patel *et al.*, 2008). Additionally, bacterial infection is the main cause of increased length of hospital stay by an average of 10 days due to the delay in recovery which results in doubling of hospital costs (Plowman, 2000). These sums were confirmed by a study undertaken in the UK. This study reported that the cost per case of HAIs was on average 2.5 times greater than for non-infected skin or surgical wounds, which is equivalent to between £1,618 and £2,398 per person (Plowman *et al.*, 1999). Similarly in the United States the cost for wound care is approximately \$2.3 billion per year (Patel *et al.*, 2008).

The above figure was only related to surgical wound infection and did not include the costs associated with chronic wound infections such as diabetic foot ulcers. It is known that infections in foot ulcers are common and disabling and frequently lead to amputation of the affected part. In the UK around 24,000 admissions a year are for patients with diabetic foot ulceration, thereby costing the NHS around £17 million. The cost for venous leg ulcers is about £400 million annually (Harding *et al.*, 2002). These sums show only the direct costs and do not reflect the untold misery, frustration, economic loss, and impaired quality of life experienced by individual sufferers and their families.

Infected wounds can cause great distress to the patient, especially with the rising prevalence of resistant bacterial strains (Ansermino & Hemsley, 2004). The state of delayed healing accompanying an infected wound is a cause for patients' concern, which adversely affects their physical and social qualities of life. Infections not only delay wound healing but also increase pain, anxiety, discomfort and costs by those patients which result in physical and psychological trauma (Kingsley, 2001).

A study by Pollard *et al.*, 2006 demonstrated the great impact of wound infection after surgery for proximal femoral fracture (PFF) on the patient in terms of mortality and social consequences, and on the National Health Service in terms of financial burden. Patients with wound infections had greatly increased hospital stays (80 days compared to only 28 days in the control group) and were 4.5 times less likely to survive to discharge. The total cost of the treatment per infected case was £24,410 compared with £7,210 for controls. 51% of the infected cases suffered from MRSA infection which increased admission length and cost compared with non-MRSA infection. These findings indicate that infected wounds require considerable healthcare interventions and that the management of these wounds costs a substantial amount of money which results in lost opportunities to provide care for other patients.

1.2.4 Wound-associated organisms

Bacteria may affect all of the processes of wound healing (from inflammation to remodelling) and they are always present in the surface and deep tissues of all wounds. Through different stages of healing, micro-organisms seem to interact with acute and chronic wounds in similar but distinct ways. During the inflammatory phase, impaired haemostasis or inflammation reportedly favours infection, for instance dirty surgical wounds with inflammatory foci (foreign body or necrotic tissue) are twice as likely to be infected as a contaminated surgical wound. In acute wounds, micro-organisms may enhance or have no effects on the granulation and epithelization stages of wound healing, although in chronic wounds they may inhibit the healing process. Acute wounds are at risk of infection during the 24-72 hours after injury while chronic wounds are often colonized with bacteria. Many variables affect the relationships between micro-organisms and the wounds they occupy, including wound features, patient conditions, organism virulence and environmental variables (McGuckin *et al.*, 2003). For example, the normal skin microbiota, if present in large numbers, can lead to sepsis in a patient with a graft/flap while a chronic leg ulcer can continue to harbour these organisms for years with minor life-threat to a patient (Bowler *et al.*, 2001).

1.2.4.1 Organisms associated with non-infected wounds

All wounds are colonized with micro-organisms at the peri-wound area by the endogenous microbiota of the body, other contaminants from the patients surrounding environment at the time of injury or micro-organisms introduced from staff or visitors (non-resident exogenous microbiota) (Edwards-Jone & Greenwood, 2003; Erol *et al.*, 2004). However, most microbial contaminants (both aerobic and anaerobic) are endogenous to the host (cutaneous, oral and gastrointestinal microbiota) but they are exogenous to the wound. Subsequently, moist, superficial traumatized tissue will embrace these organisms, amongst many others that have disseminated into deeper wound tissue (Bowler *et al.*, 2001).

Normally, the indigenous or normal body microbiota can be hugely beneficial to the host and play an important role in keeping the bacterial balance and

protecting the host from pathogenic microbes. Although if the immune system becomes compromised, or the skin is broken, or after local changes within the body occurs due to prolonged antibiotic treatment or pH unbalance in vagina or stomach, the potential pathogens of the microbiota overgrow and invade the tissues (Mims *et al.*, 1998).

Normal microbiota that may infect wounds includes skin and mucous membrane microbiota such as coagulase-negative staphylococci (CNS) (e.g. *Staph. epidermidis*) (Bowler & Davies, 1999a), *Staph. aureus* (also colonize nose), *Corynebacterium* spp., micrococci, propionibacteria, streptococci, *Pseudomonas aeruginosa*, and *Candida* spp.; members of the oral microbiota such as *Streptococcus mutans*, *Bacteroides* spp., *Fusobacterium* spp., streptococci; and members of the gut microbiota for instance *Enterococcus faecalis*, coliforms, *Bacteroides* spp., *Clostridium* spp., *Pseudomonas* spp., *Klebsiella* spp., enterobacteria, *Lactobacillus* spp., *Escherichia coli* (Bowler *et al.*, 2001; Ratliff *et al.*, 2008).

The microbes that contaminate wounds are diverse and depend on the type, location and chronological age of the wound. Both anaerobic and aerobic bacteria contaminate wounds; the anaerobe to aerobe ratio is estimated to be 1000:1. Consequently, the resulting polymicrobial ecosystem colonizing a wound is unique and complex (Bowler, 2003).

White & Cutting, (2006a) reported that a chronic wound can be colonized but not infected with one or more of certain bacteria among them *Morganella* spp, *P. aeruginosa*, and *Peptostreptococcus* spp. These wounds may exhibit erythema and delayed healing without a traditional or even evident host response. Scenarios involving these organisms, and possibly others yet to be identified, have been used to postulate the concept of critical colonization, which was defined as multiplication of organisms with no invasion yet hampering wound healing (Edwards & Harding, 2004).

In a fast healing wound there is only a short time for skin contaminants to colonize the wound. However, in a slowly healing chronic wound there is continuous exposure of the devitalized tissue to contaminants which is likely to

facilitate the colonization and establishment of a wide variety of endogenous micro-organisms. For instance, anaerobes constitute up to 95-99 % of the gut microbiota which are estimated at 10^{11} micro-organisms/g of tissue (Mims *et al.*, 1998). *Bacteriodes* spp. are a major component of the faecal material that is often isolated from contaminated wounds (Bowler *et al.*, 2001).

Most of the microbiological studies carried out to determine the microbial population present in wounds classify wounds according to the level of chronicity or according to the level of contamination (non-infected or infected). The predominant organism isolated from clean, non-infected chronic wounds was *Staph. aureus* (Ratliff *et al.*, 2008). Another study by Bowler and Davies, (1999b) reported that 53% of the isolates from non-infected leg ulcers were staphylococci, mainly *Staph. aureus*. In addition, anaerobes represented 36% of the total microbial population in these wounds. It is evident from this study that leg ulcers are populated by a wide variety of endogenous anaerobes as well as the more commonly recognized aerobic/facultative organisms.

Burn wound microbial colonization and the indigenous microbiota isolated from 51 patients was studied weekly for a period of 3 weeks (Altoparlak *et al.*, 2004). In an acute non-infected wound, the predominant genus was *Staphylococcus*; 63 % of the isolates were CNS and 19.7 % were *Staph. aureus*. The rest included 2% *P. aeruginosa*, 1.6 % *Enterobacter* spp., 1.2 %, *Candida* spp., 3.1 % diphtheroids, 1.2 % *Escherichia. coli*, 1.6 % pneumococci, 2.3 % β -haemolytic streptococci, 2.0 % *Bacillus* spp. and 0.4 % non-haemolytic streptococci (Altoparlak *et al.*, 2004).

The microbial species found in the normal microbiota of the gut or oral cavity closely correlate with the micro-organisms present in wounds in close proximity to those sites (Brook & Frazier, 1997; Brook & Frazier, 1998a). De Paula *et al.*, (1991) analyzed the bacterial isolates from anal wounds after open haemorrhoidectomy. Specimens were collected from the patients' open wounds, intraoperatively, and on the 6th, 13th and 20th postoperative days for bacteriologic examination. The most commonly identified bacterium was *E. coli*, followed by *Staph. aureus* and *Staph. epidermidis*. *P. aeruginosa*, *E.*

faecalis, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Proteus mirabilis* were also identified.

Larson *et al.*, (1998) conducted a prospective observational study on 40 nurses (20 with diagnosed hand irritation and 20 without). They reported that nurses with damaged skin did not have higher microbial counts, but did have a greater number of colonizing species. Nurses with damaged skin were significantly more likely to be colonized with *Staphylococcus hominis* of which 59 % percent were resistant to methicillin compared with 27% of isolates from those with healthy skin. 20 % of nurses with damaged hands were colonized with *Staph. aureus* compared to none of the nurses with healthy hands. Nurses with damaged hands were also twice as likely to have gram-negative bacteria, enterococci, and *Candida* spp. present on the hands.

Bacteria may inhibit a number of key processes in wound healing, from angiogenesis to re-epithelization (Jones *et al.*, 2004). However, in acute wounds, bacterial colonization could play a vital part in initiating a degree of inflammatory response. Theoretically, bacteria may accelerate wound healing with beneficial effects on each phase of wound healing, including increased granulation tissue formation and angiogenesis and increased tensile strength of the wound, with increased infiltration of polymorphonuclear leukocytes (PMNL) and macrophages, increased levels of prostaglandin, and an increase in collagen formation (Jones *et al.*, 2004). Also, bacteria produce proteolytic enzymes such as hyaluronidase which contribute to wound debridement and stimulate neutrophils to release proteases (Schultz *et al.*, 2003).

1.2.4.2 Wound-infecting organisms

The complex polymicrobial population that colonizes wounds is likely to consist of mixed aerobic and anaerobic bacteria, many of which may be antibiotic-resistant and/or pathogenic (Bowler, 2003). Although the infective dose of micro-organisms is a crucial factor in wound infection, the synergistic mechanism by which multi- species communicate within a wound may increase the degree of the pathogenicity of these organisms even if they are below the infective dose (Edwards & Harding, 2004). Consequently, this polymicrobial

population not only provides an infection threat to the wound, but also presents a serious cross contamination risk, particularly in the hospital environment.

The increase in microbial burden can endanger the wound especially under local environmental conditions that favour microbial reproduction (e.g. moisture, nutrition, and a low oxygen tension). In this favourable but abnormal environment, micro-organisms thrive (particularly if the local circulation is compromised), and their relationship with the host may change from symbiotic to pathogenic in order to challenge other colonizing micro-organisms and resist elimination by the host's immune system. This may involve bacteria increasing the production of specific enzymes or toxins, producing cell adhesion and cell-protecting components (e.g. biofilms), or interacting with other bacteria in order to gain a competitive advantage over the host. The density and diversity of the wound microbiota are likely to influence communication strategies within and among species that subsequently intensify the expression of virulence factors essential for microbial survival. The net effect is a probable increase in microbial pathogenicity; hence a greater challenge to the host (Bowler, 2003).

Many microbiological studies have reported that *Staph. aureus* (including MRSA), streptococci (Brook, 1989; Brook & Frazier, 1998b) and *P. aeruginosa* (Agnihotri *et al.*, 2004; Nasser *et al.*, 2003; Giacometti *et al.*, 2000; Guggenheim *et al.*, 2009) are the most frequently identified aerobic pathogens causing invasive wound infection. In bite wounds, aerobic or facultative bacteria were isolated from all clenched-fist injury (CFI), 17 of 18 human bite (HB), and 33 of 39 animal bite (AB) wound cultures (Goldstein *et al.*, 1978). The α -haemolytic streptococci were the organisms most frequently isolated from all types of bite wounds. *Staph. aureus* was isolated from 18 wounds. However, in human bites, group A streptococci, *Staph. aureus*, and *Eikenella corrodens* were usually associated with infection (Goldstein *et al.*, 1978).

Staph. aureus is a key human wound-infecting pathogen that interferes with cell functions of the host and causes delayed epithelial closure of the wound, possibly due to its interaction with fibronectin and inhibition of keratinocyte migration (Cooper, 2005). In accordance with Athanasopoulos *et al.*, 2006, impaired healing observed in *Staph. aureus* infected wounds was attributed to

the extracellular adherence protein (EAP). This study demonstrated that EAP is a potent anti-inflammatory and anti-angiogenic agent that has the ability to interact with adhesion molecules especially those of endothelial cells as well as with adhesive proteins in the extracellular matrix (ECM). Thus it prevents migration of both inflammatory and endothelial cells to the wound site, in turn inhibiting neovascularisation.

In a general study of the microbiology of acute and chronic wounds, 61 acute wounds including abscesses, surgical and traumatic wounds were examined. 14 of the 61 acute wounds were clinically infected and 65% of these isolates were aerobes. In these infected acute wounds, *Staph. aureus* was the most frequently isolated facultative bacterium (isolated from 43 % of abscess wounds) and often existed as a pure culture or in combination with other Gram-positive aerobes. Anaerobes were quite rare in infected acute wounds (with the exception of *Peptostreptococcus* spp.). 12 of 45 chronic wounds (primarily leg ulcers, pressure and foot ulcers) were infected. Anaerobic bacteria (*Peptostreptococcus* spp., *Bacteroides* spp., non-pigmenting *Prevotella* spp., pigmenting *Prevotella/Porphyromonas* spp., *Clostridium* spp.), coliform bacteria, and faecal streptococci were common in the infected chronic wounds. However, none of these wounds were colonized by *Staph. aureus* or *P. aeruginosa*, the most frequently implicated bacteria in wound infection (Bowler & Davies, 1999a).

1.2.4.2.1 Surgical wound infection

The pathogens present in an acute surgical wound can be directly correlated with the surgical procedure. For example, the usual cause of infection in a clean surgical procedure is *Staph. aureus* from an exogenous source or from the patient's own microbiota. In a clean-contaminated, contaminated, or dirty/infected surgical site, polymicrobial aerobic and anaerobic microbiota, resembling the endogenous microbiota of the resected organ, are the most frequently isolated pathogens (Nichols, 1991).

According to data from the National Nosocomial Infections Surveillance System (NNIS), the incidence and distribution of pathogens isolated from infections during the last decade have not changed. However, an increase in multiple-

resistant bacteria, such as MRSA, has been noted. In addition, several studies have reported that surgical site infection (SSI) rates after discharge ranging from 20% to 84% (Thomas *et al.*, 2004; Oliveira *et al.*, 2007; Whitby *et al.*, 2007). *Staph. aureus* was the major cause of SSI after orthopaedic and cardiac surgery, which account for approximately 20% of all SSIs. The Hospital in Europe Link for Infection Control Surveillance (HELICS) network reported that *Staph. aureus* accounted for 48.6% of the pathogenic bacteria isolated from 59,274 hip prostheses. Similarly, *Staph. aureus* represented 43.7% of pathogens after coronary artery bypass grafting (Saadatian-Elahi *et al.*, 2008).

1.2.4.2.2 Burn wound infection

Infection remains the major cause of morbidity and mortality in burn patients in spite of the significant improvements in burn management (Howard *et al.*, 1999). The type and amount of micro-organisms colonizing the burn wound influence the frequency of invasive burn wound infection and the clinical characteristics of such infections (Pruitt *et al.*, 1998; de Macedo & Santos, 2005; Church *et al.*, 2006).

The most common pathogens isolated from a burn wound are *Staph. aureus* (75%) *P. aeruginosa* (25%), *Strep. pyogenes* (20%), and various coliform bacilli (5%) (Lawrence, 1992). Also streptococci, anaerobic organisms and fungi (*Candida albicans* and *Aspergillus fumigatis*) can also cause burn infection (Lawrence, 1992). Gram-positive bacteria such as *Staph. aureus* are likely to infect small surface area burns whilst the larger burns become infected with both *Staph. aureus* and *P. aeruginosa*. Staphylococci are the most prevalent bacteria identified from burn wound cultures on admission but are superceded within a week, mainly by *P. aeruginosa* (Alttoparlak *et al.*, 2004; Nasser *et al.*, 2003). Both studies concluded that the micro-organisms causing burn infections can change over time and this should be taken into consideration when planning antimicrobial therapy for burn patients.

1.2.4.2.3 Traumatic wound infection

A broad study of the micro-organisms causing infection following trauma was performed from 1973 to 1988 (Brook & Frazier, 1998a). A total of 368

specimens obtained from 340 trauma patients showed bacterial growth. The traumas included lacerations (163), blunt trauma (76), penetrating trauma (65), bites (20), and open fractures (10). The types of infections included abscesses (109), bacteraemia (32), bites (13), empyema (10), osteomyelitis (21), peritonitis (52), thrombophlebitis (12), and wounds (116, including post-traumatic wounds, cellulitis, stump wound, decubitus ulcers, myositis, and fasciitis). Anaerobic bacteria were isolated from 32% of the total specimens whilst aerobic bacteria from 16%, and mixed aerobic-anaerobic microbiota from 52%. These data highlighted the role that multimicrobial species play in many infections following trauma. A total of 444 anaerobic species were recovered which included *Bacteroides fragilis* group, *Peptostreptococcus* spp, *Clostridium* spp, *Prevotella* spp, and *Fusobacterium* spp. Also 267 aerobic or facultative bacteria were isolated. These included *E. coli* (83), *Staph. aureus* (61), *Streptococcus pyogenes* (27), group D streptococci (16), and *Klebsiella pneumoniae* (16). *Staph. aureus* was isolated at all body sites. Yet, organisms of the oral microbiota predominated in infections that originated from head and neck wounds, abscesses or bites, those of the gut microbiota predominated in infections that originated from that site such as peritonitis and abdominal abscesses (Brook & Frazier, 1998a).

Table 1-3 summarizes some of the studies carried out to provide microbial analyses of acute infected wounds.

Table 1-3: Studies involving the microbial analysis of acute infected wounds

Author	Description of the study	Predominant aerobes	Predominant anaerobes
Isibor <i>et al.</i> , 2008	isolate aerobic bacteria and <i>Candida albicans</i> from infected post-operative wounds	<i>Staphylococcus aureus</i> (35.0%), <i>Pseudomonas aeruginosa</i> (26.0%), <i>Escherichia coli</i> (13.0%), <i>C. albicans</i> (9.3%), <i>Klebsiella aerogenes</i> (7.4%), <i>Proteus</i> spp. (7.4%) and <i>Streptococcus</i> spp. (1.9%).	Not applicable
Nasser <i>et al.</i> , 2003	To verify the pattern of microbial colonization of burn wounds, throughout the study period starting from 1999 till 2001.	<i>Pseudomonas aeruginosa</i> 21.6 % <i>Klebsiella pneumoniae</i> 15.2 % <i>Escherichia coli</i> 13.6 % <i>Staphylococcus aureus</i> 13.2 % Coagulase-negative <i>Staphylococci</i> 11.6 % <i>Streptococcus pyogenes</i> 8.3 % <i>Enterobacter</i> species 6.6 % <i>Streptococcus faecalis</i> 5.9 % <i>Candida albicans</i> 3.6 %	None
Appelgren <i>et al.</i> , 2002	A 3-year prospective study for all infections presenting in the burns unit	<i>Staphylococcus aureus</i> <i>Pseudomonas aeruginosa</i> <i>Streptococci</i> group A, B, G Coagulase-negative <i>staphylococci</i> <i>Enterobacter</i> spp.	
Brook and Frazier, 2000	Bacterial isolates for 18 spinal fusion postoperative wound infections	<i>Enterococcus</i> spp. , Nonhemolytic streptococci, <i>Staph. aureus</i> , <i>Proteus mirabilis</i> , <i>Proteus vulgaris</i> , <i>E. Coli</i> , <i>Klebsiella pneumoniae</i> , <i>P. aeruginosa</i> , <i>Enterobacter</i> spp., <i>Serratia marcescens</i>	<i>Peptostreptococcus</i> spp., <i>Veillonella parvula</i> , <i>Propionibacterium acnes</i> , <i>Clostridium perfringens</i> , <i>Bacteroides</i> spp., <i>B. fragilis</i> , <i>Bacteroides distasonis</i> , <i>Bacteroides vulgates</i> , <i>Bacteroides thetaiotaomicron</i>
Bang <i>et al.</i> , 1998	Determination of the bacteria causing septicaemia in burn patients	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) 40.7 %, Methicillin-resistant <i>Staphylococcus epidermidis</i> (MRSE) 14.4%, <i>Pseudomonas</i> 12.7%, <i>Acinetobacter</i> 10.2 %, <i>Streptococcus</i> 3.4%, <i>Enterococci</i> 3.4%, <i>Klebsiella</i> 1.7%, and 12.7% to more than one organism.	None
Brook, 1989	65 specimens from 74 patients with postthoracotomy sternal wound infection	<i>Staphylococcus epidermidis</i> (28 isolates), <i>Staphylococcus aureus</i> (21 isolates), and <i>Enterobacteriaceae</i> spp.(14 isolates)	<i>Peptostreptococcus</i> spp. <i>Peptostreptococcus magnus</i> <i>Peptostreptococcus prevotii</i> <i>Peptostreptococcus anaerobius</i> <i>Propionibacterium acnes</i> <i>Propionibacterium</i> spp. <i>Clostridium</i> spp. <i>Clostridium perfringens</i> <i>Bacteroides</i> spp. <i>Bacteroides fragilis</i> <i>Bacteroides thetaiotaomicron</i> . <i>Bacteroides melaninogenicus</i> .
Brook & Finegold, 1981	Analysis of 209 cutaneous abscesses in children for aerobic and anaerobic microorganisms.	β -hemolytic <i>Streptococcus</i> group A & B Group D <i>Streptococcus</i> α - hemolytic <i>Streptococcus</i> Nonhemolytic <i>Streptococcus</i> <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i> <i>Neisseria gonorrhoeae</i> <i>Proteus</i> spp <i>P. aeruginosa</i> <i>E. coli</i> <i>K. pneumoniae</i> <i>Enterobacter</i> spp <i>Haemophilus parainfluenzae</i> <i>Eikenella corrodens</i> <i>Candida albicans</i>	<i>Peptococcus</i> spp <i>Peptostreptococcus</i> spp <i>Veillonella</i> spp <i>Eubacterium</i> spp <i>Bifidobacterium</i> spp <i>Lactobacillus</i> spp <i>Propionibacterium acnes</i> <i>Clostridium</i> spp <i>Bacteroides</i> spp <i>B fragilis</i> group <i>B melaninogenicus</i> group <i>Fusobacterium</i> spp

1.2.4.2.4 Chronic wound infection

In addition to the aerobic cocci (*Staph. aureus* and haemolytic streptococci), Gram-negative bacilli (e.g. *P. aeruginosa*, *E. coli*, and *Klebsiella*, *Proteus*, *Acinetobacter*, and *Enterobacter* spp.) tend to appear in the upper layers of open wounds, approximately a few weeks from initiation. They do not penetrate into deeper tissue, albeit they increase the wound bioburden and pathogenicity. Gram-negative bacilli can combat phagocytes and can attach to the host cells as they are capable of producing extracellular polysaccharides (biofilm matrix). They also produce endotoxins and some exotoxins, therefore it is very difficult to eliminate them. These toxins can result in a prolonged chronic inflammation (Gilbert & Allison, 2004).

One of the most invasive opportunistic organisms isolated from chronic wounds is *P. aeruginosa*. *P. aeruginosa* produces pyocyanin, a highly diffusible pigmented toxic secondary metabolite (Bianchi *et al.*, 2008) and can cause delayed wound healing (Muller *et al.*, 2009). Pyocyanin causes cell apoptosis which inhibits many cell functions and impairs host defenses (Denning *et al.*, 2003; Lau *et al.*, 2004; Look *et al.*, 2005). An *in vitro* study has shown that *P. aeruginosa* affects host immunity by inducing early apoptosis of immune cells such as macrophages and neutrophils consequently resulting in escalating inflammation (Zychlinsky & Sansonetti, 1997). This is assumed to be a clinically important mechanism of *P. aeruginosa* persistence in human tissue (White & Cutting, 2006a).

Foot infections are a major complication in diabetic patients, contributing to the development of gangrene and lower extremity amputation (Frykberg, 2003). Chronic wound infection was likely evoked by polymicrobial infection as in the case of severe diabetic foot infections, while mild infections are often monomicrobial. Gram-positive cocci predominate in most infections, but Gram-negative rods and anaerobic organisms are also frequently isolated from threatening infections (Frykberg, 2003; Lipsky *et al.*, 2006).

Brook and Frazier, (1998b) demonstrated that chronic venous leg ulcer (CVLU) infection resulted from the presence of aerobic and anaerobic micro-organisms. In the 22 specimens where a polymicrobial aerobic–anaerobic infection was

observed, several bacterial combinations were isolated. *E. coli* was recovered with *Bacteroides fragilis* and with group D streptococci. In addition, *Peptostreptococcus* spp. were recovered with *Staph. aureus*. Aerobic or facultative bacteria alone were present in 42% of CVLU specimens, just anaerobic bacteria were detected in 7%, and a mixed aerobic–anaerobic microbiota was present in 51% of CVLU specimens. The predominant aerobic organisms were *Staph. aureus* (26 isolates), group D streptococci (5), and *E. coli* (5). The predominant anaerobes were *Peptostreptococcus* spp. (15), *Bacteroides fragilis* group (6), *Propionibacterium acnes* (4), and *Prevotella* spp. (3). Similarly, Slater *et al.*, (2004) reported that Gram-positive cocci were the major isolates from infected diabetic foot wounds. Some anaerobes were found, but only in conjunction with aerobes, otherwise the presence of anaerobes was relatively rare.

Many studies have been conducted to identify the pathogens associated with chronic wound infections. Some of these studies paid great attention to developing the most appropriate method to analyse the microbial residents in chronic infected wounds (Table 1-4).

All organisms associated with a range of skin and soft tissue infections (SSTI) in hospitalised patients in several countries worldwide were studied (Jones *et al.*, 2003). Data was reported by clinical laboratories in the USA, France, Germany, Italy and Spain which participated in The Surveillance Network (TSN) during 2001. The analysis demonstrated that *Staph. aureus*, *Enterococcus* spp. and CNS were the most prevalent Gram-positive bacteria whilst *E. coli* and *P. aeruginosa* were the most widespread Gram-negative species in all countries. 44.4, 34.7, 12.4, 41.8 and 32.4% of *Staph. aureus* detected were MRSA in each country, respectively. The majority of MRSA were cross-resistant to other antibiotic classes (Jones *et al.*, 2003).

The presence of resistant species in a wound bed has serious outcomes in terms of the increased risk of cross contamination to both the community and the environment. Resistant species raise the mortality and morbidity rate among those patients, as well as impairing the patients' functions as a result of the open non-healing wounds. Consequently, these outcomes increase the

financial cost and burden on the health care resources to deal with such wounds. Thus this worldwide gradual increase in the development of antibiotic-resistant bacteria, drives the clinician and scientist to find alternative or adjacent therapy.

Table 1-4: Studies involving a detailed microbial analysis of chronic infected wounds

Author	Description of the study	Number of wounds	Predominant aerobes	Predominant anaerobes
Senneville <i>et al.</i> , 2009	Assessment of both swab technique and bone biopsy of specimens for patients with diabetic foot osteomyelitis.	76	<i>Staphylococcus aureus</i> (include MRSA) Streptococci spp. Corynebacteria Coagulase-negative staphylococci Enterococci <i>Escherichia coli</i> <i>Pseudomonas</i> spp. <i>Klebsiella</i> spp. <i>Proteus</i> spp. <i>Acinetobacter</i> spp. <i>Enterobacter</i> spp.	<i>Bacteroides</i> spp. <i>Peptococcus</i> spp. <i>Propionibacterium acnes</i>
Kessler <i>et al.</i> , 2006	To study prospectively two methods for the bacteriological diagnosis of osteomyelitis related to diabetic foot ulcer	21	<i>Staphylococcus</i> <i>Streptococcus</i> of Lancefield groups B and G <i>Streptococcus anginosus</i> and <i>Enterobacter aerogenes</i>	<i>Peptostreptococcus</i>
Shankar <i>et al.</i> , 2005	to study the bacterial aetiology of diabetic foot ulcers infection in South India	77	<i>Pseudomonas aeruginosa</i> Coagulase-negative staphylococci <i>Staphylococcus aureus</i> (include MRSA) <i>Escherichia coli</i> <i>Klebsiella pneumoniae</i> <i>Proteus mirabilis</i> <i>Proteus vulgaris</i> <i>Citrobacter koseri</i> <i>Citrobacter freundii</i> <i>Klebsiella oxytoca</i> <i>Edwardsiella tarda</i> <i>Klebsiella ozaenae</i> <i>Enterobacter aerogenes</i>	<i>Bacteroides fragilis</i> <i>Peptostreptococcus</i> spp.
Slater <i>et al.</i> , 2004	Comparison between accuracy of swab cultures vs. deep tissue cultures in infected diabetic wounds	60	<i>Staphylococcus aureus</i> Coagulase-negative Staphylococci Streptococci Enterococcus Diphtheroids Proteus Klebsiella <i>E. coli</i> <i>Pseudomonas</i> <i>Acinetobacter</i> <i>Citrobacter</i> <i>Enterobacter</i>	Anaerobic cocci Anaerobic rods <i>Bacteroides</i>
Pellizzer <i>et al.</i> , 2001	Compare the microbial isolates from limb-threatening infected foot ulcer using swabbing vs. deep tissue biopsy	29	<i>Staphylococcus</i> spp. <i>Proteus mirabilis</i> <i>Escherichia coli</i> <i>Pseudomonas aeruginosa</i>	<i>Peptostreptococcus</i> spp. <i>Bacteroides fragilis</i>

1.2.5 Management of wound infection

The persistence and growth of micro-organisms within a wound depends on their ability to evade the body's immune system and whether a nutritious, moist environment is provided for their growth (White *et al.*, 2006). A state of critical colonization or wound infection may become established once microbial contaminants persist and flourish within a wound (Kingsley, 2003). What happens next depends on the complex interaction between both host and microbial factors (Frank *et al.*, 2005).

The approach to manage wound infection depends on whether the wound is acute or chronic. Many factors determine the response of infected wounds to treatment with antimicrobial agents. These include, the competency of the host immune system to maintain a microbial balance within the wound; local perfusion (the delivery of nutrient & O₂ through arterial blood to tissue bed); the location, surface area, and the stage of a wound; and the presence of foreign bodies (i.e, slough and/or eschar) that offer a particularly hospitable environment for colonization because the dead tissues provide a ready source of nutrients for bacterial reproduction (Heinzelmann *et al.*, 2002). Proliferating bacteria are not only present on the surface of the wounds or in nonviable tissue, but may also invade healthy, viable tissue to such a depth and extent that they elicit a widespread immune response from the host – this makes wound management very complex (White *et al.*, 2006).

The development of infection within a wound delays the healing rate due to the expression of bacterial virulence factors. The underlying mechanisms for delay of wound healing and tissue damage following infection can be summarized as follows: (1) Micro-organisms compete with host cell responsible for wound repair for nutrients and oxygen (White *et al.*, 2006); (2) Bacteria may produce exotoxins and/or endotoxins. Exotoxins are soluble proteins produced by Gram-positive and Gram-negative bacteria for example *P. aeruginosa* exotoxin A, elastase, or alkaline protease. Exotoxins produced by common wound bacteria (e.g. staphylococci, streptococci, *Pseudomonas* spp.) have a broad mechanism of action attacking many types of cells and tissues, resulting in generalized tissue necrosis at the wound surface (Ovington, 2003). Endotoxins (lipopolysaccharides) are released from the cell walls of Gram-

negative bacteria particularly after lysis. Endotoxins stimulate the production of inflammatory mediators such as tumour necrosis factor (TNF- α) and interleukins, which in turn induce the production of endogenous MMPs. Increased levels of MMPs are observed in many types of non-healing wounds and are believed to contribute to the local destruction of growth factors, receptors, and tissue components (White *et al.*, 2006). Moreover, bacterial endotoxins have a detrimental effect on wound tensile strength due to decreased collagen deposition and cross-linking (Jelinek & Driver, 2006); (3) the production of large quantities of inflammatory cytokines and proteases by the host cells in response to bacterial infection can degrade the ECM of the wound bed (Ovington, 2003); (4) Anaerobes release short-chain fatty acids which impair white blood cell functions, reduce fibroblast production and decrease collagen deposition hence decreasing scar tensile strength (Stephens *et al.*, 2003).

For all the above-mentioned reasons, the management of wound infections has long tested clinicians' ingenuity. Clinical studies emphasize the importance of minimizing the bacterial load in both chronic and acute wounds. The advent of antibiotics in the 1950s revolutionised the control of bacterial infections, but the recent escalating prevalence of antibiotic-resistant bacteria in both hospital and community environments is driving the development of innovative antimicrobial modalities (Taylor & Stapleton, 2003; Moffatt, 2006). There has been renewed interest in the use of topical antimicrobials, particularly silver, iodine, honey and larval therapy (Cooper, 2004). However, there are great controversies among clinicians regarding the application of some of these agents due to probable host cellular toxicity (White *et al.*, 2006).

When wound infection occurs, immediate intervention to avert accelerating infection is essential. The main lines of treatment for infected wounds must focus on the three components of local wound care: debridement, maintaining of bacterial and moisture balance (Sibbald, 2003). The first stage of infection control is to identify the species residing within a wound. The second stage is to assess the level of penetration of the bacterial bioburden within the wound. If the bacteria invade only the superficial layers, the wound can be treated using topical agents (either antibiotics or other antiseptic agents). If the

bacteria have disseminated into deeper tissue, systemic antibiotics or related antibacterial compounds should be used. Lastly, a careful assessment of the underlying causes that might be associated with the infection should be performed (Sibbald, 2003).

1.2.5.1 Debridement

Wound debridement is the removal of the dead tissue, slough and foreign material that are present in and around a wound to expose healthy tissue - this facilitates healing and eliminates infection. It can be classified into surgical, mechanical, chemical or enzymatic and autolytic (Leaper, 2002). Surgical debridement (also known as sharp debridement) uses a scalpel or scissors to remove necrotic tissue from a wound and it is the quickest method of debridement. It is the preferred method if there is rapidly developing inflammation of the body's connective tissues (cellulitis) or a more generalized infection (sepsis) that has entered the bloodstream (Leaper, 2002).

Mechanical debridement is one of the oldest methods of debridement and it has been replaced by other modalities. Simply, a saline-moistened dressing is allowed to dry overnight and adhere to the dead tissue. When the dressing is removed, the dead tissue is pulled away too. It is very painful because the dressing can adhere to living as well as nonliving tissue and it may remove healthy granulation tissue when the dry gauze is lifted from the wound base. Some practitioners also have used irrigation (pulsed lavage) and hydrotherapy (Sibbald *et al.*, 2000). Chemical debridement takes advantage of certain proteolytic enzymes to lyse nonviable tissue. It is more selective than mechanical debridement. In fact, the body makes its own enzyme, collagenase, to break down collagen, one of the major building blocks of skin. A pharmaceutical version of collagenase is available and is highly effective as a debridement agent (McGuckin *et al.*, 2003).

Autolytic debridement depends on keeping the wound moist, which can be accomplished with a variety of dressings such as hydrogels, hydrocolloids, alginates, and transparent films. These dressings help to trap wound fluid that contains growth factors, enzymes, and immune cells that promote wound healing. Autolytic debridement is more selective than any other debridement

method, but it also takes the longest to work (Sibbald *et al.*, 2000). It is inappropriate for wounds that have become infected. Maggot therapy is a form of biological debridement known since antiquity. The larvae of *Lucilia sericata* (green bottle fly) are applied to the wound as these organisms can digest necrotic tissue and pathogenic bacteria. The method is rapid and selective (Courtenay *et al.*, 2000).

1.2.5.2 The role of antimicrobial agents in controlling wound infection

The application of topical antimicrobial agents comes immediately after debridement. Topical antimicrobial agents play an important role in regaining the bacterial balance within an infected wound. These agents include antibiotics, antiseptics and disinfectants (Scanlon, 2005). The choice of antimicrobial agents to minimize or eliminate micro-organisms from a wound must be influenced by the specificity and efficacy of the agent, its cytotoxicity to human cells, its potential to select resistant strains and its allergenicity (Vowden & Cooper, 2006). The common topical antimicrobial agents currently in use for treating both acute and chronic infected wounds are summarized in Tables 1-5 & 1-6.

1.2.5.3 Antiseptics, disinfectants and topical antibiotics

Both antiseptics and disinfectants are agents that can destroy micro-organisms or limit their growth in the non-sporing or vegetative state. However, antiseptics are usually applied merely to living tissues, while disinfectants may also be applied to equipment and surfaces (Scanlon, 2005). Antiseptics and disinfectant have different modes of action from antibiotics. Antiseptics and disinfectants target multiple sites on or within bacterial cells and so produce a broad spectrum of action against bacteria, fungi, viruses, and protozoa, unlike antibiotics that act selectively on a specific target on or within a bacterial cell and have a narrower spectrum of activity (Drosou *et al.*, 2003). They include alcohols (ethanol), anilides (triclocarban), biguanides (chlorhexidine), bisphenols (triclosan), chlorine compounds, iodine compounds, silver compounds, peroxygens, and quaternary ammonium compounds. The most commonly used products in clinical practice today include cadexomer iodine,

chlorhexidine, alcohol, acetate, hydrogen peroxide, boric acid, silver nitrate, silver sulfadiazine, and sodium hypochlorite (Drosou *et al.*, 2003).

Topical preparations may be divided into two classes, according to their function. One group consists of lotions with bactericidal properties, used to irrigate or clean wounds. These generally have only a short contact time with the wound surface, unless they are applied as a pack or soak. They include the hypochlorites (e.g. Edinburgh University Solution of Lime, an extemporaneous formulation of hypochlorite; Eusol[®]), hexachlorophane (a constituent of some soaps and other skin cleansers), and substances such as potassium permanganate and gentian violet (both used in solution for skin cleansing). The second group consists of preparations designed to stay in contact with the wound surface for a longer period of time, ideally until the next dressing change (O'Meara *et al.*, 2000). These include creams, ointments and impregnated dressings. Most topical antibiotics come into this category, and include mupirocin (available as 2% ointment), which has a wide range of activity, and fusidic acid (available as impregnated dressing, or ointment, cream or gel, all 2%) for staphylococcal infections. Neomycin sulphate, available as a cream (0.5%) or ointment (0.25%), is used to treat bacterial skin infections (O'Meara *et al.*, 2001; O'Meara *et al.*, 2008). If large areas of skin are treated, ototoxicity is a possible adverse effect. Silver based products, such as silver sulphadiazine (1% cream and impregnated dressing), have a broad-spectrum action against both Gram-negative and Gram-positive organisms, and also yeasts and fungi (Fleck, 2006).

Some products that are available in different forms fall into both categories. These include povidone iodine (available as 10% solution, 10% ointment, 5% cream, 2.5% dry powder spray and impregnated dressing), chlorhexidine (available as 0.05% solution, 5% ointment and medicated tulle dressing; it is also a constituent of skin cleansers), benzoyl peroxide (available as lotions, creams and gels in various strengths) and hydrogen peroxide (available as 3% and 6% solutions and 1% cream) (O'Meara *et al.*, 2000).

The use of antiseptics on wounds is a very common practice but at present their efficacy is in doubt due to the results from *in vitro* (Teepe *et al.*, 1993;

Rabenberg *et al.*, 2002; Wilson *et al.*, 2005) and *in vivo* (Brennan & Leaper, 1985) studies. These have shown that antiseptics are toxic not only to bacteria and other micro-organisms but also to human cells essential to the wound healing response. Topical antibiotics are not preferred due to the risk of emergence of resistant bacteria (O'Meara *et al.*, 2008). Also some topical antibiotics, such as neomycin, may cause hypersensitivity reactions (Sheth & Weitzul, 2008).

1.2.5.4 Systemic antibiotics

The use of systemic antibiotic is not recommended in cases of localized wound infection. Even though the emergence of antibiotic-resistant bacteria is a great concern, systemic antibiotics still can be used where there is clear evidence of widespread infection manifested in the form of cellulitis, lymphangitis or systemic-related complications (e.g. bacteraemia and sepsis). Only in this situation antibiotic treatment is indicated (Sibbald *et al.*, 2000; Hernandez, 2006).

Table 1-5: Topical antibiotics used to control wound infection

Agents	Mechanism of action	Antibacterial activity	Side effects
Aminoglycosides Gentamicin sulphate Neomycin sulphate	Interfere with the binding of formylme-thionyl-transfer RNA (fmet-tRNA) to the ribosome and thereby prevent inhibit protein synthesis.	<ul style="list-style-type: none"> • Active against Gram-negative infections especially that caused by <i>P. aeruginosa</i>. • <i>Staph. aureus</i> • Not active against streptococci or anaerobes 	<ul style="list-style-type: none"> • They induce contact sensitivity (i.e. dermatitis include increased erythema, oedema, and pruritus). • Nephrotoxic and ototoxic • Emergence of resistant bacteria
Fusidic acid	Inhibit protein synthesis	Active against Gram-positive cocci but should be applied with other antibiotics	Showed rapid emergence of resistance
Metronidazole	Interact with and break the DNA of the bacterial cell	Anaerobic coverage only	Rarely can cause peripheral neuropathy
Mupirocin	Pseudomonic acid inhibits the isoleucyl tRNA synthetase of staphylococci and streptococci	Very active against Gram-positive cocci including MRSA	Recent emerge of resistant strain.
Polypeptides Polymyxin B sulphate Bacitracin	Inhibit cytoplasmic membrane synthesis and suppress its function	Active against Gram-negative organisms except <i>Proteus</i> spp.	<ul style="list-style-type: none"> • Development of resistant bacteria • Hypersensitivity

Table 1-6: common antiseptics and disinfectants used to control wound infection

Agents	Mechanism of action	Antibacterial activity against	Examples
Biguanides Chlorhexidine Polyhexanide	<ul style="list-style-type: none"> It damages outer cell layers and the semi-permeable cytoplasmic membrane to allow leakage of cellular components. It causes coagulation of intracellular constituents, depending on concentration also denaturate enzymes. 	<i>Staph. aureus</i> , <i>P.aeruginosa</i> and a range of clinical isolates has been documented, however in MRSA, resistance has been observed.	Chlorhexidine as diacetate, digluconate and dihydrochloride; the digluconate is most applied in wounds.
Honey	<ul style="list-style-type: none"> It has an osmolarity and acidity sufficient to inhibit microbial growth. It has an enzyme that produces hydrogen peroxide upon dilution The presences of unidentified phytochemicals contribute to the antimicrobial effect of honey. 	58 clinical isolates of MSSA 82 epidemic strains of MRSA 20 isolates of <i>Pseudomonas</i> 56 strains of vancomycin-resistant enterococci (VRE) 7 strains of vancomycin-sensitive enterococci	Manuka honey from New Zealand
Hypochlorites	<ul style="list-style-type: none"> Initiation of autolysis due to structural and functional changes led to release of wall components. 	It has a broad spectrum of activity against Gram-positive and Gram-negative bacteria	Dakin's solution EUSOL
Iodine compounds Cadexomer iodine Povidone iodine	It involves multiple cellular effects by binding to proteins, nucleotides and fatty acids. Iodine is thought to affect protein structure by oxidizing S-H bonds of cysteine and methionine, reacting with the phenolic groups of tyrosine and reacting with N-H groups in amino acids (such as arginine, histidine and lysine) to block hydrogen bonding. It reacts with bases of nucleotides (such as adenine, cytosine and guanine) to prevent hydrogen bonding, and it alters membrane structure by reacting with C=C bonds in fatty acids.	It has a broad spectrum of activity against bacteria, mycobacteria, fungi, protozoa and viruses.	Cadexomer iodine is available as an ointment, as well as a dressing while povidone iodine is available commercially in several formulations solution, cream, ointment, dry spray or dressings
Peroxygens Hydrogen peroxide	It functions as an oxidising agent by producing free radicals that react with lipids, proteins and nucleic acids to affect cellular constituents non-specifically.	It has a broad spectrum of activity against bacteria, with greater effect on Gram positive species than Gram negatives one.	A 3-10 % clear, colourless liquid that decomposes in contact with organic matter.
Silver compounds Silver sulfadiazine Silver nitrate Silver (ionized)	<ul style="list-style-type: none"> Silver ions bind to negatively charged components in proteins and DNA, thereby result in denaturation in bacterial cell walls, membranes and nucleic acids that influence viability and lead to cell death. Silver ions bind to DNA block transcription and those bind to cell surface components interrupt bacterial respiration and ATP (adenosine triphosphate) synthesis. It inhibits bacterial oxidative enzymes (e.g. yeast alcohol dehydrogenase, causes metabolite efflux and inhibits DNA replication. 	It possesses broad-spectrum antibacterial, antifungal, and antiviral activity.	Flamazine cream Silver-impregnated dressings

1.2.5.5 Disadvantage of topical antimicrobial Agents

1. Topical antibiotics (e.g. gentamycin and tobramycin), which are also used systemically may induce the development of resistant organisms.
2. Neomycin, a topical antibacterial agent contains the allergens neosamine sugar and the deoxystreptamine backbone. The neosamine sugar is the same allergen found in framacetin. The deoxystreptamine backbone is also present in the aminoglycoside antibiotics: gentamicin, amikacin, and tobramycin. It is considered one of the top five cutaneous sensitizers. Other common sensitizers include lanolin, often present in tulle dressings. Bacitracin recently also has been identified as a common allergen (Sheth & Weitzul, 2008).
3. Many of the commonly used antiseptics result in high cellular toxicity in healable wounds (e.g. povidone iodine, chlorhexidine, and hydrogen peroxide) (Drosou *et al.*, 2003).
4. Silver sulfadiazine (SSD) is an antimicrobial agent that is generally recognized as safe, with minimal adverse effects, that has broad-spectrum antibacterial coverage against Gram-positives, Gram-negatives, and anaerobes as well as yeasts and some viruses. Yet, SSD has been shown to preserve viable dermal tissue with the formation of pronounced granulation tissue and an abundance of myofibroblasts in a pig burn model still it may delay re-epithelialization. Also irritation and argyria (irreversible discoloration of the skin resulting from subepithelial silver deposits) have been reported (Payne *et al.*, 1992; Flohr *et al.*, 2008).

1.2.5.6 Emergence of antibiotic resistance in wounds

The growing emergence of multidrug-resistant (MDR) bacteria isolated from wounds raises the alarm for clinicians to re-evaluate the use of antimicrobial agents that are available to treat wound infection. Colsky *et al.*, (1998) highlighted the problem of the development of resistance due to the use of antibiotics by comparing the antibiotic-resistance profiles from data collected from inpatients with skin wounds in a 4 year- period (1992 to 1996). In superficial skin wounds, *Staph. aureus* constituted 77% of isolates whilst in leg ulcers, the frequencies of *Staph. aureus* and *P. aeruginosa* were approximately

equal, constituting 43% and 42% respectively. The results have shown an alarming drift toward increasing antibiotic resistance, revealing that 50% of *Staph. aureus* isolates from leg ulcers were resistant to oxacillin, while 36% of *P. aeruginosa* isolates were resistant to ciprofloxacin. Over time, there was a marked increase in oxacillin and ciprofloxacin resistance in *Staph. aureus* and *P. aeruginosa*. In leg ulcers isolates, an increase from 24% to 50% oxacillin resistance in *Staph. aureus* and from 9% to 24% ciprofloxacin resistance in *P. aeruginosa* was noted. While in superficial wounds, *P. aeruginosa* resistance to ciprofloxacin grew from 24% to 36%.

1.2.5.7 Antimicrobial agents to control wound infection - an area of controversy

Since the discovery of antibiotics, bacteria have been shown to be highly adaptable and rapidly develop resistance to each new agent that comes along (Tenover & McGowan, 1996). Resistance to an antimicrobial agent can arise either by 'intrinsic' or 'acquired' mechanisms (Russell, 1990). Intrinsic resistance is a phenotype demonstrated by micro-organisms before the use of an antimicrobial agent, i.e. some species are naturally resistant to antibiotics. Intrinsic resistance to antimicrobial agents may be either because of the lack of the susceptible target or because the nature and composition of the bacterial cell wall may act as a permeability barrier, thus reducing uptake of the compound, and also by constitutively synthesized enzymes that may lead to degradation of the compound. Acquired resistance can take place by either chromosomal mutation or the acquisition of various types of genetic material in the form of transmissible plasmids and transposons (jumping genes). Acquired resistance to a wide range of antibiotics has been observed in a variety of micro-organisms (Percival *et al.*, 2005).

The mechanism by which micro-organisms exhibit antibiotic-resistance depends on the site and mode of action of the antibiotic (Perera & Hay, 2005). Mechanisms of resistance can be broadly classified into three main categories:

1. Altered target - the target enzyme in the organism may be altered or alternatively an additional target enzyme may be synthesised so that the organism has a lower affinity for the antibacterial agent.

2. Altered uptake - the organism may decrease the amount of the drug that reaches the target by either altering entry, for example by diminishing the permeability of the cell wall, or by pumping the drug out of the cell (i.e. efflux mechanism).
3. Drug inactivation - the organism may produce enzymes such as β -lactamase that modify or destroy the antibacterial agent (Mims *et al.*, 1998).

Wound-infecting organisms may exhibit resistance to topical and systemic antibiotics by one or more of these mechanisms. For example, *Staph. aureus* is resistant to penicillin via the production of β -lactamase. The penicillin binding proteins (PBPs) occur in the bacterial cell wall and have an enzymatic role in the synthesis of peptidoglycan. PBPs normally possess a high affinity for β -lactam antibiotics; in MRSA this affinity is reduced resulting in antibiotic resistance. By contrast, *P. aeruginosa* is resistant to many antimicrobial agents not only due to its ability to produce β -lactamase but also due to mutations in porin genes which result in a decrease in permeability of the outer membrane to the drug (Perera & Hay, 2005). Furthermore, Gram-negative rods demonstrate resistance to aminoglycosides through alterations in cell wall permeability or in the energy dependent transport across the cytoplasmic membrane. They also produce aminoglycoside-modifying enzymes that change the structure of the aminoglycoside molecule, thereby altering its uptake by the cell. The genes encoding these enzymes are often plasmid-mediated and transferable from one bacterial species to another (Mims *et al.*, 1998).

Though mupirocin is the topical antibiotic of choice to treat MRSA-infected wounds, some MRSA strains exhibit reduced susceptibility to this agent through acquiring a multiple-resistance plasmid encoding high-level mupirocin resistance (Pérez-Roth *et al.*, 2006). Resistance was reported for the first time in UK, USA, Australia and Spain (Gilbart *et al.*, 1993; Janssen *et al.*, 1993; Udo *et al.*, 1994; Alarcón *et al.*, 1998). Despite mupirocin-resistance, particularly plasmid-mediated, being rare, the increasing worldwide usage of this agent may result in extensive cross-infection with such strains. Thus, long-term use of this antibiotic should be thoroughly regulated by antibiotic policies and routine sensitivity testing (Alarcón *et al.*, 1998).

Silver compounds have been widely used as wound antiseptics especially for burns, traumatic wounds, and ulcers for more than a century. SSD and silver nitrate (AgNO_3) are among the most commonly used products. SSD is an established treatment for burn patients (Percival *et al.*, 2005); however, concern about its effectiveness arose with the emergence of sulphonamide-resistant bacteria following treatment of extensive burn patients with SSD (Lowbury *et al.*, 1976). Silver-resistant bacteria have been reported since 1965 (Lowbury *et al.*, 1976; Bridges *et al.*, 1979). Silver resistance is linked to genes located on plasmids (Gupta *et al.*, 2001), but can also be encoded on the chromosome (Silver & Phung, 1996 & 2005). On occasions these plasmids give rise to multiantibiotic-resistance. Resistance to silver in *Salmonella* has been located in a cluster of nine genes in three discretely transcribed units. The gene products were deduced to be a periplasmic protein that binds silver ions and two efflux pumps that export silver ions from the bacterial cell (Gupta *et al.*, 1999; Gupta *et al.*, 2001; Silver, 2003; Silver *et al.*, 2006).

In the past, biocides such as alcohol, silver nitrate, peroxide, iodine, and mercury compounds were used extensively on chronic wounds. While a few wounds will heal, more frequently these agents impair wound healing. However, their application on open wounds remains an area of debate due to reports of toxicity and damage to host tissues (Teepe *et al.*, 1993; Hülsmann & Hahn, 2000; Witton & Brennan, 2005) and the development of resistance (McDonnell & Russell, 1999; Chapman, 2003).

Bacteria vary in their susceptibility to biocides, with bacterial spores (e.g. *Bacillus subtilis*, *Clostridium difficile*) being the most resistant, followed by mycobacteria (e.g. *Mycobacterium chelonae*), then Gram-negative organisms such as *P.aeruginosa*, *Providencia* spp. and *Proteus* spp. Cocci are generally the most sensitive to biocides although some (e.g. enterococci and antibiotic-resistant strains of *Staph. aureus*) show low-level resistance (Russell, 1998). The vast majority of biocides act on bacterial cell membrane components and/or the cytoplasmic membrane. Bacterial spores, mycobacteria and Gram-negative bacteria resist biocides through reducing the uptake of the agent. The spores' coats and cortex, the arabinogalactan and possibly other components of the mycobacterial cell wall and the outer membrane of Gram-negative

bacteria limit the concentration of active biocide that can reach the target sites in bacterial cells (McDonnell & Russell, 1999). Also, constitutive degradative enzymes may lead to degradation of the biocide. Thereby intrinsic resistance, a chromosomally controlled property of a bacterial cell, enables it to evade the action of an antiseptic or disinfectant. Additionally, acquired resistance to biocides may arise by cellular mutation or by the acquisition of genetic elements. Plasmid-mediated resistance to biocides in Gram-negative bacteria and staphylococci has been noted (Chapman, 2003). Also, the emergence of plasmid/transposon-mediated resistance to inorganic and organic mercury compounds by hydrolases and reductases has been extensively studied (Russell, 1998; McDonnell & Russell, 1999; Chapman, 2003).

1.2.6 Biofilm resistance to antimicrobial agents

A particular situation was found with bacteria present in a biofilm, this displays reduced susceptibility to the antimicrobial agents. The biofilm can act as an intrinsic resistance mechanism resulting from physiological (phenotypic) adaptation of the cells (Russell, 1998).

When cellular clusters or microcolonies of bacteria attach to a surface (e.g., implanted medical device, catheter, damaged host tissue, pipes), they can encase themselves in a matrix of an extracellular polymeric substance (EPS), forming a slime layer (Donlan, 2000; Stewart & Costerton, 2001). The presence of bacteria embedded in EPS provides protective mechanisms that make them 100–1000 times more resistant to antimicrobial agents compared to planktonic cells (Spoering & Lewis, 2001). There are several mechanisms which have been proposed to explain this phenomenon of bacterial resistance to antibiotics and biocides within biofilms, including:

1. The slow or incomplete penetration of the antimicrobial agent into the biofilm extracellular matrix. It is suggested that the antimicrobial agent either reacts chemically with the extracellular components of the biofilm or attaches to the anionic polysaccharides (i.e. positively charged agents bind to negatively charged polymers in the biofilm matrix) (Stewart & Costerton, 2001). Some antibiotics still can diffuse into the bacterial biofilm; however, the deactivation of the antibiotic on the

surface of the biofilm by bacteria can retard their penetration. For example, ampicillin can penetrate through a biofilm formed by a β -lactamase-negative strain of *K. pneumonia* but not a biofilm formed by the β -lactamase-positive strain (Anderl *et al.*, 2000). In addition, hypochlorite and hydrogen peroxide are deactivated in the superficial layers of the biofilm at a faster rate than their penetration (Costerton *et al.*, 1999);

2. The occurrence of physiological changes within the biofilm micro-environment. An example of this is the accumulation of bacterial acidic waste products leading to a drop in the pH to a value as low as 1; this low pH can antagonise the action of antibiotics. Also, the consumption of oxygen by micro-organisms within a biofilm provides a highly anaerobic environment in the deep layers of the biofilm. For example aminoglycoside antibiotics are clearly less effective against the same micro-organism in anaerobic than in aerobic conditions (Stewart & Costerton, 2001). This would suggest that the response to antibiotics will vary according to the location of specific cells within a biofilm ecosystem;
3. The slow growth rate of bacteria within the biofilm due to depletion of oxygen and nutrients may render them less susceptible to antibiotics (i.e. they change from being physiologically active in the planktonic state to stationary state within the biofilm) (Brown & Gilbert, 1993; Patel, 2005). It is known that antibiotics are more effective at killing cells when they are actively growing. Antibiotics, such as penicillins, are not able to kill non-growing cells (Ashby *et al.*, 1994). Cephalosporins and fluoroquinolones, however, are able to kill non-growing cells but are more effective at killing rapidly growing bacterial cells (Brooun *et al.*, 2000);
4. Altered gene expression exhibited by organisms attached to surfaces (Brown & Gilbert, 1993) or a general stress response of a biofilm have been documented as factors known to reduce susceptibility to antibiotics (Stewart & Costerton, 2001);

5. Induction of a biofilm-specific phenotype in a subpopulation of micro-organisms within the biofilm (i.e. a spore-like state). These subpopulations have been shown to express protective mechanisms to reduce the efficacy of antimicrobial agents (del Pozo & Patel, 2007);
6. Quorum sensing (QS) has been recognized as one of the mechanisms involved in antibiotic resistance, but its role is unclear due to lack of research in this area (Mah & O'Toole, 2001; Davies, 2003). Bacterial populations within a biofilm communicate with each other through the production of signalling molecules (e.g. N-acyl homoserine lactones) to coordinate biological activity and orchestrate target gene expression once a critical population density (quorum) has been reached (Atkinson & Williams, 2009). Normally, a dense population of multiple bacterial species dwell together in a wound that provides an ideal condition for QS to take place. Communication via QS among bacteria present in a wound mainly aims to help bacteria to adapt to their new environment. Adaptive responses of bacteria within a wound environment depend on nutrient availability, competition among micro-organisms, and the evasion from host defence mechanisms. This may involve the secretion of protective EPS and enzymes that facilitate tissue invasion (Percival & Bowler, 2004).

1.2.6.1 Biofilms in wounds

Increasing evidence shows that bacteria and yeast can adhere to implanted medical devices or damaged tissue and become the origin of persistent infections (Costerton *et al.*, 1999). Microbial biofilms that form in the human body share certain characteristics with biofilms from other environments. Some well-known examples of biofilm infections include cystic fibrosis pneumonia, periodontitis, osteomyelitis, otitis media and infection of catheters and prosthetic joints (Percival & Bowler, 2004).

The wound environment encourages the formation of bacterial biofilms due to the availability of substrates and surfaces for biofilm attachment (Percival & Bowler, 2004). The presence of bacterial biofilms in wound tissue is proposed to be one of the reasons that chronic wounds fail to heal (Bjarnsholt *et al.*,

2008; James *et al.*, 2008). James *et al.*, (2008) reported that 30 of 50 chronic wound specimens contained biofilms; only 1 of 16 acute wounds contained biofilm. The bacteria were present in the form of densely aggregated colonies surrounded by an extracellular matrix, consistent with the morphology of the bacteria in biofilms (James *et al.*, 2008). Overall, these data indicated that biofilms were prevalent in chronic wounds and rare in acute wounds.

Serralta *et al.*, (2001) provided evidence that *P.aeruginosa* forms biofilms in a porcine wound model. This study showed that biofilms could have a major effect on inflammation, infection, and healing of these wounds. Bjarnsholt and co-workers reported that *P. aeruginosa* biofilms had the ability to produce rhamnolipid, a factor that protects these bacteria from phagocytosis, by eliminating the host PMNs. This is regulated via a QS communication system (Bjarnsholt *et al.*, 2008). This mechanism may explain the failure of antibiotics to clear persistent wound infections and the failure of the wounds to heal.

1.3 Photodynamic therapy

The current worldwide increase in resistant bacteria and the simultaneous downward trend in the discovery of novel antibacterial agents to combat resistant strains is a serious threat to the treatment of life-threatening infections. The existence of bacteria resistant to topical and systemic antibiotics dramatically reduces the possibilities of treating infected wounds effectively and results in delayed wound healing and complications such as septicaemia that may lead to death (Vazquez, 2006). Therefore there is a drive to develop non-invasive and non-toxic novel antimicrobial strategies that act more efficiently and faster than the current antibiotics, to which pathogens will not easily develop resistance (Taylor *et al.*, 2002). One promising alternative to current antimicrobial agents is lethal photosensitization (LP). The application of LP to the treatment of a disease is known as photodynamic therapy (PDT).

LP is defined as the application of a non toxic dye known as a photosensitizer (PS), which can be localized in the cells, and then photo-activated with light of the appropriate wavelength in the presence of oxygen to generate cytotoxic species (singlet oxygen and/or free radicals) (Shackley *et al.*, 1999). These

can damage proteins, lipids, nucleic acids and other cellular components in target cells (Phoenix *et al.*, 2003; Tegos & Hamblin, 2006).

There are three main components involved in the LP process; the PS, light and tissue oxygenation (Kurwa & Barlow, 1999; Hopper, 2000). The process is initiated when a ground state PS absorbs light of an appropriate wavelength and is converted into an excited singlet state. The PS molecules in this state readily decay back to the ground state with the emission of light (fluorescence) or heat (Green *et al.*, 1988) or are transformed into an excited triplet state. The excited state PS can do one of two things; it can pass the energy on to a substrate such as water to produce radical ions which in turn react with oxygen to produce cytotoxic species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and/or hydroxyl radicals (OH^\cdot) which is termed a type I reaction or it can react with molecular oxygen generating singlet oxygen (1O_2) a process which is termed a type II reaction. The singlet oxygen or other reactive oxygen species cause damage to bacterial cells through several mechanisms. These include oxidation of membrane lipids and amino acids in proteins, cross-linking of proteins and oxidative damage to nucleic acids with the consequent disturbance of the normal functioning of the pathogen (Figure 1-2) (Ochsner, 1997; Lukšienė, 2003).

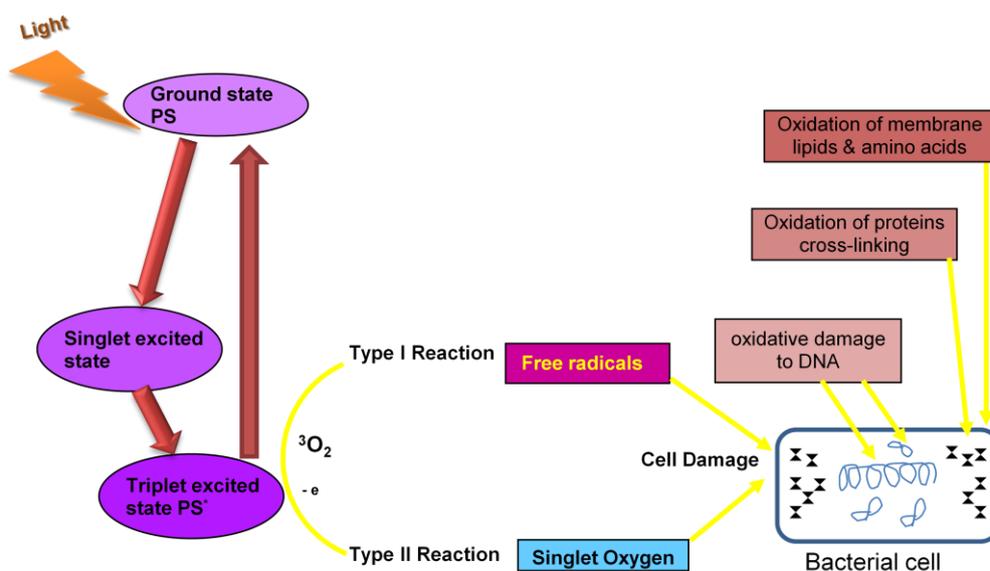


Figure 1-2: Schematic diagram explains the mechanism of action of PDT

1.3.1 The components of photodynamic therapy

1.3.1.1 Light

The ancient cultures of Egypt, India and China used the therapeutic effect of sun light to treat diverse diseases including, vitiligo, rickets, psoriasis, cancer and even psychosis. The application of light for the treatment of diseases is known as heliotherapy or phototherapy. The Greek physician Herodotus established the use of heliotherapy to restore health through whole-body sun exposure. At the start of the last century, the field of phototherapy was enriched by the work of, the Danish physician, Niels Finsen, who was awarded a Nobel Prize for inventing carbon arc lamp for the treatment of cutaneous tuberculosis. Also he described the use of red light to prevent the suppuration of abscesses (Ackroyd *et al.*, 2001).

Photo-chemotherapy is the combination of light application and either inherited pigment or locally administered PS that is localised in the target tissue to cause damage to abnormal or harmful cells (e.g. malignant or bacterial cells). The history of this therapy goes back to the ancient Indians and Egyptians when they used light in combination with the naturally occurring plant psoralens to treat skin diseases (e.g. vitiligo, leucoderma). In 1900, the first report on the lethal effect of light and acridine red had on the paramecium *Infusoria*, was a chance discovery by Oscar Raab, a medical student working with Professor Herman von Tappeiner in Munich. The experiment was performed during an intense condition of lighting (thunderstorm). Later on, he postulated the concept of cell-induced death resulting from light interacting with chemical's fluorescence via the transfer of energy from the light to the chemical, similar to that seen in plants after the absorption of light by chlorophyll. Shortly afterwards, von Tappeiner and Jodlbauer discovered the importance of oxygen in the development of photosensitization reactions, as a result in 1907 the PDT field was established when they introduced the term "photodynamic action" to describe this phenomenon (Mitton & Ackroyd, 2008).

The choice of light source to photo-inactivate pathogens depends primarily on the depth of the infected or targeted tissues into which the light should penetrate. The depth of light penetration increases in the visible and near-Infrared (NIR) spectral regions. The selected wavelength also has to match the

absorption spectrum of the PS of choice. The optimal wavelength should give maximal yield of $^1\text{O}_2$ at maximal depth. There are different types of light sources that are used to photo-activate the photosensitizing agents in use. For photodynamic antimicrobial therapy coherent and non-coherent light and ultraviolet light sources have been used, still now.

1.3.1.1.1 Coherent Light (Laser)

Laser is an acronym of light amplification by stimulated emission of radiation. Only laser devices can produce very intense beams of light which differ from the light that is produced by non-laser sources in a number of important aspects. Most notably, laser light differs from incoherent light by being monochromatic, collimated and coherent. Monochromaticity is the most important feature of laser light. The light produced by a laser source is of a single wavelength which means that each photon emitted from a given laser would have the same quantal energy. The monochromaticity permits irradiation with the exact wavelength at which a certain PS has its absorption maximum. The wavelength specificity of absorption is a key factor in PDT. Collimation of the laser light beam refers to the high degree of parallelism of its wave. Hence, a highly collimated laser beam can sustain a small spot size over a large distance which results in maintaining a great energy output. A laser beam consists of highly synchronized waves. The means by which such light is produced is spontaneous emission of radiation (Baxter *et al.*, 1994). The advantage of such a trait is that the light photons are in phase and unidirectional which gives rise to the power of this light so shorter exposure times can be employed to kill microbes (Wilson, 1993). Consequently, the light generated can be easily delivered down an optical fiber to inaccessible contaminated locations within the body for treatment and light dosimetry can be easily calculated. Accordingly, these characteristics make laser light a desirable candidate for clinical applications. In addition to the physical properties of laser light, irradiation with this light is known to exert many attractive photobiological effects within the body tissue such as bactericidal, regenerative and vasodilative effects. The underlying mechanism behind these bio-stimulatory effects of laser light was explained by Vladimirov *et al.*, 2004 (Figure 1-3).

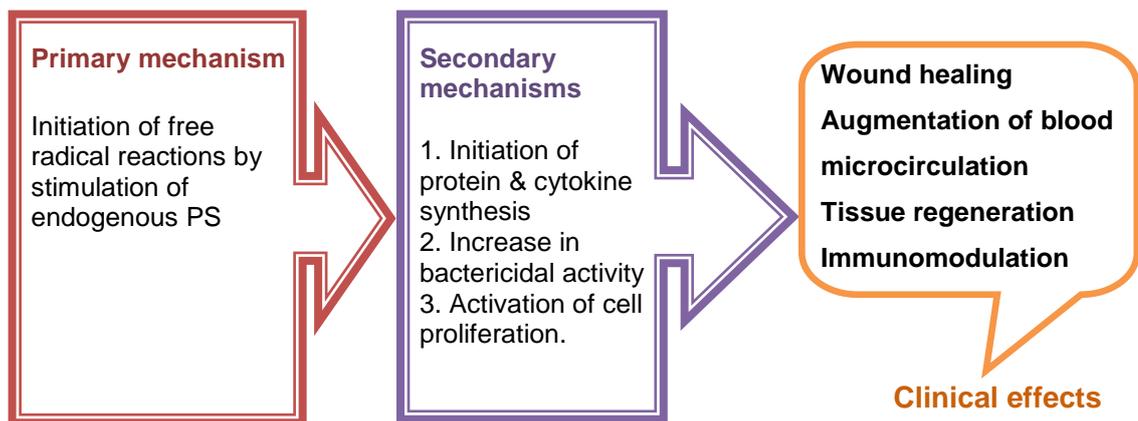


Figure 1-3: Underlying mechanism of action of laser light irradiation in the tissue
Modified from Vladimirov *et al.*, 2004

A variety of lasers have been used over the years; these are described in terms of the lasing medium used to generate the beam which may be a gas, dye, crystal or semiconductor diode. Examples include the helium/neon (He-Ne), carbon dioxide, gallium aluminium arsenide (GaAlAs), Argon dye and neodymium yttrium aluminium garnet (Nd : YAG) lasers (Wilson, 1993). The most popular of these has been the Argon dye laser, whose wavelength can be modified to match that of the optimum absorption wavelength of a desired PS. Unfortunately, these are large systems that are expensive, require an external water cooling system and separate power supply as well as a lot of maintenance (Brancaleon & Moseley, 2002). More recently, two laser systems have been used widely for photodynamic inactivation of bacteria: the He-Ne laser and the semiconductor diode lasers. These have the advantage of being relatively cheap, of smaller size, portable and reliable. Yet, the wavelength generated is not amenable to manipulation, like that of the pumped dye lasers, and therefore has to be matched for a chosen PS (Calin & Parasca, 2009). The He-Ne laser radiates at 632.8 nm while other semiconductor diode lasers emit at longer wavelength of 630- 950 nm. The most effective irradiation is that in the red and NIR range of the spectrum. This is based on the fact that wavelengths shorter than 600 nm are absorbed mainly by haemoglobin (Hb), whilst longer wavelengths can penetrate deep into the infected tissue (Vladimirov *et al.*, 2004). Moreover the beam of longer wavelength is less scattered than that of a shorter one (Niemz, 2007).

Nevertheless the He-Ne was the commonly used laser for tissue regeneration (Hawkins *et al.*, 2005); the NIR lasers have the most pronounced bactericidal

effect even in the absence of exogenous PS. Schultz *et al.*, (1986) demonstrated the bactericidal effect of the high power neodymium: YAG laser light against the wound-infecting bacteria *Staph. aureus*, *E. coli*, and *P. aeruginosa*, in the presence or absence of dyes. The Neodymium: YAG laser emits light at 1,060 nm. *P. aeruginosa* was the most sensitive bacterium while *E. coli* was the most resistant. Further studies have confirmed the bactericidal effect of the neodymium: YAG laser light on *Staph. aureus* (Yeo *et al.*, 1998) and on α -haemolytic streptococci, *B. fragilis*, *Neisseria* spp., *Streptococcus salivarius*, *Staph. aureus*, and *Candida albicans* (Meral *et al.*, 2003).

The different wavelengths of laser light used in dentistry to disinfect the periodontal pocket and root canal include 2940, 2780, 1064, 980, 810, 532 nm (Walsh, 2003). In a recent study, the effect of 4 laser systems that emit at 810, 1064, 2780, and 2940 nm on infected root dentin with either *E. coli* or *E. faecalis* was compared. All laser systems were capable of inducing significant reductions in the viable counts of both bacteria. The viable count of *E. coli* was reduced by at least three logs, with the erbium:YAG 2940 nm laser completely eradicating *E. coli* in 75% of the samples at 1 W. *E. faecalis* was more resistant to irradiation but the diode 810 nm and the Er:YAG 2940 nm light eradicated *E. faecalis* at 1.5 W (Schoop *et al.*, 2004). *Streptococcus sanguis* and *Porphyromonas gingivalis* were killed by light from the CO₂ laser at 286 J/cm² and 245 J/cm², respectively. There was no reported surface change, rise of temperature, serious damage to connective tissue cells located outside the irradiation zone, or inhibition of cell adhesion to the irradiated area (Kato *et al.*, 1998). Similarly, Lee *et al.*, (1999) recommended the use of the CO₂ laser emitting at 10600 nm to sterilize infected-surgical sternal wounds to reduce the morbidity, mortality and cost associated with surgical wound infection. The diode laser emitting at 810 nm in combination with a light dose of 210 J/cm² can be used to disinfect bioluminescent *E. coli* infected-cutaneous wounds in rats (Jawhara & Mordon, 2006). Using the same wavelength, Nussbaum *et al.*, (2003) found that illumination of *P. aeruginosa* at an irradiance rate of 0.03 W/cm² and radiant exposures in the range of 1-80 J/cm² resulted in a significant inhibition of bacterial growth.

1.3.1.1.2 Incoherent light

Ordinary light (from the Sun, from light bulbs, etc) consists mainly of light waves of different wavelengths, which are not in phase and typically radiate in all directions. Non-laser light sources are used primarily in dermatology. These sources are regular lamps that produce incoherent light with a large thermal component, whose output was defined by using filters (Mitton & Ackroyd, 2008). There are many varieties of lamps that emit incoherent light and also have been used to lethally photo-inactivate some of the pathogens associated with wound infections. Incoherent light sources include lamps with continuous spectrum (incandescent lamps, xenon arc lamps, etc.) or sources with the spectrum in bands (gas discharge lamps or metallic vapour lamps) (Calin & Parasca, 2009).

For example, a halogen lamp in combination with porphyrin was used successfully to inactivate *E. coli*. In this study the authors used a wavelength range between 350–800 nm at an intensity rate of 90 mW/cm² (Caminos *et al.*, 2006). Tegos and Hamblin, (2006) used a halogen lamp in combination with cationic phenothiazinium dyes Toluidine blue O (TBO), methylene blue (MB), and 1,9-dimethylmethylene blue (DMMB) as well as Rose Bengal (RB) and poly-L-lysine–chlorin e6 conjugate (pL-ce6) to photosensitize *Staph. aureus*, *E. coli* and *P. aeruginosa*. The bacterial suspensions were irradiated at 100 mW/cm² and the wavelength was adjusted according to the peak of each selected PS (540 -660 nm). The wild-type of the tested species were killed, yet there was selective survival of strains with increased MDR expression levels, underlining the possibility of bacteria developing resistance to phenothiazinium-based PDT.

Candida albicans, *Staph. aureus* and *P. aeruginosa* were effectively inactivated in phosphate buffered saline (PBS) when illuminated with white light from a 500 W halogen lamp at 30 mW/cm² in the presence of porphyrin chloride (TriP[4]) as the PS. Human blood plasma and serum albumin (HSA) inhibit the photodynamic inactivation of the three tested pathogens in a dose dependent manner (Lambrechts *et al.*, 2005a). Another example of the use of incoherent light in bacterial LP is the use of a xenon arc lamp (300–600 nm) at 5.4 mW/cm² to kill *E. coli* using RB as a PS (Schäfer *et al.*, 2000). A high-intensity

narrow-band blue light in the range of 407–420 nm, from a metal halide lamp was used along with an antioxidant substituted porphyrin to eradicate the Gram-negative bacteria *E. coli* and *Acinetobacter baumannii* using illumination of 20 mW/cm². Eradication of *A. baumannii* was attained at a low fluence of 7.5 J/cm², and a low porphyrin concentration of 10 µM. While a higher fluence of 37.5 J/cm² was needed to eradicate *E. coli* at the same concentration (Ashkenazi *et al.*, 2003a).

The observation that light alone has bactericidal properties has been documented for *P. acnes* which has endogenous porphyrins (Kjeldstad & Johnsson, 1986). The successful killing of *P. acnes* has been attributed to the production of coproporphyrin III, for which the absorption spectrum peak is at 415 nm. Therefore, irradiation of this organism with blue light leads to photosensitization of intracellular porphyrins, excitation of which leads to the production of reactive species, mostly singlet oxygen (¹O₂), and consequently, cell death (Papageorgiou *et al.*, 2000, Kawada *et al.*, 2002; Ashkenazi *et al.*, 2003b; Noborio *et al.*, 2007). Similarly, Hamblin *et al.*, (2005) and Ganz *et al.*, (2005) demonstrated that *Helicobacter pylori* can be killed when exposed to an endoscopically delivered blue light due to the accumulation of endogenous porphyrins. Oral black-pigmented bacteria have been found to be susceptible to inactivation using visible-light only (Feuerstein *et al.*, 2004; Soukos *et al.*, 2005). Maclean *et al.*, (2008) demonstrated that *Staph. aureus* including MRSA can be photo-inactivated using a xenon white-light source (400 to 500 nm) in the absence of exogenous PSs. Both *Staph. aureus* and *P. aeruginosa* were photosensitized with a combination of 405-nm and 880-nm light emitted by a cluster of Super Luminous Diodes (SLDs) (Guffey & Wilborn, 2006).

1.3.1.1.3 Ultraviolet

UV light in the form of sun light exerts cidal effect against a wide range of micro-organisms. This effect is attributed to the ability of microbial proteins and nucleic acids to absorb light with wavelengths less than 300 nm, which can induce cell killing through bacterial DNA dimerization (Wilson, 1993). UV is rarely used as a source of light in LP of micro-organism in human infections due to its mutagenic effects (Wilson, 1993). However it has been popularly investigated for disinfection of clinical, industrial and hospital surfaces

(Andersen *et al.*, 2007) and also for drinking/waste water treatment and disinfection (Ubomba-Jaswa *et al.*, 2009). The use of UV light to photo-activate exogenous PS in order to kill bacteria is well documented. Both *E.coli* and *Staph. aureus* were susceptible to photosensitization by psoralens (8-methoxypsoralen [8-MOP]) photo-activated with long wave length ultraviolet (Oginsky *et al.*, 1959), however, *E.coli* exhibited more resistance. Recently, Vermeulen *et al.*, (2008) demonstrated that UV irradiation of *E. coli* at 265 nm was the most efficient at killing the bacterium. A 100% kill was achieved at a dose of 1.17 mJ/cm².

1.3.1.2 Photosensitizers

The most frequently used PSs to photo-inactivate wound-associated organisms are activated by light from the visible part of the spectrum (400-700 nm). PSs should possess the ideal properties which are:

- (1) Low dark toxicity to human cells, chemically pure, water soluble stable in solutions at physiological pH and rapidly cleared from the body;
- (2) Of minimal skin sensitivity;
- (3) High quantum yield for ¹O₂ generation;
- (4) High absorption of light at longer wavelengths in the NIR region of the spectrum (700-950 nm) in order to penetrate deep in the tissues;
- (5) Selective uptake by the target pathogens (MacRobert *et al.*, 1989; Allison *et al.*, 2004; Detty *et al.*, 2004).

Some additional desirable features for PSs used as antimicrobial agents are:

- Broad spectrum of action against bacteria, fungi, yeasts, viruses, and parasitic protozoa especially those resistant to conventional antibiotics.
- Efficacy independent of the antibiotic-susceptibility of the target organism.
- Low probability of promoting the development of mutagenic processes.
- Ability to cause reduction in pathogens communities with minimal damage to the host tissues.
- Prevention of the pathogens regrowth after multiple treatments.
- Availability of formulations allowing localized delivery of the PS to the infected tissue (Jori *et al.*, 2006).

Existing PSs do not meet all the above-mentioned criteria; some are close, and with some modification could be appealing candidates as antimicrobial PSs. There are several groups of PSs that are in current use to photo-inactivate micro-organisms and can be applied to treat infectious diseases (Table 1-7). These include phenothiaziniums, acridines, cyanines, porphyrins, phthalocyanines, chlorins, psoralens and fullerenes. An overview of indocyanine green (ICG) and the widely used antimicrobial PSs is discussed in this section.

Table 1-7: Photosensitizers absorption maxima

Photosensitizer	λ_{max} range in buffer (nm)
Acridine	400–450
Cyanine	350–800
Phenothiazinium	620–660
Phthalocyanine	670–780
Porphyrin	600–650
Psoralen	300–380

1.3.1.2.1 Phenothiaziniums

Phenothiaziniums are compounds which have a core structure consisting of a planar tricyclic heteroaromatic ring with the formula $S(C_6H_4)_2NH$ (Phoenix & Harris, 2003), with an absorption range of $\lambda_{\text{max}} = 620\text{--}660$ nm (Wainwright, 1998). The lead compound of phenothiaziniums is MB. Indeed, within the growing field of PDT, there was a great need for the development of novel analogue preparation such as TBO, DMMB and new methylene blue (NMB). The minimal toxicity of these dyes to human cells, plus their ability to produce high quantum yields of singlet oxygen, has evolved a great interest in testing the potential of these PSs as photo-activated antimicrobial agents (Wainwright, 2005). MB and TBO are the most extensively studied phenothiazinium based PSs for their antibacterial activity.

The mechanism of action of these PSs is very interesting; these compounds are generally cationic at physiological pH, which enable them to target bacterial membranes of both Gram-positive and Gram-negative bacteria (Jori *et al.*, 2006). Most of them have amphiphilic properties (i.e. chemical compound possessing both hydrophilic and lipophilic properties) which are important for interaction with the membrane and enable the molecules to become

internalized into the cell. The chemistry of some phenothiaziniums has suggested that quinonemine species might be intermediates in phenothiazinium based PS uptake: NMB, TBO, although cationic at neutral pH, tend to form the neutral quinonemine species at lower pH. Thus, the low pH of the membrane interface could lead to the formation of an inactive neutral species, promoting its cellular uptake, with the possibility of subsequent intracellular regeneration of the cationic form of the PS (Phoenix & Harris, 2003).

In 1998, Wainwright and co-workers tested the light-mediated antibacterial properties of phenothiazinium dyes against several pathogenic strains of *Staph. aureus*, including MRSA. For MB, NMB and DMMB illumination at a fluence rate of 1.75 mW cm² resulted in a 4-fold increase in bactericidal activity (Wainwright, *et al.*, 1998). The lead work carried out by Wilson and colleagues demonstrated the efficacy of a range of PSs, including MB and TBO, against a diverse range of micro-organisms relevant to human diseases (especially oral cavity infections) (Sarkar & Wilson, 1993; Wilson & Mia, 1994; Soukos *et al.*, 1996; Bhatti *et al.*, 1997; Komerik *et al.*, 2003). Phenothiazinium dyes, and derivatives were effective against *E. coli* and *Staph. aureus*. The dyes showed inherent dark toxicity (minimum lethal concentrations: 3.1-1000 µM) (Phoenix *et al.*, 2003).

The phototoxicity of the commercially available phenothiaziniums is high against both Gram-positive and Gram-negative bacteria, thus indicating clinical potential in local disinfection. These antibacterial properties of phenothiazinium dyes have been translated into practical applications such as photodecontamination of blood plasma (Mohr *et al.*, 1997; Wainwright, 2000) and elimination of cariogenic and periodontopathogenic bacteria from diseased lesions in dentistry (Wilson, 2004).

1.3.1.2.2 Acridines

Acridine, C₁₃H₉N, is an organic compound and a nitrogen heterocycle. The acridine derivatives proflavine and acriflavine were first used as antibacterial agents during World War I when Browning and his collaborators introduced “dye therapy” to treat wound infections. This discovery led to the introduction

of the less toxic aminacrine (9-aminoacridine) in the 1940s. The photoactivation of intercalated acridines is known to cause oxidative damage to nucleic acid (Wainwright, 2008).

The photosensitizing effect of acridine dyes (acridine orange, acridine yellow, diflavine, proflavine etc.) against a wide range of bacteria is well established (Wainwright *et al.*, 1997). They are generally not considered appropriate for PDT in humans due to their light absorption properties: acridines absorb maximal wavelengths of light ranging from approximately 400-450 nm in the UV spectrum (Fukui & Tanaka, 1996). Acridines are now well accepted as antiviral and anticancer drugs based on their anti-nucleic acid effects (Goodell *et al.*, 2006). However, this property has made it difficult to use active acridines in the antimicrobial-PDT field due to fears over possible mutagenic effects on human DNA.

1.3.1.2.3 Cyanines

Cyanine is a non-systematic name of a synthetic dye family belonging to the polymethine group. Cyanines have many uses as fluorescent dyes, particularly in biomedical imaging. Depending on the molecular structure, they cover the spectrum from UV to IR (e.g. merocyanine 540, 1,19-Diethyl-4,49-carbocyanine iodide [CY-II] 480 nm, ICG 775nm) (Kassab, 2002; Abd El-Aal & Younis, 2004). The cyanines, like acridines, are well known for their strong antiseptic effect on wide range of bacteria and were introduced mainly to disinfect wounds (Browning *et al.*, 1924). In spite of this only very few studies have been carried out in this area. A detailed investigation using several dyes of the cyanine group confirmed the antimicrobial effects of these dyes against a wide range of bacteria and fungi (Abd El-Aal & Younis, 2004). In addition, O'Brien *et al.*, (1992) described the photo-inactivation of blood-borne enveloped viruses such as human herpes simplex virus and human cytomegalon virus by merocyanine 540.

With the continuous expansion of the PDT field beyond oncological applications, there is rising need to investigate the potential use of new PSs to treat a variety of human diseases including infections. Cyanines appear to possess interesting properties since they include a large variety of chemical

structures, which can be chemically modified (i.e. can be functionalized with groups differing in hydro/ lipophilicity and electric charge). The availability of a wide range of cyanines that can be photo-excited by different wavelength bands, allows variable penetration depths into human tissues. These criteria are of particular interest in terms of their potential phototherapeutic applications (Kassab, 2002). Yet, the use of these dyes has not received a great deal of attention as a prospective light-activated antimicrobial agent.

1.3.1.2.3.1 Indocyanine green

The concept of selecting a photosensitizing agent that has antimicrobial activities and simultaneously displays intense absorption in the NIR spectral regions, where the transparency of human tissues to the incident light is maximal, would make the ideal combination for antimicrobial PDT to treat wound infections. A dye of the cyanine family meets these criteria and has potential for use as a PS in PDT is ICG (also known as cardiogreen or foxgreen).

ICG is a water-soluble tricarbocyanine dye which is a negatively charged due to the presence of SO_3^- group (Figure 1-4). Its absorption spectrum exhibits a strong absorption band around 800 nm, and a photo-oxidative effect can be triggered with a laser emitting at 805 nm (Saxena *et al.*, 2003). The penetration depth is approximately 8 mm at 800 nm where light penetration is most effective and where the wavelength of light is still energetic enough to produce $^1\text{O}_2$ (Detty *et al.*, 2004). ICG is a United States Food and Drug Administration (U.S. FDA) approved dye, that has been mainly used for medical diagnosis such as retinal angiography, measurement of plasma volume, and cardiac output, assessment of burn depth, liver function, exercise physiology and guiding biopsies (Malicka *et al.*, 2003). This dye has a very low dark toxicity. ICG PS has already been introduced into clinical practice for the treatment of AIDS-associated Kaposi sarcoma (Abels *et al.*, 1998; Szeimies *et al.*, 2001) and acne vulgaris in humans (Lloyd & Mirkov, 2002; Tuchin *et al.*, 2003, Nouri & Ballard, 2006). Also, PDT with ICG for choroidal neovascularisation (CNV) is a well-known method for treating this condition (Costa *et al.*, 2001). ICG is under investigation as a PS for anti-cancer PDT; *in vitro* studies have shown an inhibitory effect of photo-activated ICG with NIR light on pancreatic cancer cells

(Tseng *et al.*, 2003), colonic cancer cells (Baumler *et al.*, 1999), human (SKMEL 188) and mouse (S91) melanoma cells (Urbanska *et al.*, 2002) and MCF-7 breast cancer cells (Crescenzi *et al.*, 2004). The application of ICG in the PDT of infections, with the exception of its topical use for the treatment of acne vulgaris, has received little attention and remains an open field of fundamental research.

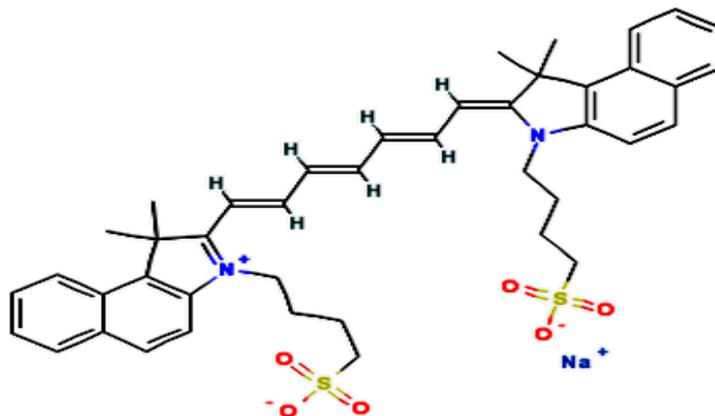


Figure 1-4: The chemical structure of ICG
Adapted from <http://pubchem.ncbi.nlm.nih.gov>

1.3.1.2.4 Macrocyclic photosensitizers

1.3.1.2.4.1 Porphyrins

With the start of the revolution of PDT in oncology, the porphyrins were the PSs of choice. A large selection of both naturally-derived and synthetic porphyrins are available for medical use (Wainwright, 1998). Two of this class of PSs have already been certified for PDT application in the USA for the treatment of cancer and in ophthalmology. These are the benzoporphyrin derivative known as Verteporfin (Visudyne[®]) and the haematoporphyrin derivative (Photofrin[®]).

Photo-activated porphyrin induces antimicrobial effects against both Gram-positive *Staph. aureus* (Ashkenazi *et al.*, 2003a) and Gram-negative *E. coli*, *A. baumannii* (Nitzan *et al.*, 1998; Ashkenazi *et al.*, 2003a). Orenstein *et al.*, (1997) assessed a deuteroporphyrin-haemin complex as an agent for the treatment of burn wounds infected with a MDR strain of *Staph. aureus*. A combination of both deuteroporphyrin and haemin exhibited potent dark toxicity *in vitro*. *In vivo*, the same porphyrin mixture was able to kill 99% of *Staph. aureus*

in infected burn wounds in guinea pigs without illumination. This effect lasted for up to 24 hours.

Banfi *et al.*, (2006) explored the antimicrobial activities of several porphyrins against *E. coli*, *P. aeruginosa* and *Staph. aureus* and concluded that *P. aeruginosa* was the most resistant. The efficacy of the tested porphyrins was increased by the presence of positively charged groups on the porphyrin frame and by the introduction of aromatic hydrocarbon side chains to cause a moderate degree of lipophilicity.

Gram-negative bacteria are reported to be more resistant to treatment with porphyrins (Nitzan *et al.*, 1992; Merchat *et al.*, 1996; Banfi *et al.*, 2006; El-Adly, 2008). This has been attributed to the inability of the dye to diffuse through the inner and outer cell membranes of these bacteria. This problem was overcome by the addition of a membrane disorganising peptide (Nitzan *et al.*, 1992).

Still, these compounds have some disadvantages which may limit their therapeutic application. For example, Photofrin[®] can cause severe skin photosensitivity which can last up to 6 weeks. Also, the longest wavelength at which the drug can be photo-activated is 630 nm. At this wavelength, effective light penetration through tissue is limited due to endogenous chromophores, mainly Hb, and light scattering in a less than ideal region of the optical spectrum (Sternberg *et al.*, 1998).

1.3.1.2.4.2 Chlorins

The chlorins are closely related to porphyrins. The reduction of a pyrrole double bond on the porphyrin periphery forms the chlorin core, and further reduction of a second pyrrole double bond on the chlorin periphery gives the bacteriochlorins. Both classes of PS have peak absorption in the far-red and NIR of the spectrum (λ_{\max} =650-670 nm for chlorins and λ_{\max} =730-800 nm for bacteriochlorins). One of the first chlorins to be investigated widely is chlorin e6 (Detty *et al.*, 2004).

Embleton *et al.*, developed three different novel targeting systems for LP of antibiotic-resistant strains of *Staph. aureus* by linking either an immunoglobulin

G protein (Embleton *et al.*, 2002) or an antibody (Embleton *et al.*, 2004) or a bacteriophage to the PS tin (IV) chlorin e6 (SnCe6) (Embleton *et al.*, 2005). These results demonstrate that effective and selective killing of MRSA was achieved using the different chlorin e6 conjugates.

Other groups have investigated the activity of various sizes of polycationic poly-L-lysine chlorin (e6) conjugates against a range of bacteria and yeast (Hamblin *et al.*, 2002a; Demidova & Hamblin, 2005). In a while, they studied the polyethyleneimine chlorin (e6) conjugates for their ability to kill a panel of pathogenic micro-organisms including the Gram-positive *Staph. aureus* and *Strep. pyogenes*, the Gram-negative *E. coli* and *P. aeruginosa*, and the yeast *C. albicans* (Tegos *et al.*, 2006).

1.3.1.2.4.3 Phthalocyanines

The phthalocyanines are tetrabenzotetraazaporphyrins or synthetic porphyrins derivatives. The aromatic character of these compounds greatly increases their absorption in the NIR region of the spectrum (670-780 nm) (Detty *et al.*, 2004). The compounds are commonly linked to a selection of metal ions include aluminium, zinc and silicon, which seem to improve the compound efficacy (Allison *et al.*, 2004). These photosensitizers are known as efficient generators of singlet oxygen, even more efficient than MB (Griffiths *et al.*, 1997a).

Phthalocyanines were developed for PDT-mediated anti-cancer treatment, but their potential application to the field of antimicrobial-PDT was rapidly spotted. There is a large body of research describing the antimicrobial activity of the phthalocyanines. Many studies report on the light-mediated virucidal activity of phthalocyanines *in vitro*, which may be applicable in the field of blood product disinfection (Ben-Hur *et al.*, 1997; Zmudzka *et al.*, 1997; Smetana *et al.*, 1998). Aluminium sulphonated phthalocyanine has been investigated for the inactivation of oral bacteria in the treatment of dental caries (Wilson *et al.*, 1995 & 1996) and inactivation of *H. pylori* associated with gastritis and peptic ulceration (Bedwell *et al.*, 1990; Millson *et al.*, 1996). Other studies have assessed the bactericidal activity of cationic water-soluble zinc phthalocyanine

(Bertoloni *et al.*, 1990; Minnock *et al.*, 1996 & 2000; Soncin *et al.*, 2002; Scalise & Durantini, 2005).

1.3.1.2.5 Fullerenes

Fullerenes (buckminsterfullerenes) are a new class of closed-cage nanomaterials made exclusively from carbon atoms that were discovered in 1985 (Kroto *et al.*, 1985). The chemical structure of this compound in the form of an extended p-conjugated system of molecular orbitals, results in a significant absorption of visible light (Mroz *et al.*, 2007).

Tegos *et al.*, (2005) compared the antimicrobial activity of six functionalized C60 compounds with one, two, or three hydrophilic or cationic groups in combination with white light against *Staph. aureus*, *P. aeruginosa*, *E. coli* and *C. albicans*. Fullerenes reduced the viable count of all tested microbes by 4–6 logs.

However, fullerene-mediated-PDT may lead to mutagenic effects. PVP-solubilized fullerene was mutagenic for *Salmonella* strains TA102, TA104 and YG3003 in the presence of rat liver microsomes when it was irradiated by visible light (Sera *et al.*, 1996). Further studies must be carried out to explore these compounds and their antimicrobial capability.

1.3.1.2.6 Naturally occurring photosensitizers

1.3.1.2.6.1 Psoralens

Psoralens are tricyclic furocoumarins which absorb UV light in the range of 320-400 nm (Ledo & Ledo, 2000). They have been used widely for the treatment of skin diseases in Egypt and India for centuries (Spikes, 1997). The use of psoralens for the phototherapy of psoriasis (PUVA) is well established (Ledo & Ledo, 2000). Due to the absorbance of blue light by psoralens, they are not appropriate for use in human infections. Other applications such as blood disinfection may be relevant due to the ability of psoralens to target nucleic acids and produce singlet oxygen upon activation by UVA (Ben-Hur *et al.*, 1996; Wainwright, 2002 & 2004).

1.3.1.3 The mode of action of photosensitizers

Larson & Marley, (1994) have described three modes of action by which light activated antimicrobial agents can interact with the cell:

1. The first is that the PS settles outside the cell, generating reactive species in solution which can diffuse into the cells of the target organism and react to induce cellular damage. An example of this mechanism is a molecule such as curcumin that produces H_2O_2 on illumination.
2. The second mechanism is that the PS binds to or becomes localized at the cell membrane (by hydrophobic or coulombic interactions) – upon light absorption the PS transfers energy (e.g. an electron, hydrogen atom etc.) to target biomolecules within the cell, resulting in ROS production that cause cell damage. Anionic porphyrins follow this mechanism of photosensitization.
3. The third possibility is that the agent penetrates into the interior of the cell and becomes associated with an intracellular target, possibly a protein (including enzymatic damage) or the nucleus (inducing genetic damage). Cationic porphyrins which bind strongly to the polyanionic macromolecules DNA, are good examples of this type of phototoxic agent.

1.3.2 Mechanism of action: photophysical and photochemical process

1.3.2.1 Light interaction with the tissue

There are many factors that affect light penetration through body tissue especially in a wound. The wound environment contains a mixture of debris, necrotic tissue, exudates and colonizing bacteria. Light dissemination in such a medium involves processes of refraction, reflection, absorption and scattering. To minimize the loss of light intensity, when light passes through the interface of two media, as a result of reflection and refraction, the light is applied perpendicularly to the tissue (Plaetzer *et al.*, 2009). Scattering of light in tissue has the most pronounced effect on light intensity and distribution. Both scattering and refraction control light beam width, and result in a loss of the light power delivered per unit area (i.e. reduction of light intensity). Absorption of light is the most relevant factor that involves the loss of light intensity with increasing penetration depth. However, the presence of chromophores in the

tissue such as oxyhaemoglobin (HbO₂) and deoxyhaemoglobin, melanin and cytochromes play an important role in light absorption. The absorption spectra of these molecules define the optical window for PDT in tissue. The absorption maxima of Hb and HbO₂ lie in the range of 500–600 nm, therefore a longer wavelength should be used for PDT (Plaetzer *et al.*, 2009). Most of the PSs in clinical use are excited at 630-670 nm, where light can penetrate tissue as deep as 3–5 mm. The use of a PS with an absorption peak at wavelengths of 800 nm or more should double the penetration depth and thus enable the treatment of deep wounds and burns. Finally, the factors by which light penetration is limited such as optical scattering, the light absorption by endogenous chromophores or by the high concentration of sensitizing agent (self-shielding) should be taken into consideration when choosing a PS (Kalka *et al.*, 2000).

1.3.2.2 Light interaction with the molecules

A PS in the ground state has two electrons with opposite spins, in this state the molecule is at its lowest energy state (S_0). Upon the absorption of a photon with the appropriate energy, one of the paired electrons is transferred to an unoccupied orbital of higher energy but keeping its spin after that converted into an excited singlet state (S_1) (Castano *et al.*, 2004; Plaetzer *et al.*, 2009). The excited singlet state is unstable and short-lived - less than 1 μ s (MacRobert *et al.*, 1989). The singlet state loses energy and decays back to the ground state via either emission of a secondary photon (i. e., fluorescence emission) or by heat dissipation (Plaetzer *et al.*, 2009). Additionally, the singlet state S_1 can go through intersystem crossing, whereby the excited electron in a higher orbit undergoes a spin conversion to transform into the excited triplet-state that has parallel electron spins (Calzavara-Pinton *et al.*, 2007). The triplet state is sufficiently long-lived for a few ms therefore, it plays the most significant role in the photochemical reactions involved in the LP process (MacRobert *et al.*, 1989; Castano *et al.*, 2004). The triplet state PS can produce chemical changes in a neighbouring molecule via two competing pathways, named type I and type II reaction (Figure 1-5). These two reactions can occur concurrently in the tissue, and the proportion of each process mainly depends upon the type of PS in use, the concentrations of the substrate and oxygen concentration.

In a type I reaction, the triplet state PS can transmit energy in the form of an electron or proton to a substrate within the cell (e.g. water in the cell membrane or the cytoplasm), to produce radical ions. These radicals may further react with oxygen to produce superoxide anion (monovalent reduction) (Hamblin & Hasan, 2004). Superoxide on its own is inactive in biological systems and does not cause much oxidative damage, but it can react with itself to produce hydrogen peroxide and oxygen, a reaction known as “dismutation” that can be catalyzed by the enzyme superoxide dismutase (SOD). In another reaction termed the Fenton reaction, superoxide acts as a reducing agent to produce the highly reactive hydroxyl radical (HO). In this process, superoxide donates one electron to reduce metal ions (such as ferric iron, copper) which in turn catalyse the transformation of hydrogen peroxide (H₂O₂) into hydroxyl radical (HO). In a further reaction, superoxide can react with the hydroxyl radical (HO) to form singlet oxygen, or another highly reactive oxidizing molecule nitric oxide NO to produce peroxynitrite (ONOO⁻). Free radicals such as H₂O₂ and HO are highly reactive since they can pass quickly through cell membranes causing oxidative damage and cannot be expelled from the cell. These highly reactive radicals can react with organic substrates (e.g. fatty acid, lipids, oxygen) in a series of chain reactions to produce more cytotoxic radicals (Castano *et al.*, 2004).

Conversely, in a Type II reaction, the triplet state PS can transfer its energy directly to molecular oxygen (³O₂) to form excited state singlet oxygen ¹O₂ (Mojzisoava *et al.*, 2007). These ¹O₂ species can travel a distance of approximately 134 nm based on its life time of ~3-4 μs in water (Skovsen *et al.*, 2005). However, in the cellular environment which is rich in reactive substrates, the life span of ¹O₂ falls to ~100 ns, hence the diffusion distance is dropped to ~75 nm (Moan, 1990; Ouedraogo & Redmond, 2003).

¹O₂ species are generated without transformation of the PS, thus allowing each single PS molecule to produce many times its own concentration of ¹O₂ (MacRobert *et al.*, 1989). Overtime, the PS will be chemically modified or even degraded due to the direct attack of ROS on the PS molecules, in a process known as photobleaching or photodegradation, which can be the final outcome of both type I and type II reactions (Plaetzer *et al.*, 2009).

Conjointly, hydroxyl radicals, superoxide, and $^1\text{O}_2$ are known as reactive oxygen species (ROS). ROS are oxidizing agents that can directly react with many biological molecules. Amino acid residues in proteins are important targets that include cysteine, methionine, tyrosine, histidine, and tryptophan.

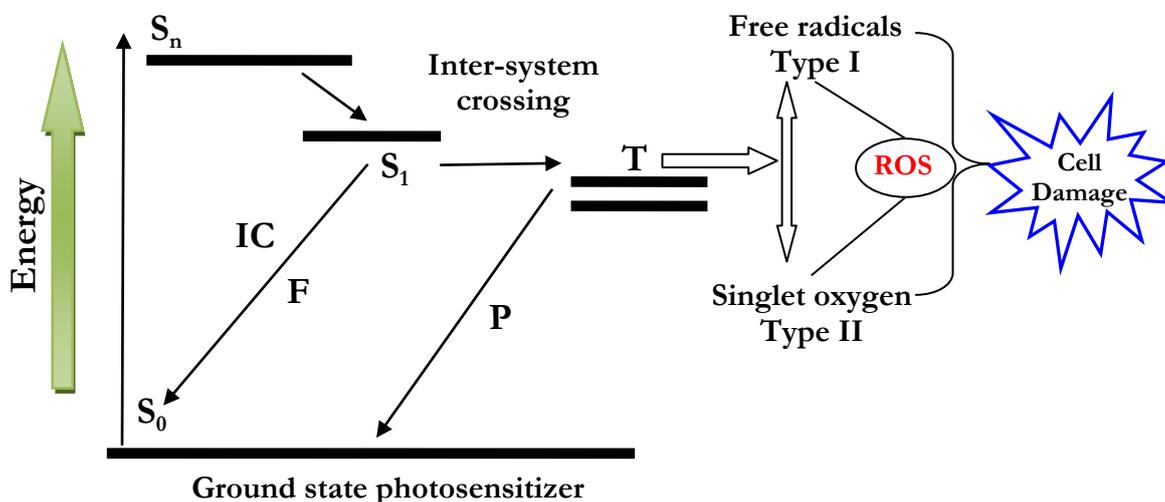


Figure 1-5: Diagram of the photophysical processes involved in photodynamic therapy S_1 singlet states, IC internal conversion, F fluorescence emission, T triplet states, P phosphorescence Modified from Plaetzer *et al.*, (2009) *Lasers Med Sci* 24:259–268

1.3.3 Photodynamic inactivation of pathogens

1.3.3.1 Site of Action

In respect to how light-activated antimicrobial agents trigger microbicidal effects, It has been hypothesized that the ROS generated from type I and II reactions can cause microbial cell damage via three main mechanisms: (1) Damage of the cell membrane (virus envelope); (2) inactivation of essential enzymes and proteins and/or (3) Damage of DNA (Hamblin & Hasan, 2004). The PDT-induced photo-damage could result in massive morphological and functional alterations of the microbial cell. Functional damage results from loss of enzymatic activities, oxidation of protein–protein cross–links and inhibition of metabolic processes (e.g. DNA synthesis, glucose transport). Morphological changes include alteration of the mesosome structure (Jori & Roncucci; 2006). Direct damage to the cell membrane leads to leakage of cellular contents and subsequent inactivation of the membrane transport system. The sites of action vary based on both the PS used and the micro-organism being studied.

The cellular structure and organization among microbes shows huge divergence. These variations influence the interaction of exogenous PS with

cell constituents and so affect the efficiency and the course of action of the photodynamic inactivation of pathogens (Jori *et al.*, 2006). A vast number of studies have focussed on clarifying which specific sites within microbes act as targets for LP.

1.3.3.1.1 Bacteria

The mechanisms by which light-activated antimicrobial agents cause bacterial cell death are complex and non-specific. Many investigators have suggested that damage to the bacterial cell membrane is one of the main mechanisms of cell death following LP (Bertoloni *et al.*, 1990; Valduga *et al.*, 1999; Bhatti *et al.*, 2002).

Differences in the cell wall ultra-structure of Gram-positive and Gram-negative bacteria (Figure 1-6) play an important role in the susceptibility of bacteria to LP. As a general rule, neutral or anionic PS molecules bind efficiently to Gram-positive bacteria and inactivate them. In contrast, they bind only to the outer membrane of Gram-negative bacteria, but do not photo-inactivate them (Bertoloni *et al.* 1990; Malik *et al.*, 1990). The high susceptibility of Gram-positive species was attributed to the cell wall structures, as their cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid that allows PS molecules to diffuse to the target sites within the cell. The cell wall of Gram-negative bacteria consists of an inner cytoplasmic membrane and an outer membrane containing lipopolysaccharide (LPS) that are separated by the peptidoglycan-containing periplasm. This negatively charged LPS hinders the permeability of many molecules in the external environment into bacterial cell (Bertoloni *et al.*, 1990; Maisch *et al.*, 2004; Hamblin & Hasan, 2004).

There are two approaches to overcome this problem and achieve broad-spectrum activity especially with neutral and anionic PSs. The first approach is to use membrane disorganizing agents to enhance their permeability such as polymyxin nonapeptide (Malik *et al.*, 1992) or ethylenediaminetetraacetic acid (EDTA) (Bertoloni *et al.*, 1990). These agents cause the displacement and the removal of the Mg^{2+} and the Ca^{2+} ions which neutralize the superficial negative charges; accordingly, electrostatic repulsion is endorsed with destabilization of

the cell wall structure, inducing the release of a large fraction of the LPS into the medium. This allows the PS molecules that are normally excluded from the cell to penetrate and cause fatal damage. The second approach is to attach a cationic polypeptide to the neutral or anionic PS molecule, to help binding to the negative charges of LPS (Soukos *et al.*, 1997&1998; Rovaldi *et al.*, 2000; Hamblin *et al.*, 2002a). Another solution is increasing the selectivity of the PS to the target micro-organism (Verma *et al.*, 2007). This can be achieved via conjugation of the PS molecule to monoclonal antibodies or bacteriophages which allow selective binding to specific structures of the target micro-organism. This approach can limit the destruction to host tissues surrounding the infected area. These techniques have been verified successfully *in vitro* against MRSA (Embleton *et al.*, 2002; 2004 & 2005) and *in vivo* against a *P. aeruginosa* skin-infection model in mice (Berthiaume *et al.*, 1994).

As early as 1959 a research work carried by Mathews and Siström, demonstrated that *Sarcina lutea* could be photosensitized in the presence of either TBO (Mathews & Siström, 1959) or 8-MOP (Mathews, 1963) upon exposure to visible light. Mathews, (1963) reported that the active site in *S. lutea* cells to LP using TBO is probably the protein of the cell membrane, as manifested by the destruction of membrane enzyme activity and the increased permeability. The killing mediated by photo-activated 8-MOP is due to alteration in the cellular DNA based on the fact that psoralen photosensitization resulted in the development of mutant strains.

Biochemical analyses performed on irradiated *E. coli* suggest that the cytoplasmic membrane is an important target of the cytotoxic species generated from photo-activation of phthalocyanine, while DNA is not the main target. This suggestion was based on the binding behaviour of the compound and the marked inhibition of ATPase activity which possibly causes cell death. This cell death is a result of depletion of energy resources disrupting cell-wall synthesis, rather than direct damage to cellular structure. This theory was supported by the fact that DNA shows no alteration in irradiated cells (Bertoloni *et al.*, 1990).

The alteration of cytoplasmic membrane proteins has been shown by Valduga *et al.*, (1999). It was reported that porphyrin exerts its phototoxic activity largely

by impairing some enzymatic and transport functions at both the outer and cytoplasmic membrane of bacteria. These alterations include a gradual attenuation of some transport proteins and a complete loss of lactate and NADH dehydrogenase activities. It was also claimed that DNA does not represent a critical target of porphyrin photosensitization as wild-type *E. coli* and strains defective for two different DNA repair mechanisms had similar photosensitivity. Furthermore, plasmids extracted from photosensitized *E. coli* TG1 cells had no detectable alterations.

Bhatti *et al.*, (2002) noted a decrease in cytoplasmic membrane fluidity of *P. gingivalis*, one of the causative pathogens of periodontitis, which was accompanied by membrane condensation and vacuolation of the cells after exposure to red light combined with TBO. This may be attributable to protein-protein and protein-lipid crosslinking within the membrane and/or peroxidation of the constituent lipids, which in turn result in the disruption of membrane functions.

The main alterations in bacterial cell functions following LP resulted from photo-oxidation of protein-protein cross linking, at the level of the cytoplasmic membrane proteins (Bertoloni *et al.*, 2000). X-ray-linked microanalysis and transmission electron microscopy (TEM) confirmed structural changes and damage to the membrane of *E. coli* and *A. baumannii*-LP treated bacteria. The changes were in the form of loss of potassium and an overflow of sodium and chloride into bacterial cells, which indicate serious damage to the cytoplasmic membrane (Nitzan & Ashkenazi, 2001). DNA damage was only observed in *Staph. aureus* (Bertoloni *et al.*, 2000), *E. coli* and *A. baumannii* (Nitzan & Ashkenazi, 2001) after a relatively long irradiation time and probably resulted from damage at multiple sites leading to a complete disruption of the cell, including DNA. Both research groups concluded that DNA may be a secondary target but not the critical target for LP of bacteria.

In contrast, other researchers have reported that the LP process may lead to direct damage of the genetic material of both Gram-positive and Gram-negative bacteria in the form of breakage in both single- and double-stranded DNA, and the disappearance of the plasmid supercoiled fraction (Fiel *et al.*, 1981; Nir *et*

al., 1991; Salmon-Divon *et al.*, 2004). A recent study have shown that *E. coli* membranes are not the key target of photodynamic action for phenothiazinium compounds, but they probably act on cytoplasmic targets, most likely DNA (Hussain *et al.*, 2006).

Another study by Caminos *et al.*, (2008) verified that LP induced DNA damage in *E. coli*. Disappearance of the supercoiled plasmid band occurred after only 27 J/cm² of illumination and that of the closed circular form plasmid DNA band occurs dramatically between 108 and 324 J/cm² of illumination. Furthermore, there was no detectable damage of the cell wall or release of intracellular biopolymers after photodynamic inactivation of *E. coli* using porphyrins. These results indicate that the photodynamic activity of these cationic porphyrins produces DNA photo-damage after a long period of irradiation. Hence, an interference with membrane functions could be the main cause of *E. coli* photo-inactivation after a short period of photosensitization.

PSs which have been found to be very effective as photo-antimicrobial agents preferentially localize in the cytoplasmic membrane (Wainwright *et al.*, 2007). One important exception is acridines, which exhibit a strong bactericidal effect against a large variety of both Gram-positive and Gram-negative bacteria due to their direct effect on DNA (Barker & Hardman, 1978; Wainwright *et al.*, 1997). The planar area of the tricyclic acridine nucleus is ideally suited to intercalate between nucleotide base pairs in the helix (Wainwright, 1998). Irradiation of this intercalated PS, can cause oxidative nucleic acid damage resulting in rapid cell death. However, the DNA damage induced by acridines may be reversible and repairable by various DNA repairing systems (Wainwright *et al.*, 1997).

In summary, LP can induce bacterial cell damage as follows; the generation of ROS causes initial membrane alterations which are generally followed by a massive influx of the PS to the intracellular constituents. As a result, a variety of targets, including protein cross linking, enzymes, DNA, undergo photo-oxidative modification at later stages of the photo-process, even though DNA damage is not directly linked with cell death. There are two solid pieces of evidence that the main target within the cell is not DNA. First of all, the

pathogen *Deinococcus radiodurans* that is very hard to kill because it has a very efficient DNA repair mechanism, is rapidly killed by LP processes (Nitzan & Ashkenazi, 1999). The second evidence is that both wild strains of *E. coli* and strains with defective DNA repair mechanisms, demonstrate similar sensitivity to photo-inactivation by a tetracationic porphyrin (Valduga *et al.*, 1999). This mode of cellular photo-changes is in accordance with the frequently observed lack of mutagenic effects induced by photosensitization of microbial cells (Jori *et al.*, 2006).

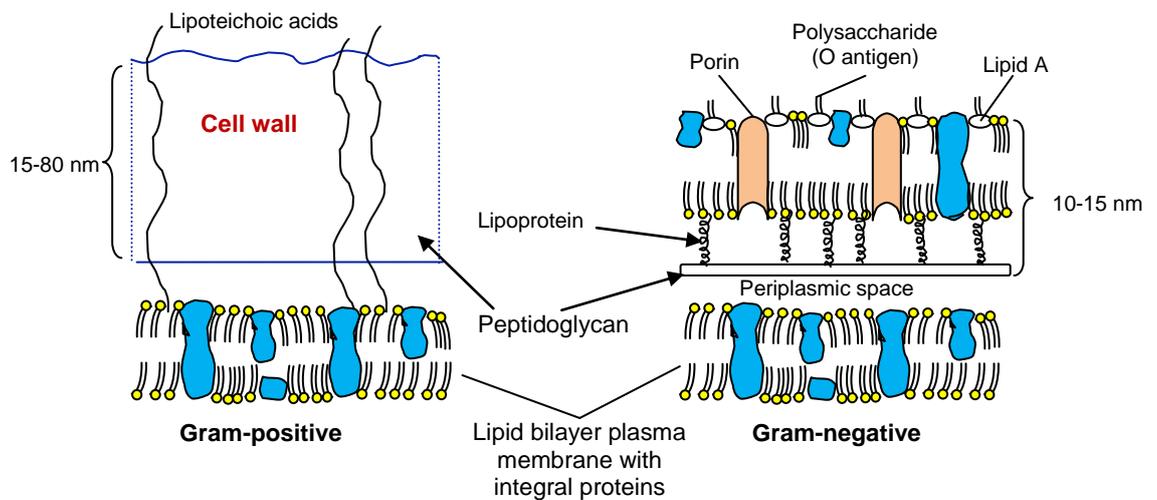


Figure 1-6: The cell-wall structures of Gram-positive and Gram-negative bacteria

1.3.3.1.2 Viruses

Photodynamic-inactivation is effective against viruses. Many authors reported successful inactivation of various viruses using a wide range of PSs encompassing many different chemical classes (Wainwright, 2004). Virus photo-inactivation has mostly been investigated in relation to blood product disinfection (Wainwright, 2000 & 2002; Wainwright *et al.*, 2007). Researchers are now interested in treating localized viral infections in humans with PDT. For example, PDT has been used to treat infectious diseases related to human papilloma virus (HPV) (Ichimura *et al.*, 2003; Herzinger *et al.*, 2006; Kacerovska *et al.*, 2007).

The structure of the virus plays a key role in effective photosensitization (Figure 1-7). In general, viruses are categorized according to whether the particle is surrounded by a protein sheath or envelope. For example, herpes viruses and human immunodeficiency virus (HIV) are enveloped, whereas HPV and

adenoviruses are non-enveloped. Enveloped viruses have been shown to be considerably more sensitive to photodynamic damage than non-enveloped viruses. Thus the viral envelope is a main target for the LP process (Wainwright & Crossley, 2004). Lipids and proteins in the envelope are assumed to act as photosensitizing binding sites; protein damage may be the mechanism underlying virus inactivation (Käsermann & Kempf, 1998).

A particular group of PSs such as merocyanine 540, hypericin and RB were found to cause cross-linking of viral membrane proteins and so destruction of the fusion function of a range of viruses such as HIV, vesicular stomatitis viruses (VSV), simplex virus type 1 and human cytomegalovirus (Lavie *et al.*, 1989; O'Brien *et al.*, 1992; Lenard *et al.*, 1993).

MB is a well known PS that can intercalate into viral nucleic acid. Viral inactivation using MB and red light is reported to induce oxidative damage within QB phage; these are 8-oxoGua formation (degraded guanine residues in nucleic acids), DNA-strand breaks, protein carbonyls and RNA-protein cross-linkages. However, the authors attributed the inactivation mainly to RNA-protein cross-linking (Schneider *et al.*, 1998). Further studies demonstrated that photodynamic treatment with MB has a high affinity for enveloped RNA viruses and can effectively inactivate various enveloped RNA viruses, including the Dengue virus, HIV, hepatitis B virus and hepatitis C virus in plasma, and the nonenveloped parovirus B19 (Mohr *et al.*, 1997; Huang *et al.*, 2004).

Also other researchers have illustrated the modification of the enzymatic activities of photo-treated viruses. Moor *et al.*, (1997) showed a rapid decrease of RNA polymerase activity upon irradiation of VSV in the presence of aluminium phthalocyanine. Another group has reported photo-damage of the viral core proteins, viral ribonucleic acid along with complete inhibition of the reverse transcriptase activity in HIV following photosensitization with MB (Bachmann *et al.*, 1995).

Surprisingly, in two recent studies, Egyeki *et al.*, (2003) and Zupán *et al.*, (2008) showed that effective inactivation of T7 phage, as a surrogate of non-enveloped viruses can be achieved using photo-activated cationic porphyrin.

The results revealed that the effective killing of the T7 phage did not depend on the DNA-binding properties of the compound and the free PS molecules were more efficient to causing viral damage. Both phage DNA and the protein capsid were affected by the photoreactions.

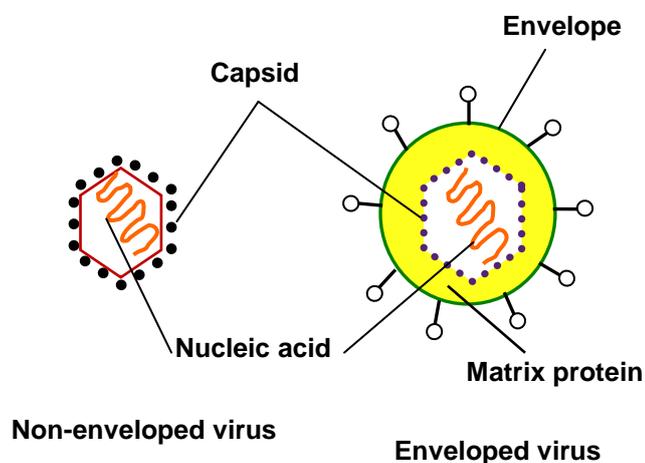


Figure 1-7: The general structure of an enveloped and non-enveloped virus

1.3.3.1.3 Yeasts and fungi

Fungal infections, mainly candidiasis, are common opportunistic infections in the oral cavity and on the skin especially in immunosuppressed patients especially AIDS and cancer patients. The growing resistance to the commercially available antifungal compounds has driven researchers to search for alternative antimicrobial modalities; photodynamic inactivation (PDI) of pathogens is a possible candidate (Wilson and Mia, 1993).

Fungi are much less susceptible to photodynamic killing than bacteria because of the large size of the fungus (10 to 50 times bigger than the bacteria), consequently the amount of ROS needed to kill a yeast cell is much greater than the amount essential to kill a bacterial cell (Demidova & Hamblin, 2005).

An enormous amount of research has documented the successful inactivation of a variety of fungi using many classes of PSs (Wilson & Mia, 1993; Jackson *et al.*, 1999; Demidova & Hamblin, 2005; de Souza *et al.*, 2006). There are a limited number of studies concerning the target site in fungi and yeasts during the photosensitization-process. Wilson and Mia (1994) showed that *C. albicans* can be photoinactivated with laser light and phenothiaziniums (TBO or MB) *in vitro* in the presence of saliva, serum, at low pH.

The examination of *C. albicans* uptake of photofrin PS indicated that the compound bound steadily to the interior of the cell surface of *C. albicans* rather than infiltrated into the fungus cells. The cells treated with irradiation in combination with the sensitizing agent showed a dose-dependent inhibition of metabolic activity in two strains of *C. albicans*. This study showed that the efficiency of killing was strain dependent (Bliss *et al.*, 2004). Moreover, Monfrecola *et al.*, (2004) provided microscopical evidence that 5-aminolaevulinic acid (ALA) PDT of *C. albicans* cells resulted in cell membrane ruptures associated with apparent cell wall swelling. This may be due to the conversion of the naturally occurring precursor metabolite ALA into porphyrins. The resulting accumulation of porphyrins in the cell produces a photodynamic reaction upon exposure to light.

A recent study described the mechanism by which killing was induced in *C. albicans* exposed to PDI by a cationic porphyrin. The fluorescence confocal microscopy and freeze-fracture electron microscopy analysis showed the following: under dark conditions TriP[4] binds to the cell envelope of *C. albicans*, and none or very little TriP[4] diffuses into the cell. Following irradiation the cell membrane is damaged and eventually becomes permeable to TriP[4]. This allows a massive influx of TriP[4] into the cell. It was found that only the vacuole membrane is resistant to PDI-induced damage once TriP[4] passes the plasma membrane. In this study increasing the incubation time did not enhance the efficacy of PDI; however, the suspension fluid had a great effect (Lambrechts *et al.*, 2005b).

Even though LP of fungi has been reported widely *in vitro*, no data are available *in vivo*, except the data presented by Teichert *et al.*, (2002) on the efficacy of MB activated with a diode laser (664 nm) for treating oral candidiasis in immunosuppressed mice. The results showed the total eradication of *C. albicans* from the oral cavity was possible using dye concentrations between 450 and 500 µg/ml, which indicate that MB-mediated PDT could be a potential treatment for oral candidiasis in immunosuppressed patients.

1.3.3.2 Biofilms

Lately, biofilms have been increasingly recognized as being a vital underlying cause of persistence in a wide range of human chronic infections. Common examples are cystic fibrosis, otitis media, gingivitis and chronic wounds. The severity associated with such infections arises from two distinct manifestations linked to all kinds of biofilms. Firstly, as described before, biofilms are highly resistant to killing and clearance by the immune system and so to treatment with antimicrobial agents (Davies, 2003). The current antimicrobial modalities may be effective to treat acute infections caused by planktonic microorganisms, but are found to be ineffective in eradicating the infections if the biofilm already exists within the tissues (Krespi *et al.*, 2008). Secondly, a sharp drop of oxygen concentration has been observed within the biofilm. Oxygen concentration can be completely diminished under a biofilm of 100 μm thick-ranging from below 1 ppm in central parts to 8 ppm in the outer portions of the biofilm (Ganesh & Radhakrishnan, 2008). These problems associated with biofilm infections have triggered the search for novel antimicrobial agents. PDT is a possible novel antimicrobial treatment that may provide a solution to biofilm chronic infections.

So far, the literature discussing the effects of LP on biofilms mainly concerns biofilms in the oral cavity (Dobson & Wilson, 1992; Wilson *et al.*, 1996; Soukos *et al.*, 2003; Zanin *et al.*, 2005 & 2006). Wilson and colleagues studied the bactericidal effect of different sensitizing agents including TBO, MB, aluminium disulphonated phthalocyanine, haematoporphyrin HCl and haematoporphyrin ester against oral biofilms. Biofilms of *S. sanguis*, *P. gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* were exposed to laser light at 632.8 nm in the presence of one of the above-mentioned agents. The results showed that TBO and MB induced detectable killing of all four target organisms after exposure to laser light for 30sec, while aluminium disulphonated phthalocyanine, haematoporphyrin HCl and haematoporphyrin ester were effective PSs of only some of the target organisms. Therefore these results imply that LP may offer an alternative to antibiotics and antiseptics in the treatment of inflammatory periodontal diseases, if effective *in vivo* (Dobson & Wilson, 1992). Afterward, the same group reported that 4-day old biofilms of *S. sanguis* can be photo-inactivated with AlPcS_2 , in a light-energy dose-related

manner from GaAIs pulsed laser light of 660 nm. Irradiation of AlPcS₂-treated biofilms at an energy density of 61.2 J/cm² resulted in complete eradication of streptococci biofilms (Wilson *et al.*, 1996). Confocal laser scanning microscopy (CLSM) evidence for the destruction of biofilm structure has been reported for dental-type biofilms treated with a cationic zinc phthalocyanine. The photosensitized biofilms were thinner and less dense with apparently reduced cell-to-cell and cell-to-matrix binding. Transmission electron microscopy (TEM) revealed that PDT induced considerable damage to bacteria in biofilms formed *in vivo*, vacuolation of the cytoplasm and membrane damage being noticeable after PDT treatment (Wood *et al.*, 1999). Later on, O'Neill *et al.*, (2002) showed that significant numbers of oral bacteria in multi-species biofilms can be killed by red light at 81.9 J/cm² in the presence of TBO. These results clearly demonstrate the potential value of PDT in the management of oral biofilms.

Lately, further studies explored the possibility of photo-inactivation of biofilms of a selection of organisms. Zanin *et al.*, (2006) showed that TBO with 85.7 J/cm² of light from a light-emitting diode produced an antimicrobial effect on 5-day old streptococcal biofilms. A significant kill of 95% was observed for *S. mutans* and *Streptococcus sobrinus* biofilms while a kill of 99.9% of *S. sanguis* biofilms was attained after photosensitization. CLSM images of *S. mutans* biofilms after exposure to HeNe laser or LED light in the presence of TBO suggested that LP affected primarily the outer layers of the biofilms, leaving some of the innermost bacteria alive, which may be due to the inability of the PS to diffuse through into these innermost layers (Zanin *et al.*, 2005). A possible explanation is that the EPS of the biofilm confines the PS molecules to the outside of the cell as a result of ionic or hydrophobic interactions and therefore reduces the amount of PS that was able to penetrate to the plasma membrane, which is one of the important sites of LP-mediated damage (Gad *et al.*, 2004a).

Staphylococcal biofilms were killed by lethal photosensitization using either merocyanine 540 (Lin *et al.*, 2004) or TBO (Sharma *et al.*, 2008). The former study revealed that the antimicrobial activity exerted on the *Staph. aureus* biofilms was enhanced with an increase in the light dose. No viable *Staph. aureus* cells were detected using 15 µg/ml of MC 540 and a light dose of 600 J/cm² or 20 µg/ml of MC 540 and a light dose of 450 J/cm² (Lin *et al.*, 2004).

The results of CLSM suggested damage to bacterial cell membranes in photo-inactivated biofilms. In addition, TEM images provided direct evidence for the disruption of biofilm structure and a decrease in the number of adherent bacteria in photosensitized biofilms. Furthermore, the treatment of biofilms with tetrasodium EDTA followed by photosensitization enhanced the bactericidal effectiveness of TBO in *Staph. epidermidis* but not in *Staph. aureus* biofilms (Sharma *et al.*, 2008).

LED irradiation at 120 J/cm² of *P. aeruginosa* biofilms treated with 20-40 mM δ -ALA, resulted in complete kill of the bacteria. However, at this concentration of the PS a dark toxicity of about 2-log reduction in biofilm density was observed. The *P. aeruginosa* biofilms started to re-grow 12 hours after receiving a single treatment of 20 mM δ -ALA-mediated PDT at 120 J/cm². Biofilms appeared to re-accumulate thereafter and reached 7 logs CFU/cm² after 48h of re-growth. Two treatments of the biofilms at interval of 12 hours completely eradicated the bacteria (Lee *et al.*, 2004). Street *et al.*, (2009) confirmed that photo-inactivation of *P. aeruginosa* biofilms is possible using PeriowaveTM and 0.01% MB at 20.6 J/cm² and treated either once or twice. Two and three logs reduction in the viable count respectively were achieved after treating 24h-old *P. aeruginosa* biofilms once or twice with MB-mediated LP. In 48h-old *P. aeruginosa* biofilms 5 and 6.5 logs kill resulted from treatment once or twice respectively.

Furthermore, it was shown that the metabolic activities of 24h-old *C. albicans* biofilms reduced gradually when exposed to photo-activated photofrin[®] with increasing light dose from 0.9 to 18 J/cm² (Chabrier-Roselló *et al.*, 2005).

To date, biofilm studies suggest that photodynamic treatment may be a useful approach for the treatment of chronic infections caused by biofilm formation *in vivo*. The inability of the PS to penetrate the inner layers of the biofilm is one potential problem associated with PDT of biofilm-related diseases. However, this could be overcome by selecting a PS able to penetrate through the biofilm matrix and by irradiating biofilms internally (e.g. via inserting an optical fiber into the biofilm itself) (O'Neill *et al.*, 2002).

1.3.4 Clinical applications of photodynamic therapy

Since the early discovery of PDT, it has been applied extensively for the treatment of malignant diseases as an alternative or adjacent to chemotherapy and/or radiotherapy (Hopper, 2000). However, more recently new PDT applications have been introduced to both clinical and experimental studies. Nowadays PDT is a promising treatment in many medical fields including dentistry, ophthalmology, dermatology and infectious diseases.

1.3.4.1 Infections

The current need for alternative antimicrobial agents, as a consequence of the increasing rate of antibiotic-resistance, has compelled researchers and clinicians to evaluate the potential of antimicrobial PDT. Thus, a plethora of PDT applications have been developed, particularly for the treatment of localized infections of the skin and the oral cavity.

1.3.4.1.1 Wound and soft tissue infections

PDT has a number of advantages over conventional antimicrobials for the treatment of infected wounds. Firstly, as the mechanism of killing is non-specific, with ROS causing damage to many bacterial components, resistance is unlikely to develop from repeated use (Jori *et al.*, 2006). The eradication of wound-infecting bacteria using LP has been studied extensively as a new strategy against antibiotic-resistant micro-organisms. So far the most extensively investigated bacteria are *Staph. aureus* (Wilson & Pratten, 1995; Griffiths *et al.*, 1997b; Zeina *et al.*, 2001; Embleton *et al.*, 2002; Nitzan *et al.*, 2004; Lambrechts *et al.*, 2005a; Embleton *et al.*, 2005; Bisland *et al.*, 2006; Banfi *et al.*, 2006; Tegos *et al.*, 2006), *P. aeruginosa* (Nitzan *et al.*, 1992; Minnock *et al.*, 1996; Hamblin *et al.*, 2003; Banfi *et al.*, 2006; Tegos *et al.*, 2006), *Strep. pyogenes* (Zeina *et al.*, 2001; Tegos *et al.*, 2006; Hope & Wilson, 2006), and *E. coli* (Minnock *et al.*, 1996; Hamblin *et al.*, 2002a & b; Lazzeri *et al.*, 2004; Nitzan *et al.*, 2004; Banfi *et al.*, 2006; Tegos *et al.*, 2006).

A wide range of microbes can be killed *in vitro* using photo-activated PS. A stream of *in vivo* studies have explored the potential of PDT for the treatment of infected wounds and related soft tissue and bone infections in the past few years (Hamblin *et al.*, 2002b, 2003; Gad *et al.*, 2004b; Lambrechts *et al.*, 2005c; Bisland *et al.*, 2006; Zolfaghari *et al.*, 2009).

Hamblin *et al.*, (2002b & 2003) demonstrated that PDT can control infection progress in excisional infected-wounds with either *E. coli* or *P.aeruginosa*. The infections in both studies were monitored using a bioluminescence imaging system. Treated wounds healed as fast as control wounds in both studies, showing that the photodynamic treatment did not damage the host tissue.

Poly-L-lysine chlorin e6 conjugate or free chlorin e6 photo-activated by red light from a diode laser was shown to be effective in controlling *Staph. aureus* soft tissue infections. Light was delivered either at a surface spot or by an interstitial fiber into the infection. There was a light dose dependent loss of bioluminescence (to <5% of that seen in control infections), but in some cases, the infection recurred (Gad *et al.*, 2004b).

Lambrechts *et al.*, (2005c) stated that PDT mediated by meso-mono-phenyl-tri(N-methyl-4-pyridyl)-porphyrin (PTMPP) was effective in the treatment of bioluminescent *Staph. aureus*-infected burn wounds in mice, achieving > 98% kills of this bacteria. However, light alone or PDT both delayed the wound healing.

Another group conducted a study to investigate the effect of PDT on osteomyelitis. A wire infected with *Staph. aureus* was implanted into the tibial medullary cavity of Sprague-Dawley rats to bring on the osteomyelitis. *Staph. aureus* infections were subject to PDT 10 days post infection. The treatment which comprised of intra-peritoneal (IP) administration of ALA (300 mg/kg) followed 4 h later by light (635 ± 10 nm; 75 J/cm^2) delivered transcutaneously via an optical fiber placed onto the tibia resulted in significant delay in bacterial growth (Bisland *et al.*, 2006).

Recently, it was shown that MB-PDT can reduce the number of epidemic methicillin-resistant *Staph. aureus* (EMRSA-16) in a mouse model of infected excisional and superficial scarified wounds (Zolfaghari *et al.*, 2009). These data suggest that PDT has the potential to rapidly reduce the bacterial load in infected wounds and can control the consequent soft tissue and bone infections.

PDT has been transferred from the stage of *in vivo* studies to clinical application in patient as case reports and clinical trials as described in this section. It has been reported that a case of chronic recalcitrant venous ulceration in the lower leg of an elderly patient who did not respond to any conventional therapies was treated successfully using ALA-PDT. The case was complicated by several episodes of cellulitis with marked colonization of MRSA. The patient had a history of multiple allergic reactions to antibiotics and her ulcer was resistant to topical antiseptics and larval therapy. Therefore, PDT treatment was the only option for this patient. PDT was delivered twice weekly over 4 weeks to the ulcer at different 5-cm diameter sites each time. 5-aminolaevulinic acid (5-ALA) was used as a topical agent and the area was subsequently irradiated with red light at a wavelength of 633 nm. The PDT was well tolerated with minimal discomfort to the patient, even without the use of topical local anaesthesia. A significant improvement was subsequently observed in the ulcer, which correlated with negative skin swabs obtained from the ulcer (Clayton & Harrison, 2007).

The treatment of chronic festering wounds and trophic ulcers proved successful in 30 patients using 5-ALA (alasers) or a mixture of sulphonated aluminium phthalocyanine (photosens). These sensitising agents were applied for 2-4 h and then the ulcers were irradiated with either laser light at 675 nm to activate photosens or by a pulsed light source to activate alasers, at a fluence rate of 30-50 mW/cm² and a total light dose of 40-60 J/cm². The treatment was effective and well tolerated by the patients without any allergic or toxic reactions (Loschenov *et al.*, 2008).

A brief case report showed the significance of antimicrobial PDT in the treatment of osteomyelitis associated with diabetic foot ulcers, and in healing of fractured bones. The PDT protocols consisted of local injection of mixtures of phenothiazines (i.e. either MB or/and TBO 2% in water) and *Hypericum perforatum* extract (10% in propylene glycol), followed by illumination for 10 min from an optical fiber and a non-coherent light sources simultaneously to the lesion's interior and exterior to allow total exposure of the bone and the soft tissues injuries. The treatment frequency was daily or every other day in the beginning, and weekly after tissue recovery began. Both patients treatment

was successful and they were spared amputation of their feet. Radiograms showed that bone had healed and that the bone's texture had improved (Tardivo & Baptista, 2009).

Furthermore, the first part of a phase II, blinded, randomised placebo-controlled, single PDT treatment demonstrated significant reduction in bacterial load in the treated group and strong trends towards wound healing. 3 months after the treatment, 50 % of PDT treated patients completed healing in comparison to only 12.5 % in the placebo group. The PS 3,7-Bis(di-n-butylamino) phenothiazin-5-ium bromide (PPA904) was topically applied to the wound concealed for 15 minutes then any excess cream was removed then irradiated with red light (50 J/cm^2) (Brown *et al.*, 2008). The second part of this clinical trial to investigate the efficacy of PDT multi-treatment in reducing bacterial burden in leg ulcers is in progress now in several medical centres in the UK (Harding *et al.*, 2009).

There is a double rationale for the treatment of wounds by PDT on account of its antibacterial effects and the prospect of accelerating wound healing. PDT, therefore, appears to represent an alternative modality for the treatment of localized microbial infections and may be an adjacent modality in treating infected ulcers, wounds and osteomyelitis. It may also reduce requirements for systemic antibiotics in the management of skin infections, thereby lessening antibiotic resistance.

1.3.4.1.2 Acne vulgaris

PDT for the treatment of acne vulgaris is a fast-growing therapeutic modality. Currently, light in combination with a variety of PSs can be applied for the treatment of the infection associated with acne vulgaris via killing the *P. acnes* bacteria that cause skin eruptions (Elman & Lebzelter, 2004).

An open-label prospective study was conducted by Hongcharu *et al.*, (2000) for the treatment of acne vulgaris using PDT and ALA. ALA is taken up by the tissue and converted into protoporphyrin, a potent PS. Acne on the back of 22 patients was treated with topical 20% ALA–PDT and 150 J/cm^2 of red light (550 to 700 nm). Even though the treatment initiated transient acne-like folliculitis,

there was evidence of acne improvement that lasted for 20 weeks after multiple treatments and 10 weeks after a single treatment. Additionally, there was a significant decrease in follicular bacteria, which lasted for at least 20 weeks after treatment. The side effects associated with the ALA-PDT included pain, erythema, oedema, superficial exfoliation, crusting, and hyperpigmentation that completely diminished over several weeks. Thus, PDT may be an effective treatment that is associated with adverse side effects (Hongcharu *et al.*, 2000). Akaraphanth *et al.*, (2007) reported that there was no significant difference between ALA-PDT with blue light and blue light only in the treatment of acne, yet at the end of 16 weeks of the treatment ALA-PDT showed 71.1% reduction in inflamed lesions and only 56.7% was observed with the blue light.

Methyl aminolevulinate-PDT (MAL-PDT) has been applied to patients with moderate to severe facial acne vulgaris in randomized, controlled and investigator-blinded trials (Hörfelt *et al.*, 2006; Wiegell & Wulf, 2006). The treatment proved successful in both studies with a reduction of 68% from baseline in inflammatory lesions in the first study (Wiegell & Wulf, 2006) or median reduction of 54% in the second one (Hörfelt *et al.*, 2006). Local adverse side effects were consistent with this modality since the patients suffered moderate to severe pain during treatment and developed severe erythema, pustular eruptions and epithelial exfoliation (Hörfelt *et al.*, 2006; Wiegell & Wulf, 2006). In contrast, the use of MAL reduced to 4% concentration, together with application of low doses of light, proved to be useful for controlling the side-effects of PDT in acne and have the same efficacy as the higher concentration whereas the count of inflammatory lesions had decreased on average by 66% and with lower costs (Mavilia *et al.*, 2007).

Unlike ALA and MAL-PDT, ICG-PDT has been effective in the treatment of acne with no obvious side effects (Lloyd & Mirkov, 2002; Tuchin *et al.*, 2003). A pilot study was carried out by Tuchin *et al.*, (2003) on the effects of ICG photodynamic and photothermolysis treatment on acne vulgaris. The authors used 1.0 mg/ml ICG lotion, which was allowed to penetrate for 5 or 15 minutes, then was photo-activated by NIR laser light. Three protocols of irradiation were applied: (1) Low-intensity protocol was accomplished at laser power densities of 10–50 mW/cm² (803 nm) on the skin surface and exposures of 5–10

minutes; (2) medium intensity protocol at 150–190 mW/cm² (809 nm) and exposure of 15 minutes; and (3) photo-thermal laser at 18 W/cm² (803 nm, 0.5 seconds) without skin surface cooling or at 200 W/cm² (809 nm, 0.5 seconds) with cooling. Single and multiple (up to 8–9) treatments were investigated. The ICG-PDT decreased the number of active elements, reduced erythema and inflammation, and considerably improved the skin state without any side effects. The successful treatment was due to the inactivation of *P. acnes* stained with ICG and irradiated by NIR light. Multiple treatments were 80% more effective than a single treatment; improvement lasted for at least a month compared to a week with a single treatment.

In an evidence-based review, it was concluded that PDT is one of the best optical treatments that possess the potential to improve inflammatory acne on a short-term basis and it resulted in the most consistent outcomes account for up to 68% improvement of the symptoms (Haedersdal *et al.*, 2008). The suggested mechanisms of action are photothermal heating of sebaceous glands and photochemical inactivation of *P. acnes*, which produces coproporphyrins and protoporphyrins. Moreover, photoimmunological reactions may possibly contribute to improve acne (Haedersdal *et al.*, 2008).

1.3.4.1.3 Oral infections

Light-activated antimicrobial agents provide a broad spectrum of action against micro-organisms responsible for caries, periodontal diseases and root canal infections. The advantages of applying this approach in oral infections are that microbes can be eliminated in very short periods of time, resistance development in the target microbes is unlikely and damage to adjacent host tissues and disruption of the normal microbiota can be limited (Wilson, 2004). PDT may be of great interest to dentists as an alternative to antibiotics and antiseptics in eliminating cariogenic and periodonto-pathogenic bacteria from disease lesions and for the disinfection of root canals. The approach has been tested in a few clinical trials. The effect of TBO-PDT in the treatment of peri-implantitis was investigated in 15 patients. The implant surface was stained with 100 µg/ml TBO for 1 min, afterwards it was irradiated with a light at 690 nm for 60 s. It was found that the TBO-PDT reduced bacterial counts by 2 logs on average (Dörtbudak *et al.*, 2001).

With regard to PDT for periodontitis, PDT using a laser light with a wavelength of 690 nm in conjunction with a phenothiazine chloride PS (10 mg/ml) was effective in the treatment of aggressive periodontitis and improving the clinical signs and symptoms accompanying the disease (de Oliveira *et al.*, 2007). Garcez *et al.*, (2008) reported the effectiveness of PDT in the treatment of root canal infections associated with necrotic pulps and periapical lesions in 20 patients. A total reduction of 3.19 log₁₀ of microbial loads after 3 treatment sessions of polyethylenimine and chlorin(e6) conjugate-PDT was achieved. Results suggested that the use of PDT added to endodontic treatment leads to an enhanced decrease of bacterial load and may be an ideal approach for the treatment of oral infections.

1.3.4.1.4 Gastric infection

H. pylori is a Gram-negative porphyrin-containing bacterium that colonizes the mucus layer of the human stomach and duodenum; the organism is a major cause of chronic gastritis, gastric ulcers, duodenal ulcers, gastric lymphoma, and gastric adenocarcinoma (Kusters *et al.*, 2006).

A preliminary clinical trial was carried out in 13 patients using orally administered 5-ALA at a dose of 20 mg/kg then after 45 minutes, a zone of the gastric antrum was illuminated endoscopically using a blue laser emitting light at 410 nm and a light dose of 50 J/cm² or white light (10 J/cm²). They demonstrated killing effect was achieved 4 hours post-irradiation when a maximum of 85 % of treated biopsies were *H. pylori* negative (Wilder-Smith *et al.*, 2002). This organism can be killed using a blue light without the use of external PSs *in vitro*. A controlled, prospective, blinded, trial of endoscopically delivered blue light to eradicate *H. pylori* in regions of the gastric antrum, showed that blue light reduced *H. pylori* bioburden by 91% in the treated patients. The treatment consisted of light at 405 nm and a light dose of 40 J/cm². This was delivered via an optical fiber to the gastric antrum to cover a spot of 1-cm diameter. The results of blue light phototherapy may represent a novel approach to eradicate *H. pylori*, particularly, in patients who have failed standard antibiotic treatment (Ganz *et al.*, 2005).

1.3.4.2 Wound healing

A few studies have reported the promising application of PDT to enhance wound healing. The effect PDT has on the healing process in excisional wounds in rats, has been studied. The hematoporphyrin derivative (HPD) or ALA PSs were applied systemically at concentration of 5 mg/Kg. The wounds treated with light from a He-Ne laser (3 J/cm^2) in combination with ALA as a PS showed complete wound closure by 12-14 days, whilst wounds treated with HPD and combined light from He-Ne and Nd:YAG lasers at (33 J/cm^2) was completely healed by 13-15 days. However, the control group of wounds that received no irradiation showed healing around 18-21 days. Both histopathological and tensile strength results did not vary significantly between control and PDT groups. The results suggested that ALA along with He-Ne light or HPD along with the combination of He-Ne and Nd-YAG lights speed up the healing process in wounded rats (Jayasree *et al.*, 2001). A recent study conducted by Silva *et al.*, (2004) established that topical PS (Chloro-Aluminium phthalocyanine-derived) in combination with laser light at 685 nm and total dose of 2.5 J/cm^2 were applied transcutaneously at four sites of the wound edge. The results clearly indicated a synergistic effect of light-PS on tissue healing. Not only did PDT not cause any healing inhibition or tissue damage during the healing process, PDT treated wounds also showed higher collagen content, improved re-epithelialization and more evident connective tissue remodelling compared to control wounds.

In contrast, Kübler *et al.*, (1996) showed that photofrin[®]-PDT resulted in serous effusion, epidermal necrosis, and weaker tensile strength in myocutaneous skin flaps. Photofrin[®] was administered IP at a concentration of 5 mg/Kg and then the flaps were irradiated at intensity range $25\text{-}75 \text{ J/cm}^2$ from an argon dye laser (630nm). The results of this study demonstrate that PDT given immediately before flap reconstruction will result in delayed wound healing. The discrepancy between these studies may be due to the difference in light dose and the type and concentration of the PS used in each study.

PDT using benzoporphyrin derivative and chloroaluminum sulfophthalocyanine at concentrations of 5-10 mg/Kg had no influence on either the rate or final

appearance of wound healing. Histologically, there were no apparent differences between treated and untreated sites, regardless of the drug, dose of light, or time of irradiation. The data confirmed that a single PDT treatment (10- 100 J/cm²) on wounded skin does not apparently alter wound healing even when PDT caused strong inflammatory reactions (Parekh *et al.*, 1999). These data suggested that the application of PDT as a modality to enhance or restore wound healing is promising, yet still needs further investigation due to the limited number of studies and the huge number of variables that could be involved in such therapy.

1.3.4.3 Other applications

Lipson in the late 1960's marked the beginning of PDT as a cancer therapy after the detection of selective tumour damage caused by localised PS in the tumour tissue and light irradiation. The use of PDT in oncology dates back to the early 1970s, when Dougherty and associates established the use of haematoporphyrin derivative (HpD) as an anti-tumour therapy and began investigating the mechanisms and clinical applications in malignant diseases (Kessel, 2004). In oncology, PDT has been used for anal, vulvar, basal cell and squamous cell carcinoma, neoplasia, Barrett's esophagus, palliation of metastatic breast cancer (Taub, 2007a), and head and neck cancer (Hopper *et al.*, 2004). Even though PDT was developed initially for the treatment of cancers, its use has increased in non-oncology field especially in ophthalmology in the treatment of macular degeneration of the retina which was approved by US FDA in 2000 and has been applied over a million times (Taub, 2007a). Also there is a variety of non-malignant diseases including psoriasis (Collins *et al.*, 1997), arthritis (Trauner & Hasan, 1996; Iriuchishima *et al.*, 2008), and atherosclerosis (Rockson *et al.*, 2000) that can benefit from the effects of PDT.

The results of the few clinical trials studying the effect of PDT on psoriasis were controversial and showed that this application needed refinement by choosing the suitable PS and light source. Based on the fluorescence emission of topical ALA in psoriasis plaque in 15 patients, Stringer *et al.*, (1996) suggested that using 100 mg of 20% ALA without occlusion followed by successive illumination after allowing the drug to penetrate for 3.5 h as a protocol for psoriasis treatment. This regimen of treatment proved aggressive when

researchers used much lower drug and light doses (1% ALA and a light dose of 5 - 20 J/cm²) and reported a severe intolerance and unsatisfactory results after the PDT treatment (Radakovic-Fijan *et al.*, 2005). This result was in agreement with a previous study conducted using even a lower light dose (8 J/cm² delivered at a dose rate of 15 mW/cm²) (Robinson *et al.*, 1999). Finally, it was concluded that ALA-PDT was unsuitable for the treatment of psoriasis (Robinson *et al.*, 1999; Radakovic-Fijan *et al.*, 2005).

1.4 Aims and objectives

This introduction has provided a summary of the problems associated with wound infections and the persistence of such infections that lead to chronicity of wounds and failure of them to heal and consequently fatal problems such as cellulitis, osteomyelitis or even septicaemia, which may follow. Also the introduction gave a brief overview about the failure of topical antimicrobials to keep the wound bacterial bioburden under control as a result of biofilm formation in wounds and the growing resistance of micro-organisms to the current antimicrobial agents. These reasons have motivated the search for novel antimicrobial agents to which micro-organisms will not easily develop resistance. PDT may offer an alternative approach to traditional wound disinfectants. PDT is an ideal modality to treat infected wounds as it may provide antibacterial as well as healing effects. In addition, the localised nature of light and PS applicability into wounded tissue will spare adjacent tissue any side effects.

Up till now, most research into the LP of microbes has employed light from either coherent or non-coherent sources with wavelengths around 630- 670 nm to activate the PSs that are currently of clinical use, such wavelengths have a limited tissue penetration maximum of 2-4 mm.

The project was mainly designed to test the potential use of the PS ICG which can be activated by laser light in the NIR region of the spectrum and so allowing maximum tissue penetration. Therefore, the main aim of this study was to investigate whether common wound-associated organisms *Staph.*

aureus, *P. aeruginosa*, *Strep. pyogenes* and *E. coli* are sensitive to LP using the dye ICG coupled with light from a NIR laser emitting at 808 nm.

To achieve this aim, the project had seven main objectives:

1. To evaluate the bactericidal effect of ICG LP on micro-organisms frequently responsible for wound infections in aqueous suspensions.
2. To conduct a comparison of the effect of pulsed versus continuous wave NIR laser light on LP of organisms frequently responsible for wound infections.
3. To investigate the possibility to enhance the LP process of *Staph. aureus* cells suspensions *in vitro*.
4. To determine whether ICG in conjunction with NIR laser light can effectively photosensitize wound infecting organisms when grown as biofilms.
5. To investigate the impact that the environmental factors may have on the efficacy of LP by conducting the LP process in the presence of horse serum that mimics wound fluid. Also to attempt the photosensitization of bacteria in a low oxygen environment. These factors reflect wound conditions that may be found in the *in vivo* situation. Additionally, to study the effect that LP may exert on bacterial virulence factors.
6. To explore the underlying mechanisms involved in the LP process.
7. To determine if ICG-PDT can reduce the bacterial load in infected wounds *in vivo*.

Chapter 2

Material and Methods

2.1 Lethal photosensitization

2.1.1 Target organisms

The organisms used in this series of experiments were: *Staph. aureus* NCTC 8325-4, epidemic methicillin-resistant *Staph. aureus*-16 (EMRSA-16), *Strep. pyogenes* ATCC 12202, *P. aeruginosa* strain PA01, and *E. coli* ATCC 25922. These bacterial strains are of clinical importance and are commonly available in microbiology laboratories. All bacteria were maintained by weekly subculture on blood agar (Oxoid Ltd., UK) supplemented with 5% horse blood (E & O Laboratories Ltd., UK), while *P. aeruginosa* strain PA01, and *E. coli* ATCC 25922 were subcultured weekly on nutrient agar (Oxoid Ltd., UK).

2.1.2 Growth conditions

All bacteria were grown aerobically in nutrient broth (NB) yet EMRSA-16 was grown in brain heart infusion broth (BHIB) (Oxoid Ltd., UK) in a shaking incubator at 200 rpm except *Strep. pyogenes* which was grown statically in an atmosphere of 5% CO₂ / 95% air in BHIB. All were incubated at 37°C for 16 hours. Overnight cultures were centrifuged and the pellet resuspended in an equal volume of PBS and the optical density was adjusted to 0.05 at 600 nm, corresponding to approximately 10⁷ colony forming units (CFU) per mL for *Staph. aureus*, EMRSA-16, *Strep. pyogenes*, ~10⁸ CFU/mL for *E. coli* and 10⁹-10⁸ CFU/mL for *P. aeruginosa*.

2.1.3 Light sources

Three near-infrared laser light sources were used for irradiation in these studies. A 0.5 W gallium-aluminum-arsenide (Ga-Al-As) NIR-laser (Thor International Ltd., UK) and a 0.4 W diode Laser (Ondine Biopharma Corp., USA). Both lasers emit light continuously with a wavelength at 808 ± 5 nm. The GaAlAs Velopex diode laser system (Medivance Instruments Ltd., UK) emits light at a wavelength of 810 ± 10 nm and its output power can be adjusted to 0.4 – 5W. The light from this system can be applied to the tissue through an optical fibre of 400 µm diameter, either in continuous, single pulse or repeated pulse duration modes which can be selected to switch on or off between 0 – 1000 msec.

The power output of each laser was calibrated immediately before each experiment using a thermopile TPM-300CE power meter (Genetic-eo, Québec, Canada), and were found to be 0.470, 0.370 W and 0.525 W respectively. For experimental purposes, the distance between the laser probe and the microtitre plate surface, in which the experiments were performed, was adjusted to give a range of fluence rates. The light dose delivered to each well was expressed as the energy density which was delivered at a certain fluence rate. The fluence rate or irradiance rate can be defined as the number of photons crossing over from all directions on a sphere of unit cross section which surrounds a point of irradiation. The energy density is the total energy delivered into a specific area of irradiation over a certain period of time. The basic equations used for light dose calculations are given below:

$$\text{Fluence/irradiance rate (W/cm}^2\text{)} = \text{Power output (W)} / \text{unit area (cm}^2\text{)}$$

$$\text{Energy density (J/cm}^2\text{)} = \frac{\text{Power output (W)} \times \text{irradiation time (sec) \{J\}}}{\text{per unit area (cm}^2\text{)}}$$

2.1.4 Photosensitizer

Indocyanine green also named 4,5-benzoindotricarbocyanine ($\text{C}_{43}\text{H}_{47}\text{N}_2\text{NaO}_6\text{S}_2$) is a negatively-charged polymethine dye. The dye was purchased from Sigma-Aldrich-UK with a purity of 90% and fresh stock solutions of 1 mg/mL were prepared in sterile H_2O , immediately prior to each experiment. After that, further dilutions were prepared in phosphate buffered saline (PBS) (Oxoid Ltd., UK) to obtain the desired concentrations, then stored in the dark at room temperature until use.

2.1.4.1 Absorption spectrum of indocyanine green

Scans (500 – 850 nm) of the absorption spectrum of 25 $\mu\text{g/mL}$ ICG either in PBS or in 6.25, 12.5 or 50% horse serum (HS) were carried out using a UNICAM UV 500 UV/Visible spectrophotometer (ThermoSpectronic, Rochester, NY, USA).

2.1.5 Experimental procedures and viable counting

50 μL of indocyanine green was added to an equal volume of the bacterial suspension in triplicate wells of a sterile, flat-bottomed, untreated 96-well plate

(Nunc, Roskilde, Denmark) and irradiated with a pre-calculated light dose of NIR laser light (L+S+), with stirring. Three additional wells containing 50 μ L ICG and 50 μ L of the bacterial suspension were kept in the dark to assess the toxicity of the photosensitizer alone (L-S+). 50 μ L PBS was also added to 50 μ L of the inoculum in a further six wells, three of which were irradiated with NIR laser light (L+S-) and the remaining three were kept in the dark (L-S-). Following irradiation/dark incubation, each sample was serially diluted 1 in 10 in PBS. 20 μ L of each dilution was plated in duplicate either on blood agar (*Staph. aureus* and *Strep. pyogenes*) or nutrient agar (*P. aeruginosa* and *E. coli*) plates and the plates incubated for 48 hours at 37°C. The surviving organisms were enumerated by colony counts.

2.2 Biofilm formation

2.2.1 Microtiter plate biofilm assay

Staph. aureus NCTC 8325-4 and *P. aeruginosa* PA01 were grown aerobically in nutrient broth (Oxoid Ltd, UK) at 37°C for 16 hours in a shaking incubator at 200 rpm. Cultures were diluted 1:100 in BHIB. Aliquots (200 μ L) of the diluted cultures were placed in individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Sarstedt, Leicester, UK) and 200 μ L of sterile BHIB (in four replicates) served as controls to check sterility. The plates were incubated statically at 37°C for 18-22 h in air. The optical density of the cultures was measured at A_{590nm} using a microplate reader (Dynex Technologies, Inc. USA). The OD_{590} values for *Staph. aureus* culture was approximately 1.0-1.1 and for *P. aeruginosa* it was approximately 1.8.

2.2.2 Lethal photosensitization of the biofilms

After 18-22 h of incubation, the medium containing unattached bacteria was decanted and wells were rinsed twice with 200 μ L sterile PBS to remove any remaining planktonic cells. The PBS was then carefully removed and 200 μ L of either ICG or sterile PBS was added. The pre-irradiation time was 15 minutes to allow the penetration of the dye into the biofilm.

The biofilm samples were exposed to a pre-calculated light dose in the presence and absence of ICG, while the wells containing the control biofilm were incubated in the dark. Later on, a semi-quantitative estimation of biofilms

adherence using spectrophotometric method was conducted as described below.

2.2.3 Crystal violet assay

The content of each well was discarded then the biofilm was fixed by adding 200 μL 99 % methanol. The plates were left for 15 minutes at room temperature. Methanol was removed then 150 μL of 0.1 % crystal violet solution was added. The plates were incubated for 5 minutes at room temperature. The wells were washed several times with H_2O by pipetting. Each microtiter plate was inverted and vigorously tapped on paper towels to remove any excess liquid. The plates were allowed to air-dry at 50°C for 25-30 min, and then 250 μL of 95% ethanol was added to solubilize the stain. The plate was agitated on an orbital shaker until the colour was uniform with no areas of dense colouration in the bottom of the wells. The samples were diluted 1:10 in 95% ethanol to give a final volume of 250 μL then the absorbance of stained adherent bacterial biofilms was read at 590 nm in a Dynex microplate reader.

2.2.4 Biofilm photosensitization and viable counting

To investigate the effect of lethal photosensitization, 200 μL of ICG was added to each well and the plates were incubated in the dark for 15 min at room temperature. Wells used as controls were incubated with PBS or ICG and kept in the dark at room temperature. ICG-treated biofilms were irradiated with a 808-nm NIR laser light using a diode Laser (Ondine Biopharma Corp., USA) to deliver a specific light dose to each biofilm. The intensity of the light source at the position of the bacterial cells was 0.3 W/cm^2 . The power output was measured by using a TPM-300CE Genetic power meter (Genetic-eo, Québec, Canada). Following irradiation, the biofilms were scraped from the wells, carefully pipetted and then agitated on an orbital shaker for 10 minutes to homogenise the samples. Treated and untreated samples were serially diluted, plated on nutrient or blood agar plates, and incubated for 48 h at 37°C in the dark. Controls consisted of biofilms treated with ICG but not exposed to light (L-S+), biofilms exposed to light only (L+S-), and biofilms treated with neither ICG nor light (S-L-).

2.2.5 Confocal laser scanning microscopy

After the irradiation, the wells were carefully evacuated from both the ICG and PBS. The bottom of each biofilm well was displaced carefully from the 96-well plate using a bench punch and was fixed in a 24-well plate. To determine the viability of bacteria within the biofilms after lethal photosensitization, a Live/Dead[®] BacLight[™] Bacterial Viability Kit (Molecular Probes, Invitrogen Ltd., UK) was used. The kit includes two fluorescent nucleic acid stains: SYTO[®] 9 and propidium iodide. SYTO[®] 9 penetrates both viable and nonviable bacteria, while propidium iodide penetrates bacteria with damaged membranes, reducing SYTO9 fluorescence. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red.

For assessing viability, 1 µl of the stock solution of each stain was added to 4 ml of PBS and, after being mixed, 500 µl of the solution was dispensed into 24-well microplates containing treated and untreated biofilms and incubated at 22°C for 15 min in the dark. Stained biofilms were examined under a Radiance 3000 confocal laser-scan head at wavelengths of 488 and 543 nm (Bio-Rad GmbH, Jena, Germany) in conjunction with a BX51 stereomicroscope (Olympus UK Ltd, Southall, UK) equipped with a 40x water immersion objective. The laser power settings used for the scan were 2–10% for 488 nm and 10–25% for 543 nm. The resulting collections of confocal optical sections were collected by Bio-Rad Lasersharp 2000 software as stacks of images. The optical sections of 6 µm were collected over the complete thickness of the biofilm. The images were subsequently analysed using ImageJ (Rasband, National Institutes of Health, Bethesda, Maryland, USA, <http://rsbweb.nih.gov/ij/notes.html>).

2.3 Effect of physiological factors

2.3.1 Kill experiments in horse serum

In order to investigate the lethal photosensitization of the bacteria in an environment similar to that which would exist in a wound, lethal photosensitization experiments were performed in the presence of either 50, 12.5 or 6.25 % horse serum (HS).

2.3.1.1 Target organisms

The organisms used in this series of experiments were: *Staph. aureus* NCTC 8325-4, EMRSA-16, *Strep. pyogenes* ATCC 12202, *P. aeruginosa* strain PA01 and/or *E. coli* ATCC 25922. All organisms were grown as described previously but bacterial cells were harvested by centrifugation and resuspended in an equal volume of HS or PBS. All bacteria were diluted in HS or PBS and standardised at an optical density of 0.05.

2.3.1.2 Lethal photosensitization in horse serum

Aliquots (50 µL) of the microbial suspensions were transferred into a 96-well plate and an equal volume of the photosensitizer was added to give final concentrations of 25, 100 or 200 µg/mL. After addition of the ICG, the wells were exposed to a measured dose of laser light for 5 minutes (L+S+). The other control conditions tested were; 1) the microbial suspension plus PBS instead of ICG and kept in dark (L-S-), 2) incubation with ICG in the dark (L-S+), 3) irradiation in the absence of ICG to determine the effect of light alone on bacterial viability (L+S-). Each experimental condition was tested in at least triplicate except for lethal photosensitization experiments in 50% HS with a concentration of 25 µg/mL, which were performed in duplicate. Each experiment was carried out on at least two different occasions. Serial 10-fold dilutions were plated in duplicate either on blood agar (*Staph. aureus* & *Strep. pyogenes*) or on nutrient agar (*P. aeruginosa* & *E. coli*). Following incubation for 48 hours, survivors were enumerated by viable counting as before.

2.3.2 Kill experiments under anaerobic conditions

2.3.2.1 Target organisms

The organisms used in this series of experiments were: *Staph. aureus* NCTC 8325-4 and *Strep. pyogenes* ATCC 12202. The bacteria were grown as described in the previous section but for the purposes of these experiments, the NB, BHI, and PBS used for bacterial growth and dilution were pre-reduced by incubation in an anaerobic chamber 24h before the experiment. Bacterial cells were then harvested by centrifugation and resuspended in an equal volume of reduced PBS. All bacteria were diluted in reduced PBS and standardised at an optical density of 0.05.

2.3.2.2 Lethal photosensitization in an anaerobic pouch incubation system

The BBL™ GasPak™ Pouch (Cat. No. 260651, Becton, Dickinson and Company, USA) was used herein, composed of a transparent impermeable bag which, when properly activated and sealed, provides an anaerobic atmosphere of less than 1% oxygen and approximately 5% carbon dioxide. Each individual GasPak Pouch system utilizes a 3.5 mL liquid activating reagent packet and an incubation Pouch with an Integral anaerobic Indicator strip. The reagent sachet contains 5 g of iron powder, calcium carbonate, citric acid and inert extender. The system was prepared by dispensing and squeezing the liquid activating reagent through the reagent channel of the incubation pouch. The anaerobic indicator strip initially is blue in the presence of oxygen which changes to white when the O₂ concentration reaches 1% 3-4 hours after sealing the pouch.

Six replicates of aliquots (50 µL) of each microbial suspension were transferred into a 96-well plate and an equal volume of the photosensitizer was added to give a final concentration of 25 µg/mL (L-S+, L+S+) or an equal volume of the reduced PBS was added (L-S-, L+S-). These were then placed inside the BBL™ GasPak™ Pouch system and heat-sealed

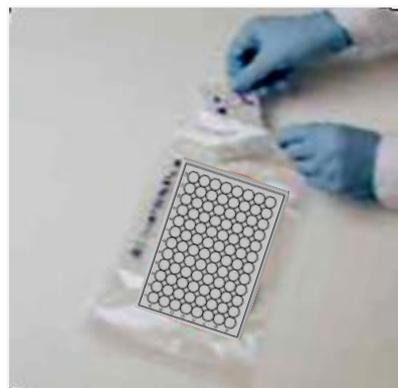


Figure 2-1: A 96-well plate inside the BBL™ GasPak™ Pouch system

as seen in Figure 2-1. One plate from each group was incubated in the dark at 22°C (L-S-, L-S+) inside the BBL™ GasPak™ Pouch. A further six replicate of each sample L+S- and L+S+ were irradiated with a measured dose of laser light for 5 minutes. Irradiation of the samples took place either immediately after incubation inside the BBL™ GasPak™ Pouch system (i.e., before anaerobic conditions were achieved and the indicator strip was blue) to find out whether irradiation in this system would affect the light dose delivered to the samples, or after 3-4 hours incubation inside the BBL™ GasPak™ Pouch in the dark at 22 °C (the anaerobic indicator strip was reduced to white). Serial 10-fold dilutions were plated in duplicate on blood agar to count the survivors.

2.4 Underlying mechanisms

2.4.1 Singlet oxygen sensor green reagent (SOSGR) Assay

A singlet oxygen sensor green reagent (SOSGR) assay was used to evaluate the singlet oxygen-generating ability of the photosensitizer. This new $^1\text{O}_2$ indicator exhibits weak blue fluorescence, with excitation peaks at 372 and 393 nm and emission peaks at 395 and 416 nm. In the presence of $^1\text{O}_2$, it emits a green fluorescence similar to that of fluorescein (excitation/emission of 504/525 nm).

A stock solution of the photosensitizer was made up in sterile H_2O at a concentration of 1 mg/mL. The photosensitizer was further diluted with PBS to a concentration of 50 $\mu\text{g}/\text{mL}$. A 100 μg vial of SOSGR (Molecular Probes-Invitrogen, UK) was dissolved in 33 μL methanol to make a stock solution of ~ 5 mM and then the stock solution was further diluted to give a concentration of 10 μM SOSGR. Solutions were prepared containing either 5 μM SOSGR and 25 $\mu\text{g}/\text{mL}$ ICG or 5 μM SOSGR in 50 % methanol/ 50% PBS – these were kept in the dark. 100 μL of each solution were transferred to a 96- well plate in four replicates and irradiated with light from the NIR Ondine diode laser for 1, 3 and 5 minutes. A further four replicates of each solution were kept in the dark. The fluorescence response of SOSGR to $^1\text{O}_2$ of the samples was measured using a spectrofluorometer (Fluoroskan Ascent FL, Labsystems, Finland) using excitation and emission wavelengths of 485 nm and 538 nm respectively. These measurements were then expressed as the relative change in the fluorescence over time for solutions containing: 5 μM SOSGR and 25 $\mu\text{g}/\text{mL}$ ICG or 5 μM SOSGR on its own as a control. The samples received light doses of 0, 18, 54 and 90 J/cm^2 .

2.4.2 Kill experiments in the presence of a singlet oxygen and free radical quencher

2.4.2.1 Target organisms

The organism used in this series of experiments was: *Staph. aureus* NCTC 8325-4. The bacterium was grown as described previously but bacterial cells were then harvested by centrifugation and resuspended in either an equal volume of H₂O and adjusted to an optical density of 0.05 then diluted in H₂O or in a range of L-tryptophan concentrations.

2.4.2.2 Singlet oxygen and free radicals scavenger

L-tryptophan, C₁₁H₁₂N₂O₂ (Sigma-Aldrich, UK) was used to quench the ¹O₂ and any free radicals produced. The scavenger was dissolved in H₂O to make a stock of 24 or 20 mM. L-tryptophan was then diluted with H₂O to give a range of concentrations - 0.02, 0.2 and 2 mM.

2.4.2.3 Detection of the minimal toxic concentration of L-Tryptophan

To detect the minimum toxic concentration of L-tryptophan for *Staph. aureus*, 50 µL of the microbial suspensions were transferred into a 96-well plate in triplicate and an equal volume of L-tryptophan was added to give final concentrations of 0.01, 0.1, 1 and 10 mM. A further triplicate set of controls containing the microbial suspension plus H₂O instead of L-tryptophan were prepared. Samples were incubated for an hour at 37 °C. To enumerate the surviving bacteria, serial 10-fold dilutions were plated in duplicate on blood agar.

2.4.2.4 Lethal photosensitization in L-Tryptophan.

To determine if singlet oxygen or free radicals were involved in the lethal photosensitization process, 10 and 12 mM L-tryptophan was used to quench any ROS generated by ICG photosensitization. ICG was added to the bacterial suspension to give a final concentration of 25 µg/mL. Samples of 100 µL in six replicate wells were exposed to pre-calculated light doses. Control wells were also prepared and were either not exposed to light but received ICG (L-S+), did not receive ICG and were kept in the dark (L-S-) or did not receive ICG and were exposed to light (L+S-). To enumerate the surviving bacteria, serial 10-fold dilutions were plated in duplicate on blood agar.

2.4.3 Kill experiments in the presence of an enhancer of singlet oxygen life span

2.4.3.1 Target organisms

The organism used in this series of experiments was: *Staph. aureus* NCTC 8325-4. The bacterium was grown as described previously but bacterial cells were then harvested by centrifugation and resuspended in either an equal volume of deuterium oxide (D₂O) (Sigma-Aldrich, UK) or sterile distilled H₂O. Bacteria were diluted in D₂O or in H₂O and standardised at an optical density of 0.05.

2.4.3.2 Lethal photosensitization in deuterium oxide

To determine if singlet oxygen was involved in the lethal photosensitization process, D₂O was used to extend the life span of any singlet oxygen generated by exposure of ICG to laser light. ICG was added to the bacterial suspension to give a final concentration of 25 µg/mL. Samples of 100 µL in triplicate wells were exposed to pre-calculated laser light doses. Control wells were also prepared and were either not exposed to the light but received ICG (L-S+), did not receive ICG and were kept in the dark (L-S-) or did not receive ICG and were exposed to the light (L+S-). To enumerate the surviving bacteria, serial 10-fold dilutions were plated in duplicate on blood agar.

2.5 Effect of light/ICG exposure on bacterial virulence factors

2.5.1 Azocasein assay

Endoproteinase Glu-C (also known as V8 protease) from *Staph. aureus* V8 was purchased from Sigma-Aldrich (UK) and stored at -20°C at a concentration of 1 mg/mL in sterile distilled H₂O. A final concentration of 5 µg/mL was obtained by diluting the enzyme in PBS. 250 µL of V8 protease was added to an equal volume of either ICG (S+) or PBS (S-) in triplicate wells of a 24-well plate and samples were irradiated with NIR laser light (L+) or incubated in the dark (L-).

After irradiation, 400 µL was removed from each well and added to 200 µL of 6% azocasein (w/v) in 0.5 M Tris buffer, pH 7 (Sigma-Aldrich, UK) in 1.5 mL Eppendorf tubes. Samples were incubated in the dark for a total of 1 h at 37°C and 150 µL aliquots were removed at 0, 20, 40 and 60 minutes. At each time

point, the reaction was stopped with an equal volume of 20% acetic acid and the samples centrifuged for 10 minutes at 10,000 rpm. 75 μ L of the supernatant was removed, in triplicate, and the optical density read at 450 nm using a microplate reader (Dynex Technologies, Inc. USA). The enzyme activity at 1 h was calculated for each sample; one unit of activity was determined as that which caused a 0.001 change in absorbance in 1 h at 450 nm.

2.5.2 Azocasein assay for total proteolytic activity of *P. aeruginosa* culture supernatant

P. aeruginosa strain PA01 was inoculated into 10 mL of nutrient broth (Oxoid Ltd, UK) and incubated aerobically at 37°C in a shaking incubator at 200 rpm for 16 hours. The suspension was centrifuged at 5000 *g* for 15 min and the supernatant was collected. Either 110 or 250 μ L of the supernatant was dispensed into wells of a 96 or 24-well microtitre plate respectively, and an identical volume of ICG solution was added. Lethal photosensitization experiments were carried out in an identical fashion to that previously described before.

After irradiation at low fluence rate, aliquots of 400 μ L were removed for the azocasein hydrolysis assay described in the previous section. Aliquots of 200 μ L were removed from each sample irradiated at a high fluence rate and added to 100 μ L of 6% azocasein (w/v) in 0.5 M Tris buffer, pH 7 (Sigma-Aldrich, UK) in 1.5 mL Eppendorf tubes. Samples were incubated aerobically in the dark for a total of 4 h at 37°C. 150 μ L aliquots from the samples exposed to a low fluence light were removed at 0, 80, 160 and 240 minutes, while the same volume was removed at 0 and 240 minutes from the aliquots exposed to a high fluence light. At each time point, the reaction was stopped with an equal volume of 20% acetic acid and the samples centrifuged for 10 minutes at 10,000 rpm. 75 μ L of the supernatant was removed, in triplicate, and the optical density was read at 450 nm using a microplate reader (Dynex Technologies, Inc. USA). The enzyme activity at 1 h was calculated as described before.

2.5.3 *Limulus amoebocyte lysate (LAL) assay*

Lipopolysaccharides (LPS) from *P. aeruginosa* serotype 10 (the source strain is ATCC 27316) was purchased from Sigma-Aldrich (UK) and stored at -20°C at a concentration of 1 mg/mL in LAL reagent water (LRW) (Associates of Cape Cod Int'l Inc., UK). After 15 minutes sonication and 60 sec vortexing, a final concentration of 5 ng/mL was obtained by diluting the LPS in LRW. 50 µL of 10 ng/mL *P. aeruginosa* 10-LPS was added to an equal volume of either ICG (S+) or LRW (S-) in triplicate wells of a pyrogen free 96-well plate (Associates of Cape Cod Int'l Inc., UK) and samples were either irradiated with NIR laser light (L+) or incubated in the dark (L-).

After irradiation, the treated *P. aeruginosa* 10-LPS was serially diluted 1:2 in LRW. Simultaneously, two-fold dilutions of the control standard endotoxin (CSE) from *E. coli* O113:H10 were prepared at an initial concentration of 5 ng/mL in LRW (Associates of Cape Cod Int'l Inc., UK). The CSE was sonicated for 15 minutes then vortexed for 60 sec before the dilution step take place.

Pyrotell[®] reagent (Associates of Cape Cod Int'l Inc., UK) was purchased in a lyophilized form of an aqueous extract of amebocytes of *L. polyphemus*. The lyophilized LAL pellet was reconstituted in LRW and the contents of the vial were swirled gently to ensure homogeneity. The remains were aliquoted and stored at -20 °C in pyrogen-free eppendorfs. Pyrotell[®] reagent can detect as little as 0.25 Endotoxin Units (EU) per mL using the gel-clot technique.

The LAL test was performed by placing 10 µL of reconstituted Pyrotell[®] to the lid of a pyrogen-free 96-well microtiter plate before 10 µL of the diluted test specimen or the CSE was added to the Pyrotell[®] spots, starting with the lowest dilution. The negative control was 10 µL LRW in triplicates. The lid was covered with another 96-well plate lid, placed in a moisture chamber and incubated immediately in a dry block incubator at 37 ± 1°C for 60 minutes. The moisture chamber consisted of a square-shaped petri dish (24 x 24 cm) with a lid, which contained moistened filter paper.

At the end of the incubation period, the chamber was removed from the incubator and 1 μ L of 0.2% MB in 70% ethanol was added to each spot. A star-like formation of the MB on the top of the spot indicated a positive result.

2.6 *In vivo* Studies

2.6.1 Animal

All animal experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and with approval of the local Ethics Committee. Twelve to fourteen-week old adult male C57 black mice (Charles River, Margate, Kent, UK), of 27–30 g body weight were housed in the local animal unit for 7 days prior to experimentation, with free access to food and water.

2.6.2 Laser source

The GaAlAs Velopex diode laser system (Medivance Instruments Ltd., UK) was used to deliver light to infected wounds through an optical fiber of 400 μ m diameter which was attached to the probe to allow light distribution to the wound.

2.6.3 Experimental procedure

2.6.3.1 Anaesthesia

Mice were anaesthetised with an intramuscular injection of ketamine-xylazine mixture (90 mg/kg ketamine, 9 mg/kg xylazine), and their backs were shaved and depilated with a commercial cream (Veet®, Reckitt Benckiser, UK). Intramuscular Carprofen (5 mg/kg) was used to provide analgesia. At 35-45 minutes post-inoculation, the mice received a second dose of the anaesthetic mixture to allow for the subsequent treatment. The skin was sprayed with 70% (v/v) ethanol and left to dry prior to wound creation.

2.6.3.2 Superficial wound model

25 mm² square shaped wounds were created in the skin of the back by scarification using a Monoject hypodermic needle 21gx1.1/2", run twenty times parallel in one direction and another twenty times perpendicular to the original tracks. Two wounds were created on each mouse's back. The wounds were

red and swollen after few minutes. 25 μ L of the bacterial suspension was placed on the wound, and incubated for one hour prior to treatment.

2.6.3.3 Photodynamic therapy

All experiments were carried out in a dim light room. PDT was performed 1 hour after inoculating the wounds with the bacterial suspension. The wounds received 25 μ L of ICG (1 mg/mL) at the start of irradiation. The wounds were irradiated immediately after the application of ICG and a pre-measured light dose was delivered to the wounds. Following the completion of the treatment, a circular area of skin and associated subcutaneous tissue of 1 cm diameter with the wound at its centre, was removed using sterile scissors. These were then placed in 0.5 mL Stuart's transport medium and protected from light and delivered on ice to the microbiology laboratory for processing and analysis within 1 hour. The animals were subsequently sacrificed in accordance with the Animal Scientific Procedures act (1986).

Control wounds were used to test the effect of ICG alone (by incubating wounds in the dark for the equivalent time period as used for irradiation (L-S+) , light alone (by illuminating wounds in the absence of ICG, L+S-). A final group of untreated control wounds received no ICG or light illumination (L-S-). PBS was used instead of ICG in the control wounds that received no ICG.

2.6.3.4 Processing of tissue samples

Using a micro-Eppendorf pestle, the tissue in Stuart's transport medium was minced to release the bacteria within the wound. Tissue samples treated with ICG were kept in the dark and on ice during processing. The contents of the Eppendorf tube were transferred into 4.5 ml of PBS. Aliquots of serial 10-fold dilutions of the suspension were plated onto half plates of the appropriate non-selective and selective agar for the three tested organisms (*Staph. aureus* NCTC 8325-4, *Strep. pyogenes* ATCC 12202 and *P. aeruginosa* NCTC PA01). Plates were incubated at 37°C in air for 48 hours before bacterial colonies were counted. Results represent the mean CFU of bacteria recovered per wound based on counts from both non-selective and the selective agar plates for each sample.

2.6.3.5 Wound temperature studies

One hour after the creation and inoculation of the partial-thickness wounds with bacteria, a 1 mm diameter thermistor, attached to the digital thermometer Digitron 2038T (Digitron Instrumentation Ltd, Sifam Instruments Ltd., Devon, UK), was tunnelled subcutaneously from an entry point 2 cm away from the wound to its centre, avoiding disruption of the wound integrity. This measurement was recorded immediately before PDT treatment and immediately after the treatment. Also the core temperature for the animals was recorded during the irradiation for all animals.

2.7 Statistical analysis

For determining whether there are significant differences among the various groups, a multiple comparison by the Univariate General Linear Model test was performed. First, a Levene test was carried out for evaluating the equality of variances. If the hypothesis that the variances are equal is accepted, Post-Hoc test in the form of a Bonferroni correction was performed. If the Levene's test rejected the equality of covariance matrixes, a Games-Howell test was performed instead of the Bonferroni. The survival colony counts (CFU/mL) were transformed into \log_{10} to normalize the data before running the Univariate General Linear Model. The mean difference (P) was significant at the level of 0.05. All statistical analysis was performed using SPSS software.

Chapter 3

**Lethal photosensitization of
organisms frequently responsible
for wound infections**

3.1 Introduction

So far, lethal photosensitization of micro-organisms has been studied extensively using PSs that absorb light in the red region of the spectrum. Some of these PSs have been reported to have photo-bactericidal activity such as porphyrins and phenothiazinium (Phoenix & Harris, 2003). Studies investigating light-activated antimicrobial agents that can absorb light at longer wavelengths (670-690 nm) have been limited to phthalocyanines (Minnock *et al.*, 1996; Griffiths *et al.*, 1997b; Segalla *et al.*, 2002; Mantareva *et al.*, 2007). Therefore, there is an increased need for studying chemically defined PSs that can absorb light in the infrared band, which allows maximal penetration of light into tissue. Recently, several *in vitro* studies have shown that ICG in conjunction with NIR light is an effective NIR-PS that can exert photocytotoxic effects against a variety of cancer cells (Bäumler *et al.*, 1999; Urbanska *et al.*, 2002; Colasanti *et al.*, 2004; Crescenzi *et al.*, 2004). Furthermore Popov *et al.*, (2003) established that NIR light combined with ICG produced a photo-bactericidal effect against *Staph. aureus*. Because ICG's adverse side effects are rare (Hope-Ross *et al.*, 1994), it is thought to be a safe PS that absorbs NIR light maximally between 775 and 810 nm. The peak absorbance of ICG lies at an isosbestic point for reduced HbO₂ and deoxyhaemoglobin (Keller *et al.*, 2002). This allows deeper tissue penetration when applied locally in humans. The localized application of both the light and the PS constitute a convenient system to treat wound infections and reduce any possible adverse systemic effects. In the present study, the efficacy of the clinically approved dye ICG and NIR laser light of 808 nm were investigated *in vitro* against a range of Gram-positive (*Staph. aureus* and *Strep. pyogenes*) and Gram-negative (*P. aeruginosa* and *E. coli*) organisms which frequently cause wound infections. The first part of the project was accomplished by carrying out the following steps:

At first, the absorption spectra of ICG, either in PBS or in horse serum (HS), were determined to detect the peak absorption of the dye. Consequently, light of an appropriate wavelength for the excitation of ICG was chosen.

Subsequently, viable counting was performed to determine the bactericidal effect of different ICG concentrations, light energy doses, fluence rates and pre-irradiation times (PIT).

The next stage was to evaluate and compare the susceptibility of the target organisms when present in mixed cultures, mimicking *in vivo* conditions where a combination of organisms can cause wound infections.

It was also important to investigate the effect of varying wavelengths of laser light energy on the photo-activation of ICG, subsequently testing their effect on the viability of *Staph. aureus*.

3.2 Materials and methods

3.2.1 Absorption spectrum of indocyanine green

The absorption spectra of 25 µg/mL ICG either in PBS or in 6.25, 12.5 and 50% horse serum (HS) were determined in the range 500-850 nm using a UNICAM UV 500 UV/Visible spectrophotometer (ThermoSpectronic, Rochester, NY, USA).

3.2.2 Target organisms and growth conditions

The organisms used were: *Staph. aureus* NCTC 8325-4, *Strep. pyogenes* ATCC 12202, *P. aeruginosa* strain PA01, and/or *E. coli* ATCC 25922. The culture conditions have been described in Chapter 2, section 2.1.2.

3.2.3 Photosensitizer preparation and irradiation system

This was described in Chapter 2, section 2.1.4.

Irradiation was carried out using the 0.5 W Ga-Al-As laser, referred to as the Thor laser, or the 0.4 W diode laser, referred to as the Ondine laser throughout the current and the succeeding Chapters. Both lasers emit continuous wave laser light with a wavelength of 808 ± 5 nm. The characteristics of each laser were described in detail in Chapter 2, section 2.1.3. Another 0.35 W diode laser which emits continuous light at 784 nm (Ondine Biopharma Corp., USA) was used for the wavelength comparison experiments.

3.2.4 The effect of photosensitizer concentration on lethal photosensitization

The first variable investigated was the effect of ICG concentration on the extent of kill achieved. The method described in Chapter 2, section 2.1.5 was followed, using various ICG concentrations ranging from 1 µg/mL to 250 µg/mL. These were exposed to a light dose of 411 J/cm² from the Thor laser or to 90 J/cm² from the Ondine laser.

3.2.5 The effect of different light doses on lethal photosensitization

The effect of the light dose on bacterial viability was studied. The light dose was altered by varying the irradiation time and the fluence rate. The light doses delivered to each bacterial suspension were calculated as shown in Table 3-1 and taking in account the following equation:

$$\text{Energy density (J/cm}^2\text{)} = \frac{\text{Power output (W)} \times \text{irradiation time (sec)}}{\text{per unit area (cm}^2\text{)}}$$

Table 3-1:The light dosimetry for the laser sources used

Laser used	Fluence rate (W/cm ²)	Irradiation time (sec)	Energy density (J/cm ²)
Thor Laser 808 nm	1.37	60	82
		180	247
		300	411
	0.048	900	43
		1800	86
		2100	147
Ondine Laser 808 nm	0.3	60	18
		180	54
		300	90
	0.05	300	15
		900	45
		1800	90
Ondine Laser 784 nm	0.05	300	15

3.2.6 The effect of pre-irradiation time on lethal photosensitization

The PIT is a variable that may affect the uptake of the PS by the bacterial cell and so affect the inactivation of the bacteria. Bacterial suspensions were incubated with 25 µg/mL ICG for 0, 10, 30 and 60 minutes in the dark at room temperature. These aliquots were exposed to a light dose of 90 J/cm² at a fluence rate of 0.3 W/cm² from the Ondine laser and the same method was followed as described in Chapter 2, section 2.1.5.

3.2.7 The photo-susceptibility of micro-organisms in a mixed culture

To prepare bacterial combinations, all bacteria were grown under the conditions described in Chapter 2, section 2.1.2. Subsequently, a suspension was made using equal volumes of each bacterial species. A homogenous mixture was attained by vigorous shaking with a vortex mixer. A mixture of Gram-positive organisms *Staph. aureus* and *Strep. pyogenes* was tested. Another mixture of the Gram-positive bacterium *Staph. aureus* and Gram-negative organisms *P. aeruginosa* and *E. coli* was also studied. The mixture of Gram-positive organisms was photosensitized with 10, 25 and 50 µg/mL ICG and a light dose of 54 J/cm². While an ICG concentration of 50, 100 and 200 µg/mL and a light dose of 90 J/cm² was used for photo-inactivation of the Gram-positive/Gram-negative mixture. The NIR light was delivered to the aliquots at a fluence rate of 0.3 W/cm² from the Ondine laser. The same experimental procedure was followed as previously mentioned in Chapter 2, section 2.1.5 but with the following modification: 20 µL of each sample was plated in duplicate on both selective and non-selective agar plates (Table 3-2) to determine the number of viable bacteria.

Table 3-2: Different types of selective and nonselective agar used

organisms	Selective medium	Non-selective
<i>Staph. aureus</i>	Mannitol salt agar (MSA)	Blood agar
<i>Strep. pyogenes</i>	Streptococcus selective columbia blood agar	Blood agar
<i>P. aeruginosa</i> & <i>E. coli</i>	MacConkey	Nutrient agar

3.2.8 The effect of the light wavelength on lethal photosensitization

A diode Laser (Ondine Biopharma Corp., USA), which emits light at a wavelength of 784 nm, was compared to the 808 nm NIR Ondine laser light in the activation of ICG in order to evaluate the numbers of *Staph. aureus* killed upon irradiation from each laser source. The power output of both lasers was adjusted to 0.32 W. A light dose of 15 J/cm² was delivered at a fluence rate of 0.05 W/cm² to photo-activate ICG. The method used was as described in Chapter 2, section 2.1.5.

3.3 Statistics

The colony counts of the survivors were transformed into logs to normalize the data. The Univariate General Linear Model was then used to determine the difference between the groups and between similar experiments performed on different occasions. A Post-Hoc Test, in the form of a Games-Howell, was applied to detect where the difference occurred. The mean difference (P) was significant at the level of 0.05 (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). The values displayed in each graph are the means of at least six replicates performed in two experiments on two different occasions. Error bars represent the standard deviation from the mean.

3.4 Results

3.4.1 Absorption spectrum of indocyanine green

The absorption spectrum of 25 µg/mL ICG in H₂O, PBS solution and in a range of concentrations of HS (6.25, 12.5 and 50%) (Figure 3-1) were obtained to detect its ability to harness the maximum quantity of light photons emitted from the NIR laser of 808 ± 5 nm which was used in this study. The peak absorbance in H₂O and PBS solution was 779 nm, however ICG still exhibited substantial absorption of light energy at the wavelength produced by the laser. Interestingly, the binding of ICG with the macromolecules in the HS shifted the peak absorbance of 804 nm at all of the concentrations tested. ICG in HS exhibited its strongest absorption band around 780-825 nm. This is in agreement with Landsman *et al.*, (1976) who reported that the peak absorption

of ICG was shifted from 775 nm in H₂O to 805 nm in plasma. This revealed that ICG is capable of capturing the highest proportion of the radiant energy emitted by the 808 ± 5 nm NIR laser *in vivo*, the main aim of this study.

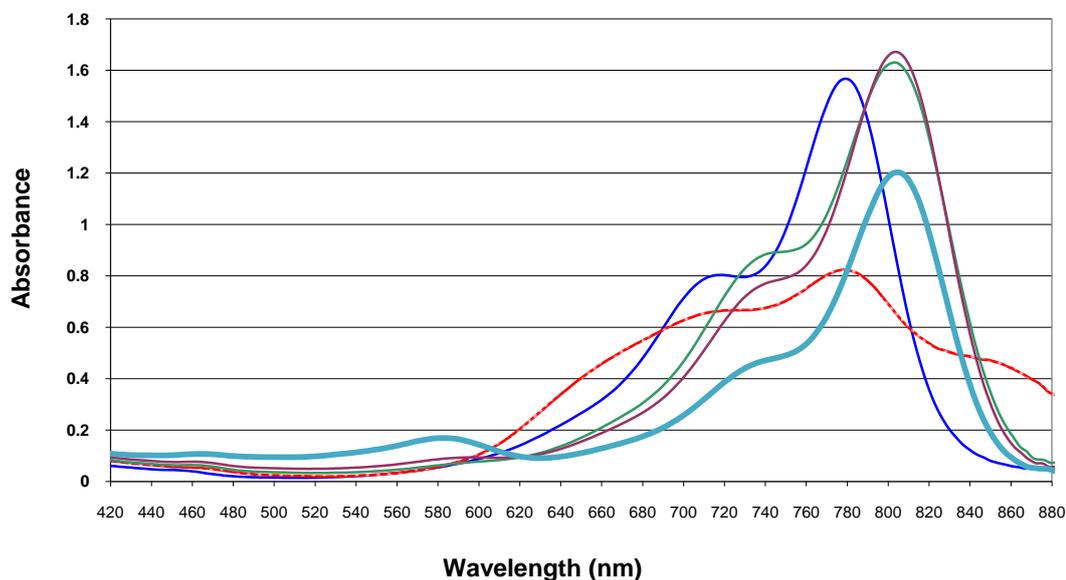


Figure 3-1: The absorption spectrum of 25 µg/mL ICG in H₂O (—), PBS (—), 6.25% (—), 12.5% (—) and in 50% (—) horse serum solutions.

3.4.2 The effect of different concentrations of indocyanine green

3.4.2.1 Thor laser

Figure 3-2 shows the effect of various ICG concentrations on the viability of (a) *Staph. aureus*, (b) *Strep. pyogenes* and (c) *P. aeruginosa*. It can be seen that *Staph. aureus* and *Strep. pyogenes* were killed in a dose-dependent manner, achieving substantial kills of > 99.99% for both organisms (P=0.0001 and P=0.00001 respectively) upon treatment with 25 µg/mL ICG and a light dose of 411 J/cm². Significant kills of *Staph. aureus* were achieved at all ICG concentrations used in conjunction with the NIR Thor laser light. Even at the lowest concentration of 1 µg/mL, 87.24% *Staph. aureus* cells were killed (P=0.001). Yet, there was a significant difference between the kill achieved at a concentration of 1 µg/mL and all the higher concentrations tested. In the case of *Strep. pyogenes*, there was no difference in the viability at all the concentrations tested except between the kill obtained at 25 and 200 µg/mL ICG (P=0.033). *P. aeruginosa* appeared to be less susceptible to lethal photosensitization at a concentration of 25 µg/mL ICG; only a 60.7% kill was

achieved. However, at higher concentrations of 100, 150, 200 and 250 $\mu\text{g}/\text{mL}$ significant proportions, 71.04, 90.65, 99.97 and 99.98% respectively, of *P. aeruginosa* were killed. Exposure to laser light alone had no effect on the viability of *Staph. aureus* or *Strep. pyogenes*. Minimal but significant dark ICG toxicity was observed with the Gram-positive bacteria at the highest concentration tested. Yet, no dark toxicity was detected in the case of the Gram-negative bacterium *P. aeruginosa*. ICG concentration of 100 and 200 $\mu\text{g}/\text{mL}$ resulted in a significant kill of less than 0.37 \log_{10} in the case of *Strep. pyogenes*, while a significant kill of 0.66 \log_{10} was observed when *Staph. aureus* was treated with 1 mg/mL ICG and kept in the dark.

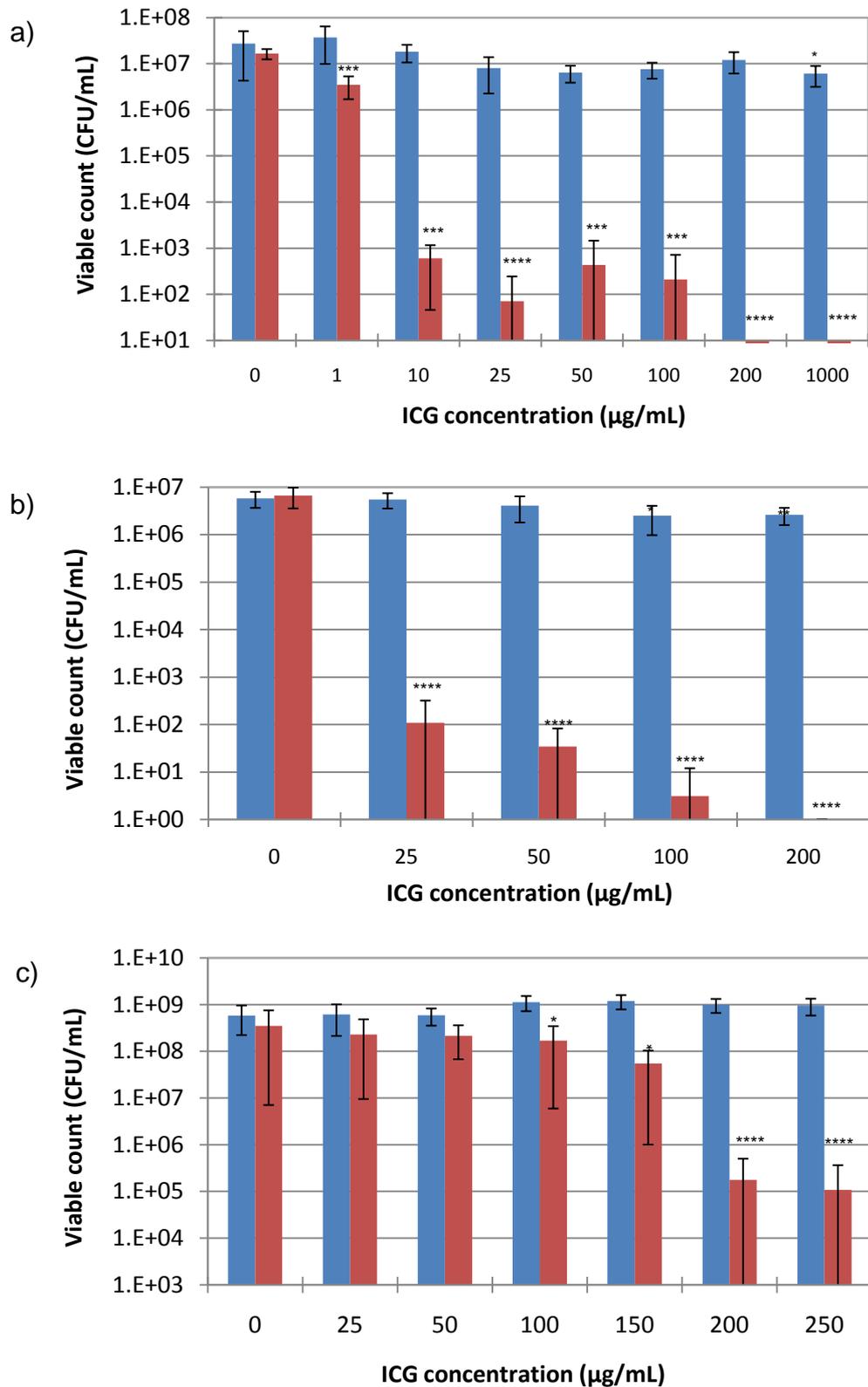


Figure 3-2: Lethal photosensitization of (a) *Staph. aureus* with 0, 1, 10, 25, 50, 100, 200 and 1000 µg/mL ICG, (b) *Strep. pyogenes* with 0, 25, 50, 100 and 200 µg/mL ICG and (c) *P. aeruginosa* with 0, 25, 50, 100, 150, 200 and 250 µg/mL ICG. Samples were irradiated with a light dose of 411 J/cm² from the NIR 808 nm Thor laser at a fluence rate of 1.37 W/cm² (■). Control suspensions were kept in the dark with or without ICG (□). Error bars represent the standard deviation from the mean.

3.4.2.2 Ondine laser

Using the 808 nm Ondine laser system, which has a lower power output compared to the Thor laser, to irradiate a range of ICG concentrations produced a statistically significant reduction in recovered viable *Staph. aureus* when compared to the controls that were kept in the dark with no PS (Figure 3-3a). The Thor laser demonstrates significant killing activity against *P. aeruginosa* in the absence of any PS (see Fig. 3.4.), most likely due to the presence of indigenous PS within this bacterial species (Bianchi *et al.*, 2008). For this reason, *E. coli*, which do not have indigenous PS, were used in experiments with the Ondine laser, to test its effects on another Gram-negative bacteria. A low concentration of 25 µg/mL ICG resulted in a significant 0.22 log₁₀ kill of *E. coli* (P=0.043). Yet, major reductions in the viable count of 3.76 and 4.83 log₁₀ were achieved when higher concentrations of 100 and 200 µg/mL ICG were used respectively to photosensitize *E. coli*. A statistically significant reduction in the numbers of this organism which were recovered was achieved at concentrations of 100 and 200 µg/mL (P=0.001, P=0.0002), estimated as >99.98 and 99.99% respectively (Figure 3-3b).

Treatment of *Staph. aureus* with 25 µg/mL ICG photo-activated with 90 J/cm² from the NIR Ondine laser eradicated 4 log₁₀ of the bacteria (P=0.0004). It was evident that the concentration of 25 µg/mL was the minimal concentration at which substantial kills of *Staph. aureus* were achieved as seen in Figures 3-2a and 3.3a. Therefore, a concentration of 25 µg/mL was considered the optimal concentration for the sensitization of the Gram-positive organisms, but 100 and 200 µg/mL were used to sensitize the Gram-negative organisms.

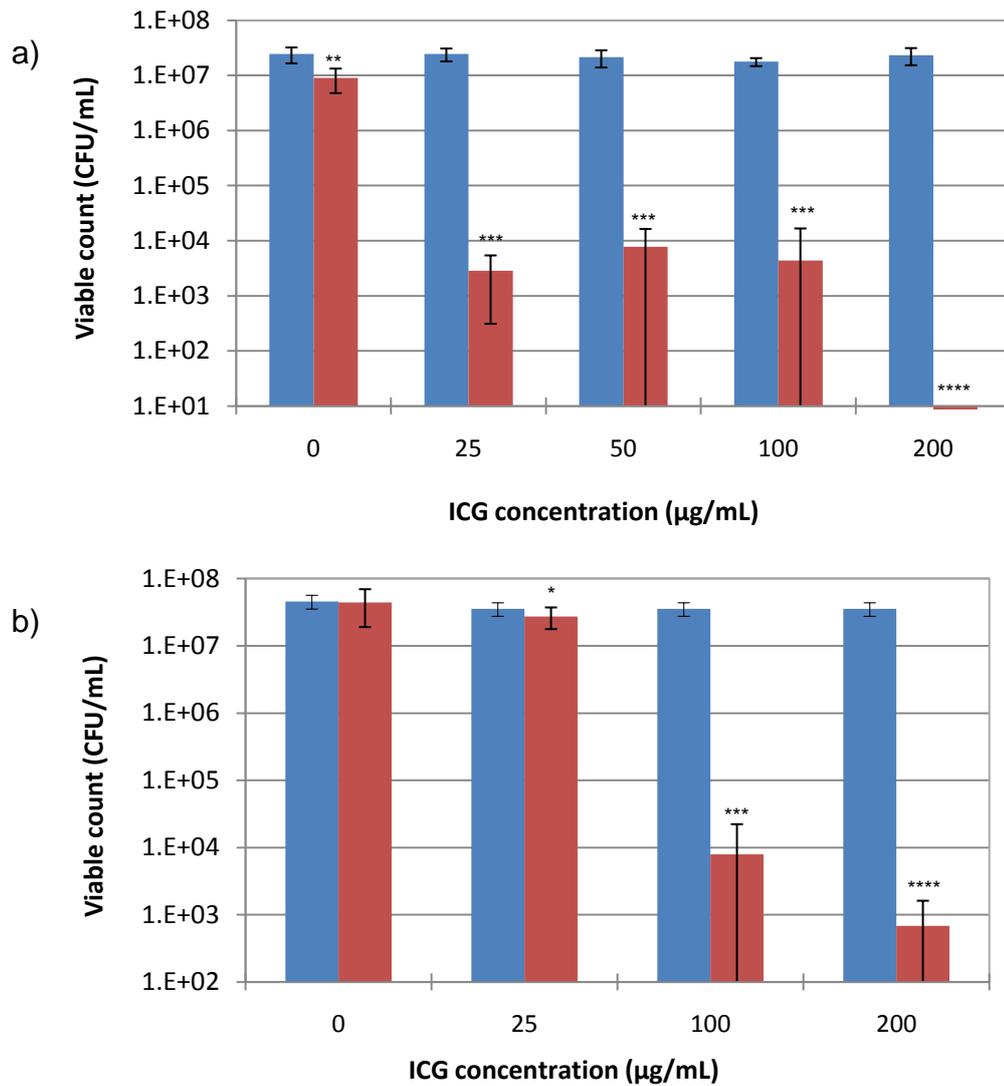


Figure 3-3: Lethal photosensitization of (a) *Staph. aureus* with 0, 25, 50, 100 and 200 µg/mL ICG and (b) *E. coli* with 0, 25, 100 and 200 µg/mL ICG. Bacterial suspensions were irradiated with a light dose of 90 J/cm² (■) from the NIR 808 nm Ondine laser at a fluence rate of 0.3 W/cm². Controls were incubated with or without ICG in the dark (□). Error bars represent the standard deviation from the mean.

3.4.3 The effect of light dose

3.4.3.1 Thor laser

The bactericidal effect was dependent on the light dose delivered. Kills of over 99.99% were achieved for all bacteria photosensitized with ICG and the highest light dose of 411 J/cm² (Figure 3-4). Significant ($P = 0.0001$) reductions of 2.5, 3.4, and 5.2 log₁₀ in the viable count of *Staph. aureus* were achieved using 25 µg/mL ICG and exposure to light energies of 82, 247 and 411 J/cm² (Figure 3-4a). Figure 3-4b shows significant ($P < 0.000001$) log₁₀ reductions in the viable count of *Strep. pyogenes* equal to 3.94, 6.82 and 6.13 log₁₀ using light energies

of 82, 247 and 411 J/cm² respectively with an ICG concentration of 25 µg/mL. A kill of 99.99% was achieved when *Strep. pyogenes* was irradiated with a light dose of 82 J/cm². Increasing the light dose to 247 or 411 J/cm² resulted in a reduction of more than 99.999% in the viable count of *Strep. pyogenes*. Even though *P. aeruginosa* was the most resistant bacterium, lethal photosensitization using the same light energies and an ICG concentration of 200 µg/mL achieved significant kills ($P < 0.001$) of 1.35, 1.75 and 4.74 log₁₀ respectively. However, in the absence of ICG, irradiation of *P. aeruginosa* with light doses of 82, 247 and 411 J/cm² also resulted in significant kills ($P < 0.01$) of 78.23, 85.93 and 95.64% respectively. These amounted to 0.66, 0.85 and 1.36 log₁₀ reductions in the viable count correspondingly, albeit not as great as those achieved in the presence of ICG (Figure 3-4c).

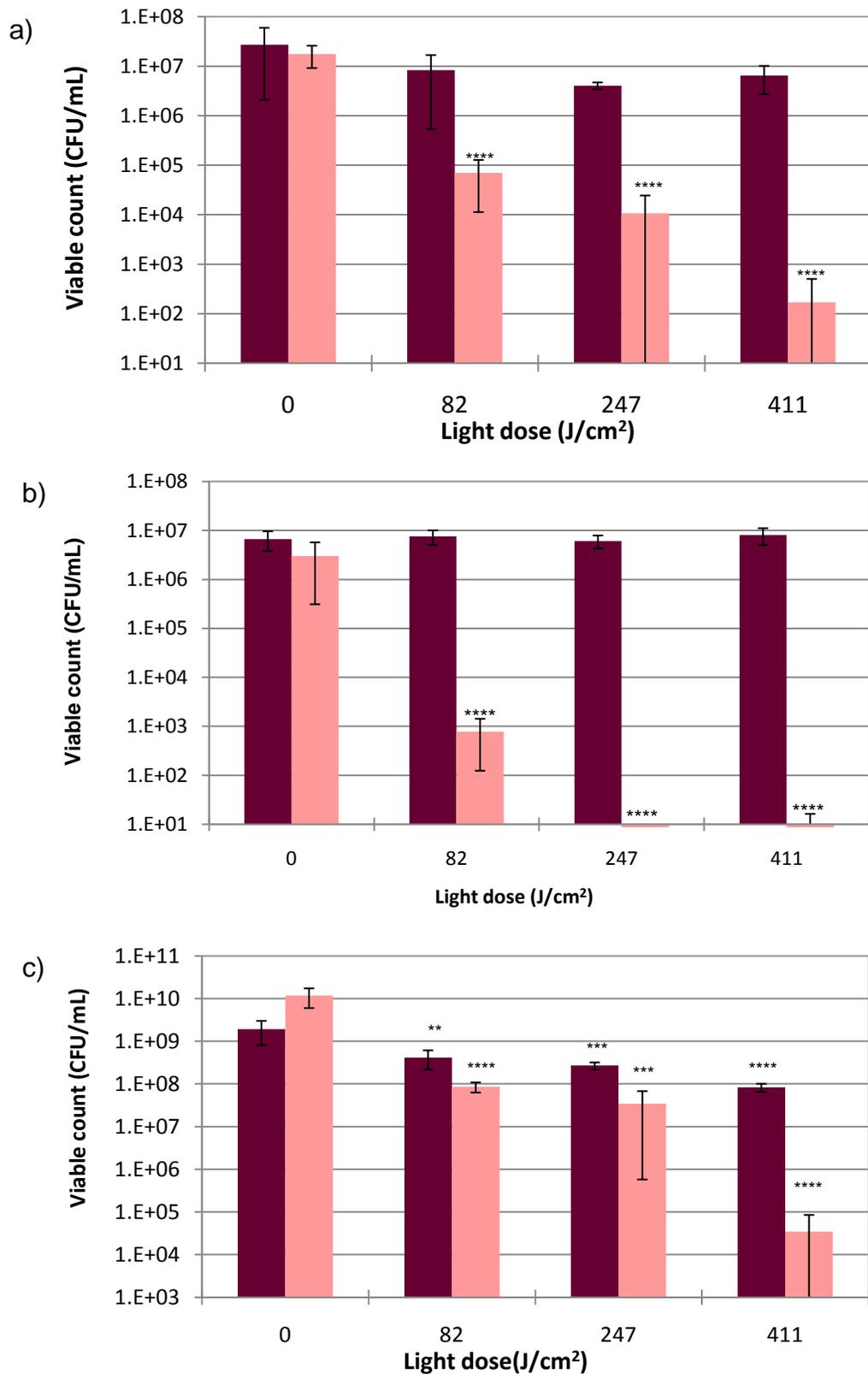


Figure 3-4: Lethal Photosensitization of (a) *Staph. aureus*, (b) *Strep. pyogenes* with 25 $\mu\text{g/mL}$ ICG and (c) *P. aeruginosa* with 200 $\mu\text{g/mL}$ ICG. Samples were irradiated at a fluence rate of 1.37 W/cm^2 and light doses of 82, 247, or 411 J/cm^2 from the NIR 808 nm Thor laser in the absence of ICG (■) or in the presence of ICG (□). Error bars represent the standard deviation from the mean.

3.4.3.2 Ondine laser

The light dose response was studied using the Ondine laser which has a lower power output than the Thor laser. Although less light energy was delivered to ICG in the case of the NIR Ondine laser, photosensitization resulted in significant kills for all bacteria (Figure 3-5). An ICG concentration of 25 µg/mL activated with light doses of 18, 54 and 90 J/cm² were able to attain significant ($P < 0.0000001$) 2.28, 3.37 and 3.80 log₁₀ reductions of *Staph. aureus* viable counts respectively. Significant ($P < 0.0001$) 2.27, 2.75 and 4.64 log₁₀ reductions in the viable count of *Strep. pyogenes* were also achieved upon exposure to light doses of 18, 54 and 90 J/cm² respectively. *E. coli* was not sensitive to the light dose of 18 J/cm² and 100 µg/mL ICG, but significant ($P < 0.00001$) reductions of 2.52 and 4.82 log₁₀ were achieved upon exposure to higher light doses of 54 and 90 J/cm², resulting in 99.69 and 99.99% kills respectively. *P. aeruginosa* showed significant ($P < 0.001$) kills at all light doses and 200 µg/mL ICG, 1.16, 3.35 and 6.11 log₁₀ reductions were achieved upon exposure to light energies of 18, 54 and 90 J/cm² respectively. Therefore, the Ondine laser was found to be as effective as the one that delivered a higher light energy in activating ICG. However, a 0.83 log₁₀ reduction in the viable count was achieved upon exposure to a light dose of 90 J/cm² in the absence of ICG, this kill was not significant.

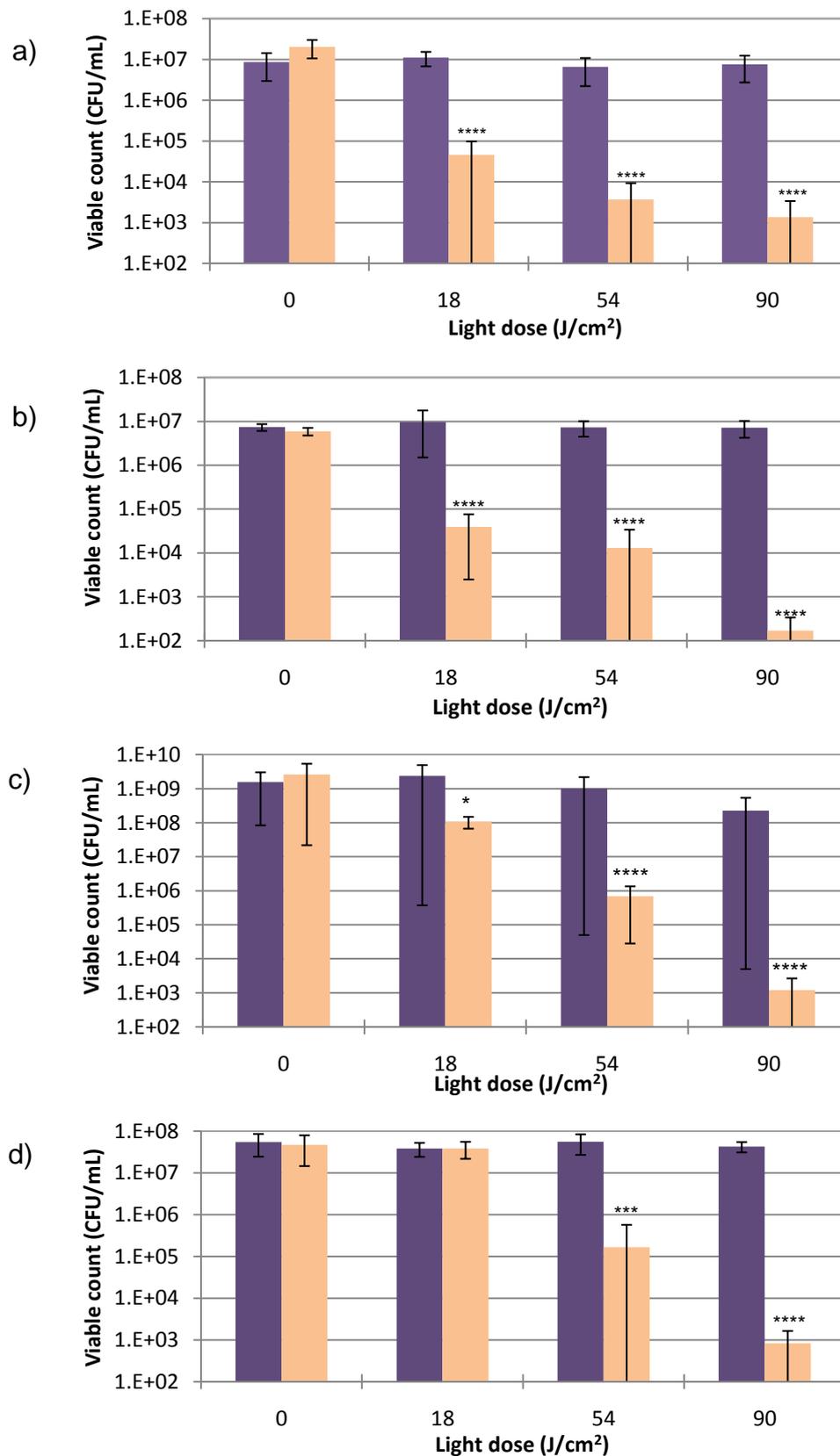


Figure 3-5: Lethal Photosensitization of (a) *Staph. aureus*, (b) *Strep. pyogenes* with 25 µg/mL ICG (c), *P. aeruginosa* with 200 µg/mL ICG and (d) *E. coli* with 100 µg/mL ICG. Samples were irradiated at a fluence rate of 0.3 W/cm² and light doses of 18, 54, or 90 J/cm² from the NIR 808 nm Ondine laser in the absence of ICG (■) or in the presence of ICG (□). Error bars represent the standard deviation from the mean.

3.4.4 The effect of irradiation at a low fluence rate on lethal photosensitization

3.4.4.1 Thor laser

The light delivered from the Thor laser at a low fluence rate to activate ICG and its effect on the photosensitization of Gram-positive bacteria is shown in Figure 3-6. Irradiation of ICG at a low fluence rate of 0.048 W/cm^2 was able to achieve considerable reductions in the viable counts of both *Staph. aureus* and *Strep. pyogenes* after irradiation for 15 and 30 minutes from the Thor laser. A light dose of 43 J/cm^2 was able to attain 99.98% kills in each case ($P = 0.0000001$ for *Staph. aureus*, $P = 0.0004$ for *Strep. pyogenes*). Doubling the light dose resulted in $> 99.99\%$ kills for both bacteria, although it was slightly more active against *Strep. pyogenes*, achieving a $5.3 \log_{10}$ reduction ($P = 0.000004$) compared with a $4.3 \log_{10}$ reduction for *Staph. aureus* ($P = 0.00005$).

In the case of *P. aeruginosa*, a fluence rate of 0.07 W/cm^2 was used. Irradiation of *P. aeruginosa* for 35 and 60 minutes from the Thor laser resulted in a significant reduction in the viable count as shown in Figure 3-7. In the presence of ICG, both a light dose of 147 and 252 J/cm^2 was able to kill 79% of *P. aeruginosa* ($P = 0.0003$ for both light doses). In the absence of ICG, a light dose of 147 J/cm^2 resulted in a significant reduction of 64% ($P = 0.015$) in the viable count of *P. aeruginosa*. Yet, increasing the light dose to 252 J/cm^2 killed $> 88\%$ of *P. aeruginosa* ($P = 0.0002$). Thus the NIR light delivered from the Thor laser alone was effective against *P. aeruginosa*, achieving approximately $1 \log_{10}$ kill.

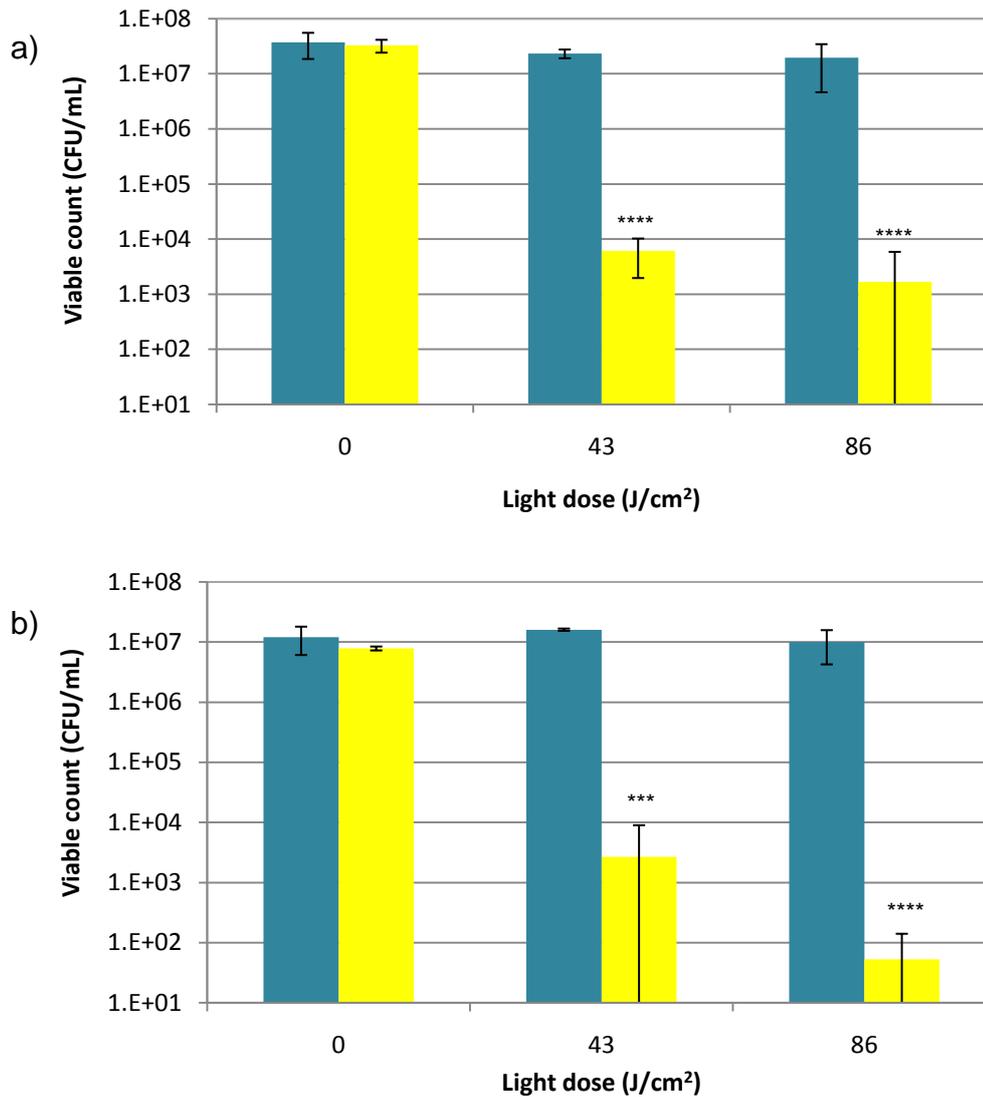


Figure 3-6: Lethal Photosensitization of (a) *Staph. aureus* and (b) *Strep. pyogenes* with 25 µg/mL ICG. Samples were irradiated at a fluence rate of 0.048 W/cm² and light doses of 43 and 86 J/cm² from the NIR 808 nm Thor laser in the absence of ICG (■) or in the presence of ICG (□). Error bars represent the standard deviation from the mean.

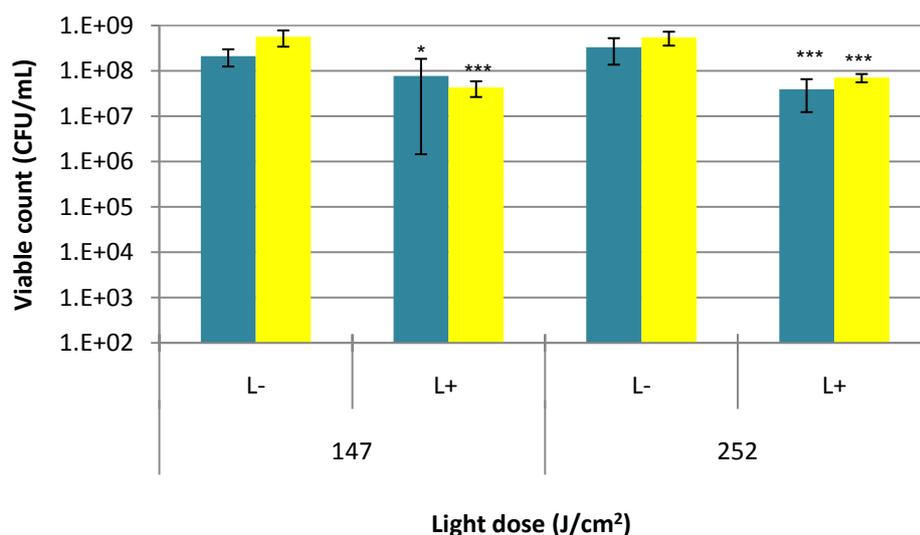


Figure 3-7: Lethal photosensitization of *P. aeruginosa* with 200 µg/mL ICG. Samples were irradiated at a low fluence rate of 0.07 W/cm² and light doses of 147 and 252 J/cm² from the NIR 808 nm Thor laser in the absence of ICG (■) or in the presence of ICG (■). L- stands for the samples which did not receive any light while L+ stands for the samples which received laser light. Error bars represent the standard deviation from the mean.

3.4.4.2 Ondine laser

Irradiation of the Gram-positive bacteria *Staph. aureus* and *Strep. pyogenes* at a low fluence rate of 0.05 W/cm² from the NIR Ondine laser resulted in significant kills for both organisms (Figures 3-8a and b). The low fluence rate of 0.05 W/cm² was as effective as the high fluence rate of 0.3 W/cm² in the excitation of ICG. Likewise, a kill of over 99.999% was attained after irradiation of the bacterial suspension (*Staph. aureus* P=0.000004; *Strep. pyogenes* P<0.0000001) with 90 J/cm² from the Ondine laser. A lower light dose of 45 J/cm² also significantly reduced the viable count of *Staph. aureus* (P=0.000004) and *Strep. pyogenes* (P=0.0001), the kills amounted to 99.98 and 99.99% respectively. At both light doses, *Strep. pyogenes* was more sensitive to photosensitization than *Staph. aureus*, achieving kills of 4.2 and 6.8 log₁₀ for *Strep. pyogenes* compared to 3.7 and 5.6 log₁₀ for *Staph. aureus*. Neither the light alone nor the ICG had an effect on the viability of *Strep. pyogenes*. However, both light doses resulted in very small but significant (P= 0.02) reductions of approximately 0.2 log₁₀ in the viable count of *Staph. aureus*.

On the other hand, a low fluence rate was not effective at killing the Gram-negative bacterium *E. coli* (Figure 3.8c). No significant kill was observed after irradiation of *E. coli* at a fluence rate of 0.05 W/cm² with a light dose of 90 J/cm². However, delivering the same light dose at a high fluence rate of 0.3 W/cm² in the presence of an identical ICG concentration resulted in a substantial kill of 99.99% of this organism (Figure 3-5d).

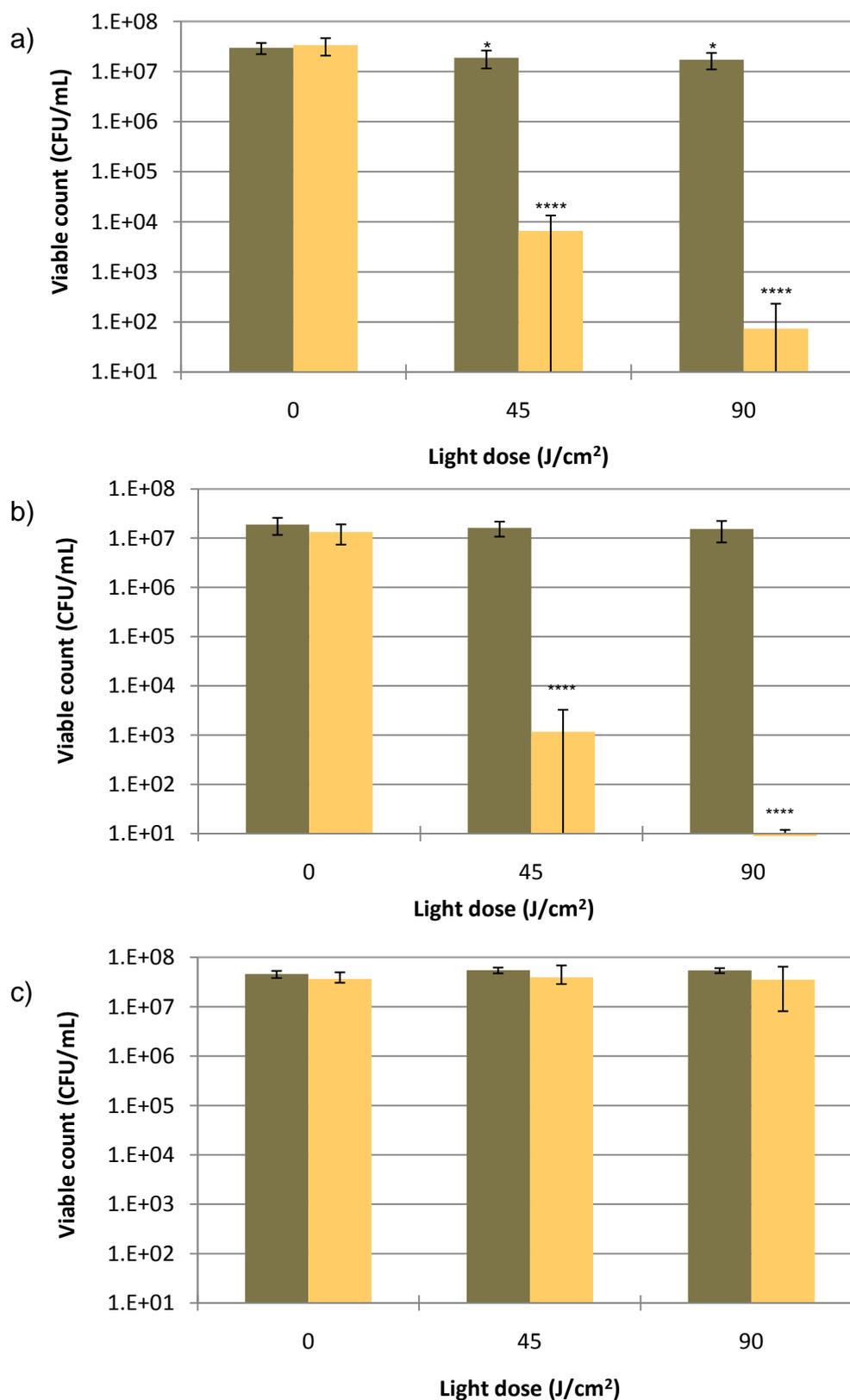


Figure 3-8: Lethal Photosensitization of (a) *Staph. aureus*, (b) *Strep. pyogenes* with 25 $\mu\text{g/mL}$ ICG and (c) *E. coli* with 100 $\mu\text{g/mL}$ ICG. Samples were irradiated at a fluence rate of 0.05 W/cm^2 and light doses of 45 or 90 J/cm^2 from the NIR 808 nm Ondine laser in the absence of ICG (■) or in the presence of ICG (□). Error bars represent the standard deviation from the mean.

3.4.5 The effect of PIT

Figure 3-9 shows the effect of varying the PIT from 0-60 minutes on the viability of the two Gram-positive bacteria *Staph. aureus*, *Strep. pyogenes* and the Gram-negative bacterium *P. aeruginosa*. Dark incubation of the bacteria with ICG for 0, 10, 30 or 60 minutes prior to irradiation with NIR laser light did not result in any significant enhancement of the bacterial kills for any of the three targeted organisms (P=0.3, 0.7 and 0.9 for *Staph. aureus*, *Strep. pyogenes* and *P. aeruginosa* respectively). A similar reduction in bacterial numbers was obtained at each PIT; approximately 6 log₁₀ reduction for *Staph. aureus*, 4.5 log₁₀ reduction for *Strep. pyogenes* and 4 log₁₀ reduction for *P. aeruginosa*.

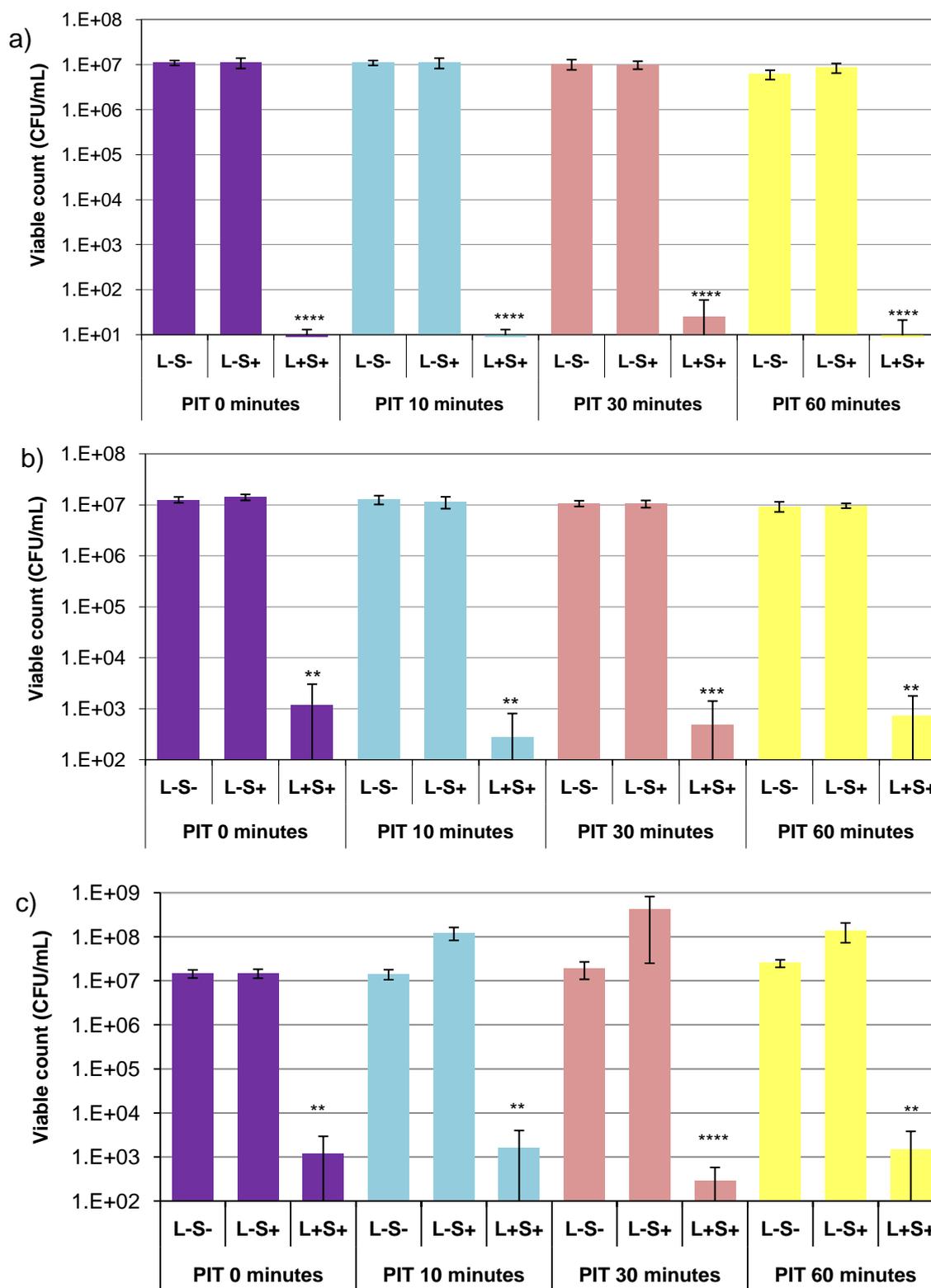


Figure 3-9: The effect of various PITs on the lethal photosensitization of (a) *Staph. aureus*, (b) *Strep. pyogenes* with 25 µg/mL ICG and (c) *P. aeruginosa* with 200 µg/mL ICG. Samples incubated with ICG for 0 (■), 10 (□), 30 (▣) or 60 (▤) minutes then irradiated at a fluence rate of 0.3 W/cm² and a light dose of 90 J/cm² from the NIR 808 nm Ondine laser (L+S+). Control suspensions were incubated in the dark with ICG (L-S+) or without ICG (L-S-). Error bars represent the standard deviation from the mean.

3.4.6 The photo-susceptibility of micro-organisms in a mixed culture

Figure 3-10 shows the photo-sensitivity of a mix of three target bacteria. The mixed cultures contained 3×10^6 of the Gram-positive organism *Staph. aureus* and approximately 1×10^7 of the Gram-negative bacteria *P. aeruginosa* and *E. coli*. Irradiation of this mixed culture at a fluence rate of 0.3 W/cm^2 and a light dose of 90 J/cm^2 combined with $50 \text{ }\mu\text{g/mL}$ ICG resulted in a substantial kill of $>99.998\%$ for *Staph. aureus* ($P=0.00001$), 99.88% for *E. coli* ($P=0.002$) and 96.97% for *P. aeruginosa* ($P=0.001$). Increasing the concentration of ICG to $100 \text{ }\mu\text{g/mL}$, achieved $> 99.99\%$ kill in all targeted bacteria ($P<0.0000001$ for both *Staph. aureus* and *E. coli* and $P=0.0001$ for *P. aeruginosa*). Likewise a concentration of $200 \text{ }\mu\text{g/mL}$ killed $>99.999\%$ of the mixed cultures. There was a significant difference between the efficacy of ICG at a concentration of $50 \text{ }\mu\text{g/mL}$ and both higher concentrations in killing *E. coli* ($P=0.004$) and *P. aeruginosa* ($P=0.023$ and $P=0.001$ for 100 and $200 \text{ }\mu\text{g/mL}$ respectively). The most sensitive bacterium was *Staph. aureus* whilst the least sensitive was *P. aeruginosa*. At the lowest concentration of $50 \text{ }\mu\text{g/mL}$ ICG, the kill rate of *Staph. aureus* was significantly different from that of *E. coli* ($P=0.002$) and *P. aeruginosa* ($P=0.00003$). When the mixed species were treated with $100 \text{ }\mu\text{g/mL}$, there was no difference in the rate of kill for both *Staph. aureus* and *E. coli*, however the rate of kill for both organisms was significantly different from that of *P. aeruginosa* ($P=0.017$ for both organisms). No significant difference in susceptibility was found between the three species at an ICG concentration of $200 \text{ }\mu\text{g/mL}$.

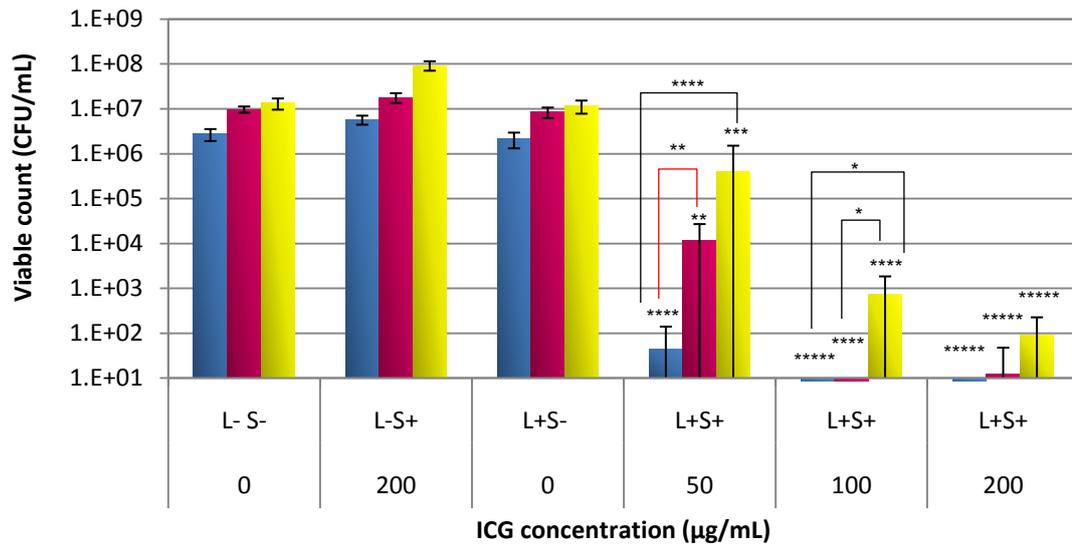


Figure 3-10: Photo-susceptibility of *Staph. aureus* (■), *E. coli* (■) and *P. aeruginosa* (■) when the three organisms were combined in a mixed culture. The mixed suspensions were irradiated at a fluence rate of 0.3 W/cm² and light dose of 90 J/cm² from the 808 nm Ondine laser in the presence of 50, 100 and 200 µg/mL ICG. Error bars represent the standard deviation from the mean.

The susceptibility of the Gram-positive bacteria *Staph. aureus* and *Strep. pyogenes* to ICG photosensitization when both were combined in a mixed suspension is shown in Figure 3-11. Upon irradiation of ICG-treated suspensions with 54 J/cm², there was a significant reduction in the viable counts of both organisms (P<0.01). Both organisms were equally susceptible to photosensitization at the various concentrations of ICG tested. Approximately 2, 3.5 and 3.7 log₁₀ reductions in the viable counts of each species were found at corresponding ICG concentrations of 10, 25 and 50 µg/mL. There was no statistically significant (P=0.06) difference between the kill obtained for both organisms at all ICG concentrations.

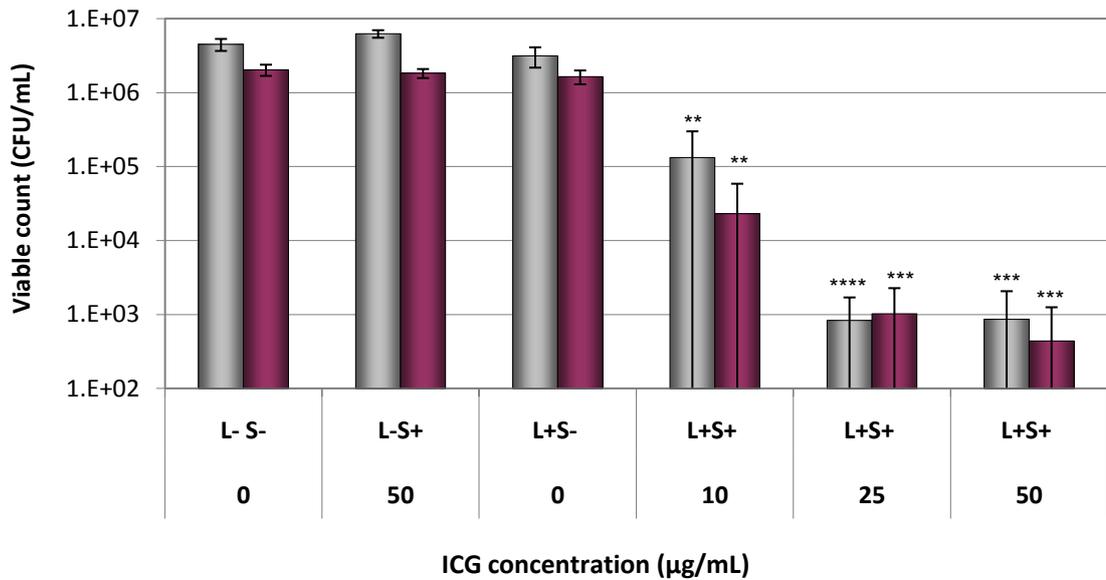


Figure 3-11: The photo-sensitivity of a mixed culture consisting of *Staph. aureus* (□) and *Strep. pyogenes* (■). The mixed suspensions were irradiated at a fluence rate of 0.3 W/cm² and light dose of 54 J/cm² from the 808 nm Ondine laser in the presence of 10, 25 and 50 µg/mL ICG. Error bars represent the standard deviation from the mean.

3.4.7 The effect of wavelength on the lethal photosensitization of *Staph. aureus*

Light with wavelengths of 808 nm and 784 nm were similarly effective in photo-activating ICG and consequently in killing *Staph. aureus* as is shown in Figure 3-12. Following the activation of ICG with light at 784 nm, a 99.97% (P < 0.0001) reduction in the viable count of a suspension containing nearly 2 x 10⁷ CFU/mL of *Staph. aureus* was achieved. Light with a wavelength of 808 was able to achieve a slightly higher kill of 99.99% (P < 0.0001) for an identical load of *Staph. aureus* following irradiation with the same light dose of 15 J/cm². However, there was no significant difference in the efficacy of both wavelengths to activate ICG and in turn photosensitize *Staph. aureus* (Figure 3-12).

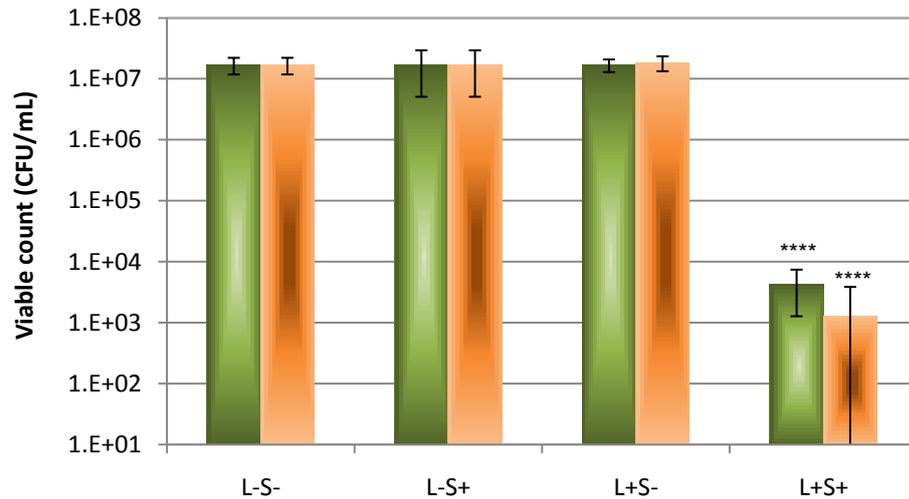


Figure 3-12: Lethal photosensitization of *Staph. aureus* using 25 $\mu\text{g/mL}$ ICG. Samples were irradiated at a fluence rate of 0.05 W/cm^2 and light dose of 15 J/cm^2 from either a 784 nm laser Ondine (■) or a 808 nm Ondine laser (■). Error bars represent the standard deviation from the mean.

3.5 Discussion

ICG has been considered as a possible PS for clinical PDT. Although the potential of ICG for use in anti-tumour PDT has been established, its application as an antimicrobial agent has, until now, received little interest and has yet to be investigated. Recently, the use of ICG combined with NIR diode laser light has been shown to be an effective method for the treatment of severe acne vulgaris (Taub, 2007b). ICG possesses several properties which could make it an ideal PS. These include: (1) water-solubility; (2) low toxicity; (3) rapid excretion from the body; (4) its use for medical diagnosis and treatment in humans at concentration of 5 mg/mL and maximum dosage of 2 mg/Kg ; (5) absorption of light of higher wavelengths within the NIR spectrum which enables deep tissue penetration (Cherrick *et al.*, 1960) and (6) an appreciable quantum yield (Kassab, 2002). In this study ICG's efficacy as an antimicrobial agent has been determined against the most common bacteria responsible for wound infections. Two Gram-positive species (*Staph. aureus*, and *Strep. pyogenes*) and two Gram-negative species (*P. aeruginosa* and *E. coli*) were treated with ICG in the presence of light from a NIR laser emitting at 808 nm. The results showed that ICG in conjunction with NIR light proved to be an effective photosensitizing agent of all targeted organisms. The lethal

photosensitization of these wound-infecting organisms with ICG was dependent on ICG concentration, the light dose and the fluence rate (i.e. light intensity).

In an effort to optimise the kills obtained, the concentration of ICG was varied and the effect on bacterial viability was compared. The concentration needed to achieve substantial kill of the targeted organisms was species- and genus-dependent. A substantial kill of over 99.99% for both Gram-positive species, *Staph. aureus* and *Strep. pyogenes*, was attained at a low concentration of 25 µg/mL ICG and exposure to a NIR laser light. Notably in the case of *Staph. aureus*, increasing the ICG concentration from 25 to 100 µg/mL did not result in any increase in the bacterial kill obtained. This result is consistent with the data documented by Bhatti *et al.*, 1997. The authors found no enhancement in the kill of *Porphyromonas gingivalis* when the bacterium was photosensitized by TBO at concentrations higher than 12.5 µg/mL. This phenomenon may be due to the accumulation of the free non-reacted PS molecules at higher concentrations, along with the absence of sufficient light photons to activate each molecule which results in a shielding effect with no more ROS generation (Bhatti *et al.*, 1997). Another possible explanation may be that at higher PS concentrations, the target sites on the bacteria are saturated and, although unbound PS may absorb light and produce ROS, these species may be quenched before reaching the cells (Komerik, 2000). This observation was not found in the case of *Strep. pyogenes*, as the kill rate was proportional to the increase in ICG concentration. However, there was no significant difference in the antimicrobial efficacy of ICG in the range of 25-100 µg/mL. The treatment of the Gram-negative species with the low concentration of 25 µg/mL ICG resulted in a very small but significant 40% kill of *E. coli*, and an insignificant kill of *P. aeruginosa*. However, increasing the concentration of ICG to 100 µg/mL resulted in a significant reduction of 99.98% and 71.04% in the viability of *E. coli* and *P. aeruginosa* respectively. Increasing the ICG concentration to 200 µg/mL eradicated more than 99.99% of *E. coli* and 99.97% of *P. aeruginosa*.

The minimum effective concentration for killing the Gram-positive organisms was 25 µg/mL whilst 100-200 µg/mL was needed to kill the Gram-negative organisms. The differing susceptibilities of the Gram-negative and the Gram-positive organisms to lethal photosensitization in this study are probably

attributable to differences in cell wall structures. Gram-negative bacteria have an outer membrane that may reduce the uptake of ROS by the cell (Jori *et al.*, 2006). In addition, the presence of lipopolysaccharide (LPS) in the outer membrane of the Gram-negative bacteria acts as a very effective permeability barrier to many of the molecules in the external environment and contributes to the development of drug resistance in these organisms (Maisch *et al.*, 2004). In contrast, Gram-positive bacteria have a porous outer layer of peptidoglycan which is a less effective as a permeability barrier (Jori & Brown, 2004). The Gram-negative bacteria are known to be relatively resistant to neutral or anionic drugs and ICG is an anionic dye (Malik *et al.*, 1990; Minnock *et al.*, 1996). It has been postulated that Gram-negative bacteria require a disturbance of the cytoplasmic membrane to render them sensitive to anionic or neutral PS (Nitzan *et al.*, 1992). However, in this study the reduced susceptibility of Gram-negative bacteria *P. aeruginosa* and *E. coli* was overcome by increasing the concentration of ICG instead of using a membrane disorganising agent. At higher ICG concentrations, no dark toxicity was detected with the Gram-negative organisms.

Both of the laser light sources used emitted light at 808 nm but the Ondine laser had a lower output compared to the Thor laser. It was evident that regardless of the power output of the laser, increasing the light energy dose enhanced the killing rate for all targeted organisms. In the case of the Thor laser, kills of approximately 99.99% of *Staph. aureus*, *Strep. pyogenes* and *P. aeruginosa* were attained upon irradiation with a light energy dose of 411 J/cm² at a high fluence rate of 1.37 W/cm². However, the same kill of 99.99% was achievable for all targeted bacteria including *E. coli* at a lower fluence rate of 0.3 W/cm² and a light energy dose of 90 J/cm² from the Ondine laser. It is well known that increasing the light energy dose plays an important role in activating PS molecules, hence augmenting the bacterial kill rate achieved (Wilson & Yianni, 1995; Komerik & Wilson, 2002; Matevski *et al.*, 2003). In these experiments, the resulting ROS was not enough to kill the initial bacterial load tested and this may be the cause for the residual population of bacteria observed following the treatment with ICG lethal photosensitization. Another explanation for this residual population may be the use of insufficient light

photons to stimulate the PS molecules, thus a rate of kill experiment may be helpful to detect the light dose that can eradicate all bacterial population.

Interestingly, both high and low intensity 808 nm light alone from the Thor laser was able to exert a cidal effect against *P. aeruginosa*. This finding is supported by the results of a previous study in which irradiation of the organism with 1–80 J/cm² laser light at a wavelength of 810 nm and using an irradiance rate of 0.03 W/cm² resulted in a significant inhibition of bacterial growth (Nussbaum *et al.*, 2003). A possible explanation for this observation is that *P. aeruginosa* has endogenous pigments (pyoverdinin and pyocyanin) that may absorb the light and result in the production of bactericidal species (Reszka *et al.*, 2006). The current data suggest that NIR laser light irradiation by itself would also inhibit growth of *P. aeruginosa* in infected wounds.

The fluence rate at which the light is delivered and the light intensity have both been implicated as factors affecting the lethal photosensitization of bacteria. It was reported that increasing the intensity of the visible light produced from a xenon lamp from 10 mW/cm² to 25 mW/cm² or 100 mW/cm² had a significant enhancement effect on *P. gingivalis* photosensitization with 50 µg/mL TBO (Matevski *et al.*, 2003). The results reported here have revealed the effectiveness of both high and low fluence rate in the photosensitization of both *Staph. aureus* and *Strep. pyogenes* in the presence of ICG. When a light dose of 90 J/cm² was delivered, either at 0.3 or 0.05 W/cm², an approximate reduction of 99.99% was detected in the viable counts of both Gram-positive organisms. Nonetheless, photosensitization of both organisms at the lower fluence rate of 0.05 W/cm² resulted in slightly higher kills of 5.6 log₁₀ and 6.8 log₁₀ for *Staph. aureus* and *Strep. pyogenes* respectively, compared to 3.8 log₁₀ and 4.6 log₁₀ at a fluence rate of 0.3 W/cm². Therefore, the lower fluence rate of 0.05 W/cm² was more successful than the higher fluence rate of 0.3 W/cm² in eradicating the Gram-positive organisms. This may be attributed to the low oxygen consumption associated with irradiation at a low fluence rate (Veenhuizen & Stewart, 1995; Dougherty *et al.*, 1998; Henderson *et al.*, 2006). In contrast, the Gram-negative bacterium *E. coli* exhibited reduced susceptibility to photosensitization upon irradiation with a light dose of 90 J/cm² at the lower fluence rate of 0.05 W/cm². An insignificant 23% kill was achieved

compared to a >99.99% kill obtained at the higher fluence rate of 0.3 W/cm². Similarly, a light dose of 252 J/cm² killed 88% of *P. aeruginosa* at the lower fluence rate of 0.07 W/cm². However >99.98% were killed at the higher fluence rate of 1.37 W/cm² and a lower light energy dose of 247 J/cm². It was clear that a high fluence rate was needed for the photosensitization of Gram-negative bacteria and a high fluence rate was, therefore, used in further investigations.

PIT effects on the photo-inactivation of numerous bacterial species have been investigated by several researchers. Their findings have shown that the effect was dependent on the PS used and the targeted species. Wilson & Pratten, (1995) reported that the kill of *Staph. aureus* upon exposure to TBO combined with visible light was independent of the PIT. Griffiths *et al.*, (1997b) confirmed the same results for the photosensitization of EMRSA-16 using aluminium disulphonated phthalocyanine. The findings presented here support these results: increasing the incubation period of the bacterial suspension with ICG for up to 60 minutes before irradiation with NIR laser light had no effect on the kills obtained for both Gram-positive organisms (*Staph. aureus* and *Strep. pyogenes*) and the Gram-negative bacterium *P. aeruginosa*. In contrast, it was shown that the numbers of *Candida albicans* killed increased markedly when the PIT was varied over the range of 1-3 min, although a further increase in the PIT did not increase the numbers killed (Wilson & Mia, 1994).

The results of this study have shown that exposure of mixed bacterial cultures to NIR laser light in the presence of ICG results in a dose-dependent decrease in bacterial viability. The kill was species-dependent. Although a concentration of 100 µg/mL ICG in combination with 90 J/cm² NIR laser light achieved a kill of >99.99% for all three species, the Gram-negative bacteria *E. coli* and *P. aeruginosa* were less susceptible than *Staph. aureus* at the lower concentration of 50 µg/mL. In contrast, when a mixture of *Staph. aureus* and *Strep. pyogenes* was exposed to 25 µg/mL ICG and irradiated with 54 J/cm² from NIR laser light, there was a similar reduction of 3.5 log₁₀ in the viable counts of both organisms. Gram-positive bacteria have generally been shown to be more susceptible to lethal photosensitization than Gram-negative bacteria, irrespective of which PS is used (Usacheva *et al.*, 2001; Phoenix *et al.*, 2003). Bhatti *et al.*, (2000) reported the reduced susceptibility of the Gram-negative

bacterium *P. gingivalis* compared to the Gram-positive bacterium *Streptococcus sanguinis* to sensitization with TBO and laser light of 632.8 nm in a mixed culture. The reduced susceptibility of Gram-negative organisms to photosensitization was proposed to be due to the barrier function of the outer membrane, which reduces the uptake of PS molecules and inhibits diffusion of the ROS to the cytoplasmic membrane (Malik *et al.*, 1990 & 1992).

Altering the wavelength of the light from 808 nm to 784 nm did not have any effect on the viability of *Staph. aureus* in the presence of ICG – both wavelengths were effective at photo-activating ICG molecules. Although the light emitted at 784 nm is closer to the peak absorbance of ICG in aqueous solutions; the light emitted at 808 nm is more convenient due to the peak absorbance shift noted when ICG binds to proteins in the serum which simulates wound fluid (Landsman *et al.*, 1976). In a similar study, Chan & Lai, (2003) showed that varying the wavelengths (632.8, 665 and 830 nm) of laser energy delivered to several oral species in the presence of MB had an effect on their viability. This dye solution shows an intense absorption peak in the visible region at 665 nm and predictably the authors found that MB coupled with light from a 665 nm diode laser was the most effective combination. Obviously, the PS should be allied with an appropriate source of light in order to enhance its efficacy as a photo-bactericidal compound. Therefore, further experiments were performed using the radiant energy emitted by the 808 nm NIR laser as it is able to photo-activate ICG effectively.

In conclusion, the results of the experiments presented in this Chapter suggest that ICG coupled with NIR laser light of 808 nm is an efficacious photosensitizing agent of the common wound-infecting organisms. This combination was found to be effective against *Staph. aureus*, *Strep. pyogenes*, *E. coli* and *P. aeruginosa* indicating that PDT could be useful in the treatment of burn and wound infections due to these organisms. It has been claimed that fractionation of the light delivered helps to maintain a high level of tissue oxygenation during PDT (Dougherty *et al.*, 1998). Hence, the investigation proceeded to determine the effect of the fractionation of NIR light delivery on the efficacy of lethal photosensitization of the most common organisms responsible for wound infections.

Chapter 4

**Comparison of the effect of pulsed versus
continuous wave near-infrared laser light on
the photo-bactericidal activity of
indocyanine green**

4.1 Introduction

In clinical practice, continuous wave light sources are most commonly used in the field of PDT (Mang, 2004). However, this may generate heat during the excitation process of the PS which in turn may induce collateral damage to the host tissue (Sawa *et al.*, 2004). This problem could be overcome, or possibly reduced, by using pulsed wave laser light rather than continuous laser light. Pulsed wave light allows a relaxation period during which the tissue would be able to dissipate the generated heat (Cotton, 2004). Therefore, the use of pulsed light may reduce any collateral damage associated with PDT.

Furthermore, it has been proposed that if the PS concentration and light fluence rate are high enough, photochemical depletion of tissue oxygen can occur (Dougherty *et al.*, 1998). For oxygen-dependent photosensitization, this results in a lower photodynamic effect and a reduced kill of the target cells. The effect may be overcome by using a pulsed irradiation regimen of light-dark cycles to allow re-diffusion of oxygen during the dark phases (Wilson *et al.*, 1997). It has been shown that during PDT a low rate of oxygen consumption and photobleaching of the PS occur when a pulsed laser is used rather than continuous laser light (Kawauchi *et al.*, 2004).

It has been reported that PDT using continuous light sources may be associated with discomfort, erythema, and localized phototoxic reactions (Alexiades-Armenakas, 2006). In contrast, the use of a long-pulsed dye laser and intense pulsed light, as alternatives to continuous laser light sources enhance PDT efficacy and provide rapid treatment and recovery, while diminishing unwanted side effects (Babilas *et al.*, 2007).

The efficacy of pulsed laser light to photosensitize micro-organisms has not been widely investigated. Therefore, in this study the ability of pulsed and continuous wave NIR laser light in the presence of ICG to photosensitize common organisms responsible for wound infections was investigated.

4.2 Materials and methods

4.2.1 Target organisms and growth conditions

The organisms used were: *Staph. aureus* NCTC 8325-4, EMRSA-16, *Strep. pyogenes* ATCC 12202, *P. aeruginosa* strain PA01, and *E. coli* ATCC 25922. The culture conditions have been described in Chapter 2, section 2.1.2, with the exception that the initial bacterial load was adjusted to approximately 10^5 - 10^6 CFU/mL for all targeted species. For the comparison of the susceptibility of *Staph. aureus* NCTC 8325-4 and EMRSA-16, an initial bacterial load of 10^7 CFU/mL was used.

4.2.2 Photosensitizer preparation and illumination system

This was described in Chapter 2, section 2.1.4.

Irradiation was carried out using The GaAIAs Velopex diode laser system (Medivance Instruments Ltd., UK) which emits light at a wavelength of 810 ± 10 nm. When the laser output power was set to 0.4 W the actual power output was found to be 0.525 W upon calibration using a thermopile TPM-300CE power meter (Genetic-eo, Québec, Canada). The light from this system was applied to the target specimens using an optical fiber of 400 μ m diameter, either in continuous or repeated pulse duration modes which were selected to switch on for 100 msec and off for 100 msec which may allow heat dissipation.

4.2.3 The effect of photosensitizer concentration on lethal photosensitization

The first variable investigated was the effect of ICG concentration on the kills achieved. The method described in Chapter 2, section 2.1.5 was followed, using ICG concentrations of 15.5-25 μ g/mL to photosensitize Gram-positive bacteria and 50-100 μ g/mL to photosensitize *P. aeruginosa*. These bacterial suspensions were exposed to light doses of 42 and/or 63 J/cm² at a fluence rate of 0.7 W/cm².

4.2.4 The effect of light energies

The effect of various light energies in combination with 100 μ g/mL ICG on bacterial viability was studied. Light doses were manipulated by varying the irradiation time while the optical fiber was held vertically at a distance of 16 mm from the surface of the bacterial suspensions. The light doses delivered were

calculated as shown in Table 4-1. In this table CW stands for the continuous mode of irradiation while PW stands for the pulsed mode. Bacterial viability was determined by viable counting.

Table 4-1: The light dosimetric parameters for the 810 nm laser light.

Laser used	Fluence rate (W/cm ²)	Irradiation time (sec)	Irradiation time (sec)	Energy density (J/cm ²)
		CW	PW	
The Velopex diode laser system	0.7	30	60	21
		60	120	42
		90	180	63

4.2.5 Lethal photosensitization of *Staph. aureus*: methicillin-sensitive strain versus methicillin-resistant strain

The photo-susceptibility of a methicillin-sensitive strain (MSSA) (*Staph. aureus* NCTC 8325-4) was compared to the methicillin-resistant strain (MRSA) (EMRSA-16). An initial bacterial load of 10⁷ CFU/mL of both *Staph. aureus* strains were photosensitized using ICG at a concentration of 100 µg/mL in combination with light doses of 42 and 63 J/cm² at a fluence rate of 0.7 W/cm².

4.2.6 Measurements of the temperature during bacterial photosensitization

One hundred microlitres of PBS, 25, 100 and 200 µg/mL ICG in triplicate were exposed to a continuous or pulsed light dose of 63 J/cm² at a fluence rate of 0.7 W/cm² to determine the temperature rise during both modes of irradiation. The temperatures of bacterial aliquots were recorded immediately before and after irradiation of the samples using an immersion thermocouple probe connected to a Fluke 179 digital multimeter (Fluke. USA).

4.3 Results

4.3.1 The effect of pulsed versus continuous wave near-infrared laser light on *Staph. aureus* and *Strep. pyogenes*

4.3.1.1 ICG concentrations

Figures 4-1 and 4-2 show the effect of delivering light energy as a continuous or pulsed wave, in the presence of different concentrations of ICG, on the viability of the Gram-positive species. Neither light of continuous or pulsed

waves alone nor dark incubation with the PS had any effect on the viability of *Staph. aureus* (Figure 4-1). At an ICG concentration of 15.5 µg/mL significant reductions in the viable count of 2.33 and 3.18 log₁₀ (P= 0.0003 and P= 0.0002) were achieved upon exposure to a light dose of 42 J/cm² delivered continuously or pulsed respectively. At the same light dose, an increased concentration of ICG of 25 µg/mL induced significant kills of 2.52 and 2.76 log₁₀ (P= 0.0001 and P= 0.001) for continuous or pulsed waves of light, respectively. The same pattern of kill was observed with 15.5 µg/mL ICG and a light dose of 63 J/cm², with reductions of 2.7 and 3.3 log₁₀ (P= 0.016 and P= 0.000001) when light was transmitted in continuous or pulsed waves, respectively. However, at 25 µg/mL ICG and a light dose of 63 J/cm², the kill increased to 3.75 log₁₀ upon exposure to continuous waves of light compared to 3.68 log₁₀ upon exposure to pulsed waves of light (P=0.001 in each case). When light was delivered as either continuous or pulsed waves, no difference in the efficacy was observed for both light doses and at all ICG concentrations tested.

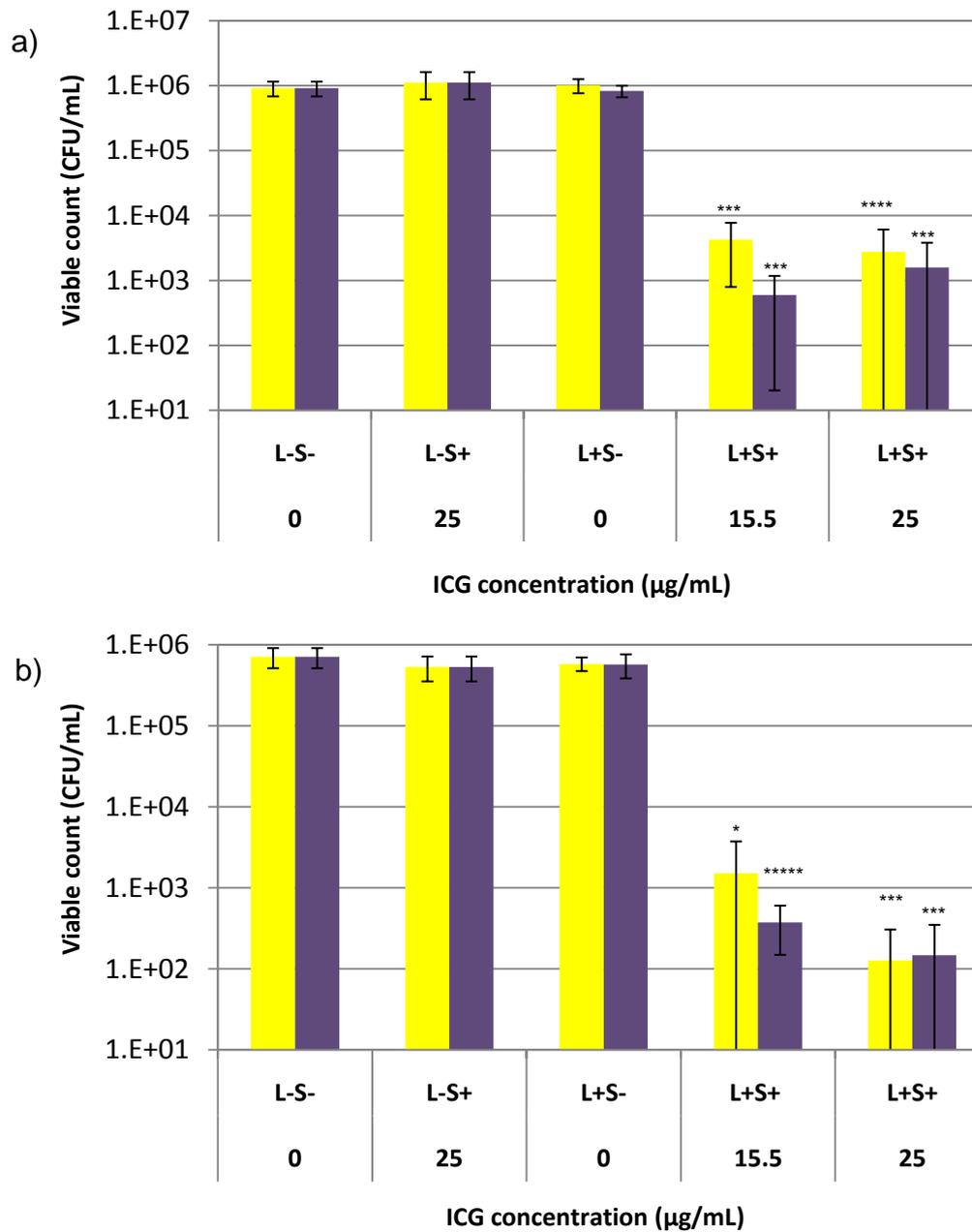


Figure 4-1: Lethal photosensitization of *Staph. aureus* NCTC 8325-4 using ICG concentrations of 0, 15.5 and 25 µg/mL. *Staph. aureus* suspensions were exposed to (a) 42 J/cm² and (b) 63 J/cm² either continuously (■) or in a pulsed mode (■). Error bars represent the standard deviation from the mean.

Figure 4-2 shows the photo-susceptibility of *Strep. pyogenes* when treated with 15.5 and 25 µg/mL ICG in combination with NIR light transmitted as continuous or pulsed waves. When a light dose of 42 J/cm² was applied in a continuous mode, a reduction of 2.8 log₁₀ (P < 0.000001) in the viable count was observed compared to 3 log₁₀ (P < 0.001) when the light was transmitted in a pulsed mode. The kills of *Strep. pyogenes* observed were similar for both ICG

concentrations. These reductions approximated to a 99.9% kill of the initial load of *Strep. pyogenes* aliquots which received neither ICG nor light.

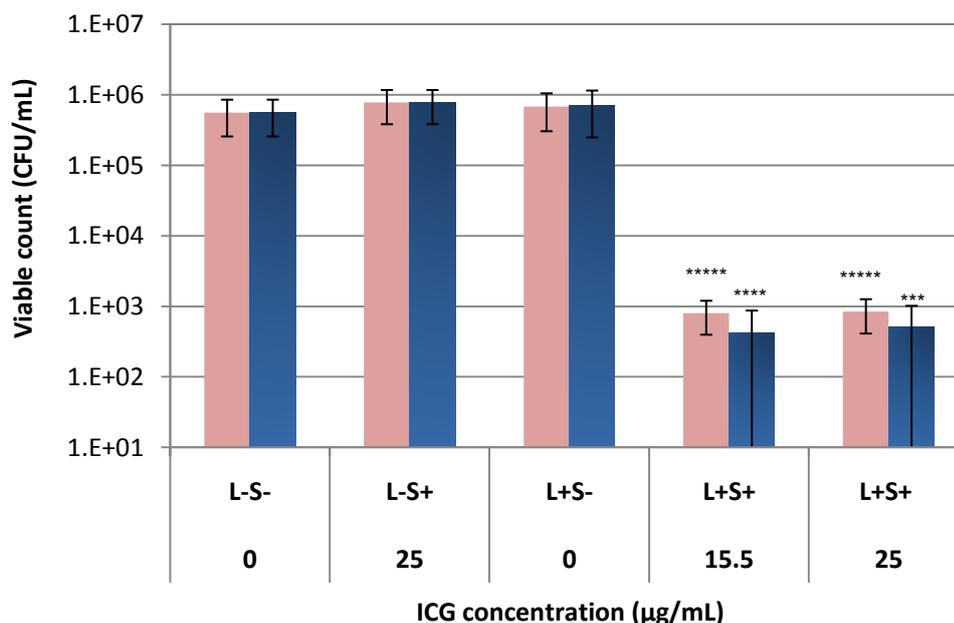


Figure 4-2: Lethal photosensitization of *Strep. pyogenes* using ICG concentrations of 0, 15.5 and 25 µg/mL. *Strep. pyogenes* suspensions were exposed to 42 J/cm² delivered either in a continuous mode (■) or in a pulsed mode (■). Error bars represent the standard deviation from the mean.

4.3.1.2 The effect of varying the light energies

Both continuous and pulsed light in conjunction with ICG were equally effective at photosensitizing *Staph. aureus* at all light doses tested as shown in Figure 4-3a. The reduction in *Staph. aureus* numbers elicited by both light delivery modes was dependent on the light dose. Significant reductions of 97.8, 99.9 and 99.999 % ($P < 0.001$, $P = 0.002$, $P < 0.00001$ for both delivery modes) were obtained upon exposure of *Staph. aureus* suspensions to light energies of 21, 42 and 63 J/cm² respectively, regardless of the transmission mode of light waves. In the presence of ICG, a greater kill was achieved upon increasing the light energy. The difference observed was statistically significant ($P = 0.027$). Neither the light nor the PS affected *Staph. aureus* viability.

As shown in Figure 4-3b both continuous and pulsed light modes resulted in a statistically significant ($P < 0.01$) reduction in *Strep. pyogenes* viable counts at all light doses. Irradiation of ICG-treated *Strep. pyogenes* with the continuous light produced a somewhat greater kill than the pulsed light. However, this

difference was not significant. When continuous light energies of 21, 42 and 63 J/cm² were used to activate ICG, kills of 99.2, 99.99, and 99.999% (P=0.0001 for 21, 42 J/cm² and P=0.0000003 for 63 J/cm²) were achieved, respectively. However, slightly lower kills of 97.56, 99.92 and 99.99% (P=0.01, P=0.0002 and P=0.00001) were observed when the former light energies were pulsed correspondingly. Neither the light nor the PS alone had any effect on the viability of *Strep. pyogenes*.

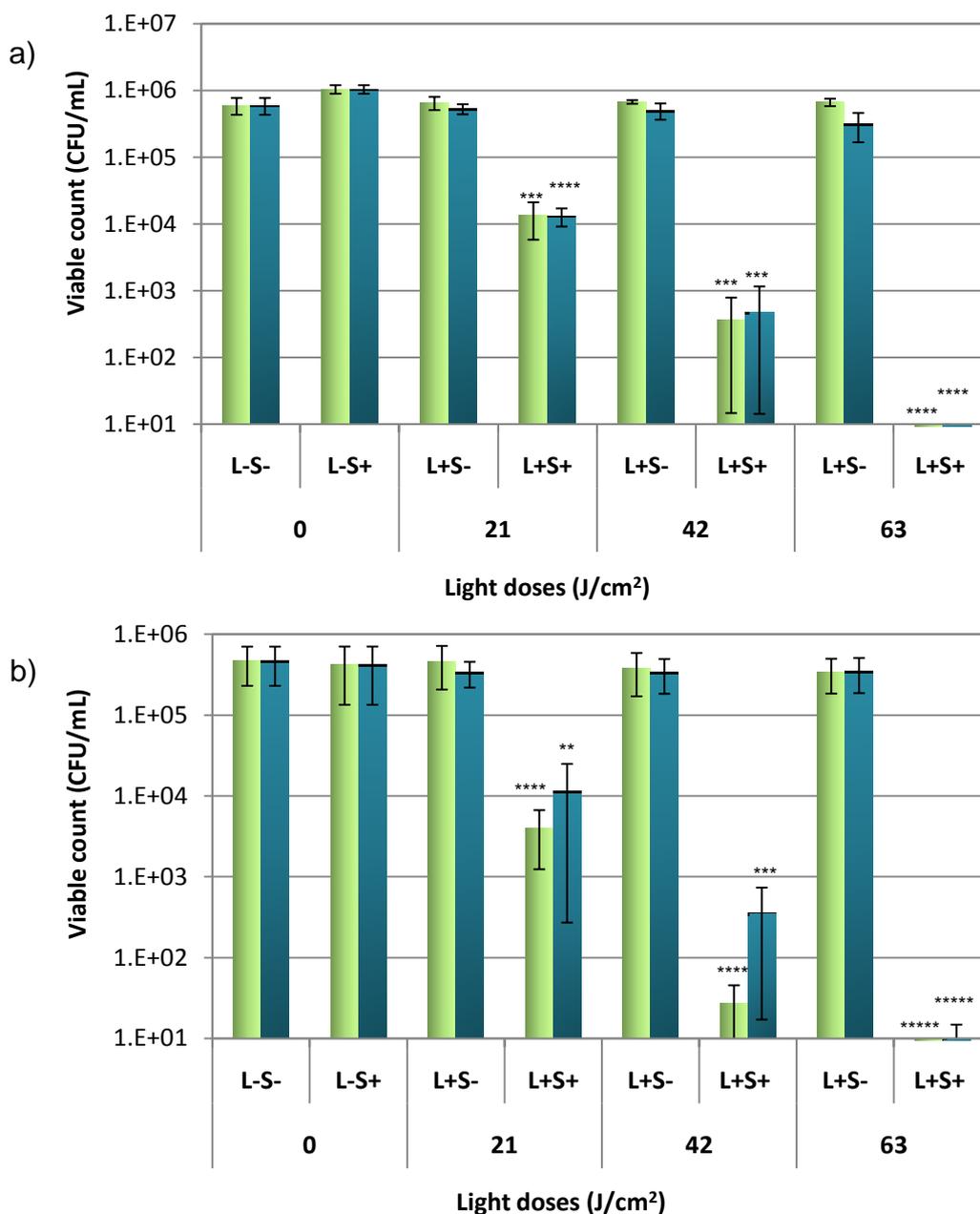


Figure 4-3: Lethal photosensitization of (a) *Staph. aureus*, (b) *Strep. pyogenes* with 100 µg/mL ICG. Bacterial suspensions were exposed to 21, 42 and 63 J/cm² transmitted either in a continuous mode (■) or in a pulsed mode (■). Controls received neither light nor ICG (L-S-). Error bars represent the standard deviation from the mean.

4.3.2 Photosensitization of methicillin-resistant *Staph. aureus*

When MRSA EMRSA-16 was subjected to lethal photosensitization using ICG and NIR laser light, the kills attained using ICG at 100 µg/mL varied with the light dose but not with the mode of light transmission used (Figure 4-4). Figure 4-4 shows that EMRSA-16 was the least susceptible, with 97.8% of the cells being killed ($P= 0.0001$), when exposed to 42 J/cm² of pulsed light. In comparison, when the cells were exposed to 42 J/cm² of continuous light, the viable count was reduced by >99.3% ($P= 0.004$). The effectiveness of lethal photosensitization using ICG was augmented by increasing the light dose to 63 J/cm². 99.98% ($P= 0.0004$) of the cells were killed upon exposure to continuous light compared with 99.96% ($P= 0.001$) when the light was pulsed. This difference in the efficacy between the continuous and the pulsed light was not significant. However, the difference in efficacy with respect to the amount of light energy delivered was significant ($P= 0.038$ for CW and $P= 0.009$ for PW).

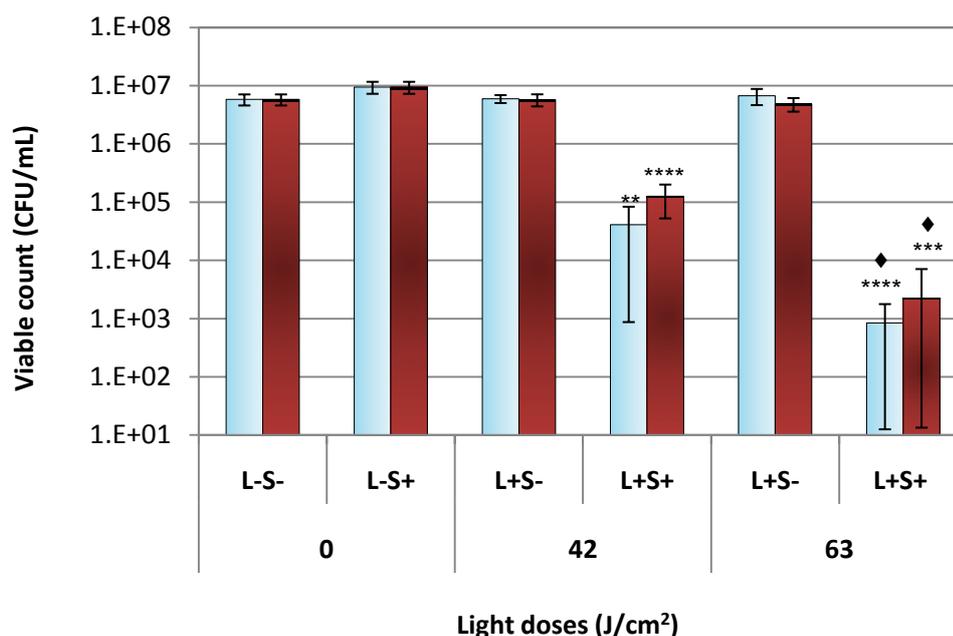


Figure 4-4: The photosensitivity of MRSA EMRSA-16 to 100 µg/mL ICG coupled with 0, 42 or 63 J/cm² transmitted either in a continuous mode (□) or a pulsed mode (■). ♦ shows a significant difference between the light doses. Error bars represent the standard deviation from the mean.

4.3.2.1 Photo-sensitivity of methicillin-resistant *Staph. aureus* compared to methicillin-sensitive *Staph. aureus*

The difference between the photosensitivity of MSSA and MRSA is illustrated in Figure 4-5. For both *Staph. aureus* strains, 100 µg/mL ICG had no dark toxicity

on bacterial viability. The kill of *Staph. aureus* was dependent on the strain and the light dose employed. Greater reductions of 2.1 and 2.3 log₁₀ in the viable count were observed in the case of MSSA, compared to 1 and 1.5 log₁₀ reductions for MRSA when exposed to ICG and 42 J/cm² delivered as continuous or pulsed waves, respectively. Increasing the light dose to 63 J/cm² enhanced the killing significantly for both strains (P= 0.002 for MSSA and P= 0.0002 for MRSA); a greater reduction in the viable count was observed in the case of MSSA. A continuous light dose of 63 J/cm² resulted in a 5.3 and 3.6 log₁₀ reduction in the viable counts of MSSA and MRSA, correspondingly. This difference in the susceptibility of the strains was significant (P= 0.038). When the light was pulsed, reductions of 4.44 and 2.67 log₁₀ for MSSA and MRSA, respectively, were achieved. The difference in the efficacy of the continuous and the pulsed wave light against MRSA was not significant at a light dose of 63 J/cm² (P= 0.051).

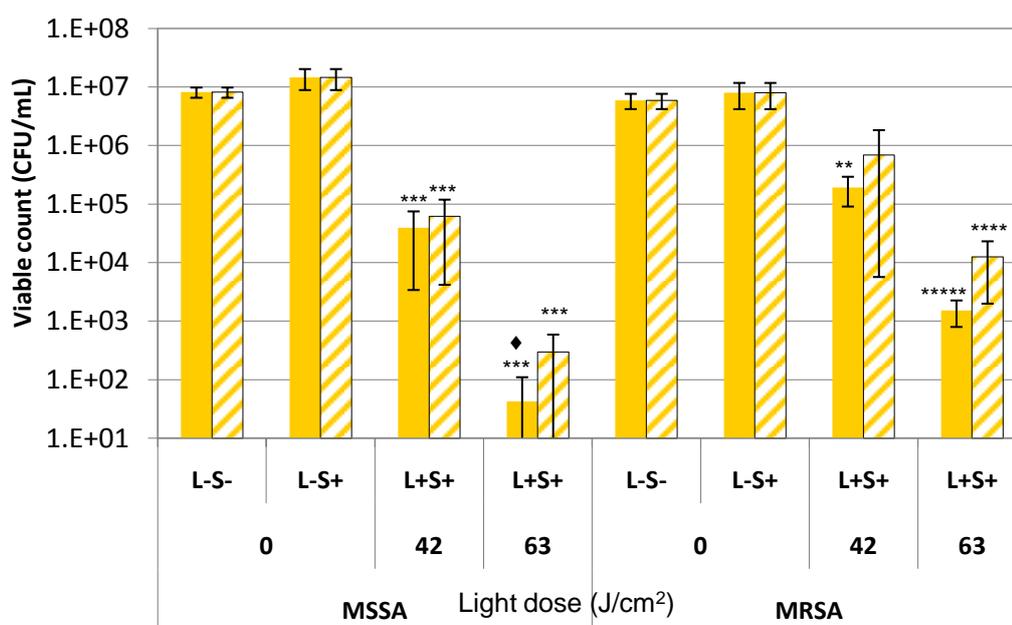


Figure 4-5: Comparison between the susceptibility of MSSA and MRSA to lethal photosensitization using 100 µg/mL ICG combined with the 810 nm NIR laser light. Bacterial suspensions were exposed to 0, 42 and 63 J/cm² transmitted either in a continuous mode (■) or in a pulsed mode (▨). Controls received neither light nor ICG (L-S-) or received ICG and were kept in the dark (L-S+). ♦ shows a significant difference between MRSA and MSSA. Error bars represent the standard deviation from the mean.

4.3.3 The Gram-negative organism: *P. aeruginosa*

P. aeruginosa did not undergo lethal photosensitization at any of the ICG concentrations when combined with pulsed light waves; a maximum kill of 45% was achieved but this was not significant ($P > 0.8$) as shown in Figure 4-6. When *P. aeruginosa* cells were treated with 50 $\mu\text{g/mL}$ ICG and exposed to 63 J/cm^2 transmitted either as a continuous or a pulsed wave, no significant kill was detected ($P = 0.11$ and $P = 0.94$ respectively). However at 50 $\mu\text{g/mL}$ ICG, irradiation of *P. aeruginosa* with continuous wave light resulted in 81% kill compared to 24% kill when the light was pulsed. At a higher concentration of 100 $\mu\text{g/mL}$ ICG, a significant kill ($P = 0.0000002$) of 99.73% was achieved upon exposure to continuous light of 63 J/cm^2 . When the light was pulsed in the presence of 100 $\mu\text{g/mL}$ ICG, there was no significant ($P = 0.83$) effect on the viability of *P. aeruginosa*. Delivering the light continuously was significantly ($P = 0.021$) more effective compared to pulsed light at killing *P. aeruginosa* using 100 $\mu\text{g/mL}$ ICG; the reductions in the viable count were 2.7 and 0.3 \log_{10} respectively.

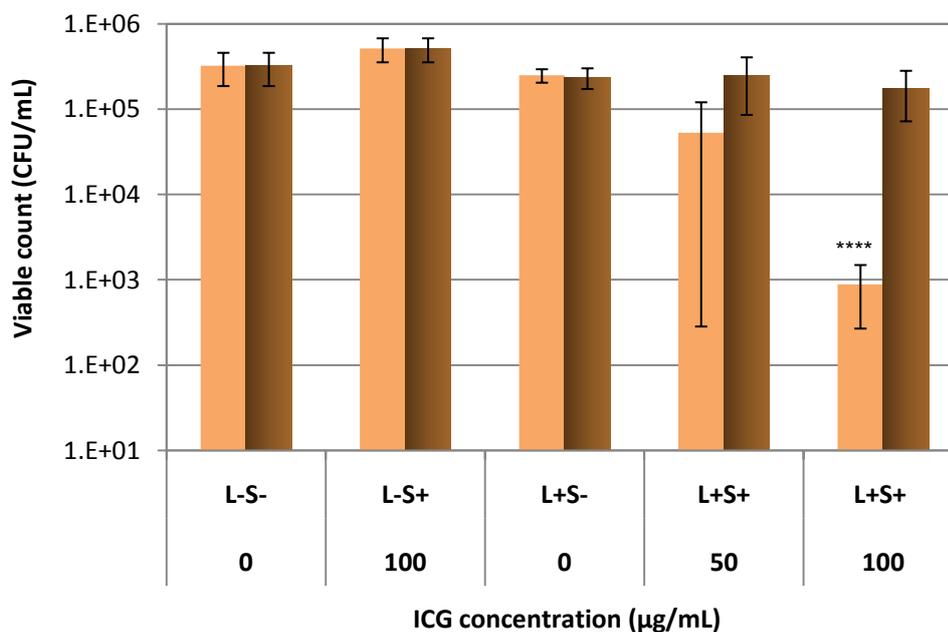


Figure 4-6: Lethal photosensitization of *P. aeruginosa* using ICG concentrations of 0, 50 and 100 $\mu\text{g/mL}$. *P. aeruginosa* suspensions were exposed to 63 J/cm^2 delivered either in a continuous mode (□) or in a pulsed mode (■).

It was evident that when the light was pulsed, *P. aeruginosa* was not susceptible to photosensitization as there was only a 0.3 \log_{10} reduction in viable cells (Figure 4-6). Therefore, an investigation of the effect of altering the light energies was carried out using continuous light. 63 J/cm^2 was the only

light dose effective at photosensitizing *P. aeruginosa* in the presence of ICG as shown in Figure 4-7. A statistically significant 99% ($P= 0.0003$) reduction in the number of viable bacteria recovered was achieved when compared to controls that received neither ICG nor light. No reduction in the number of viable *P. aeruginosa* cells was observed when exposed to lower light doses of 42 and 21 J/cm^2 and 100 $\mu\text{g}/\text{mL}$ ICG.

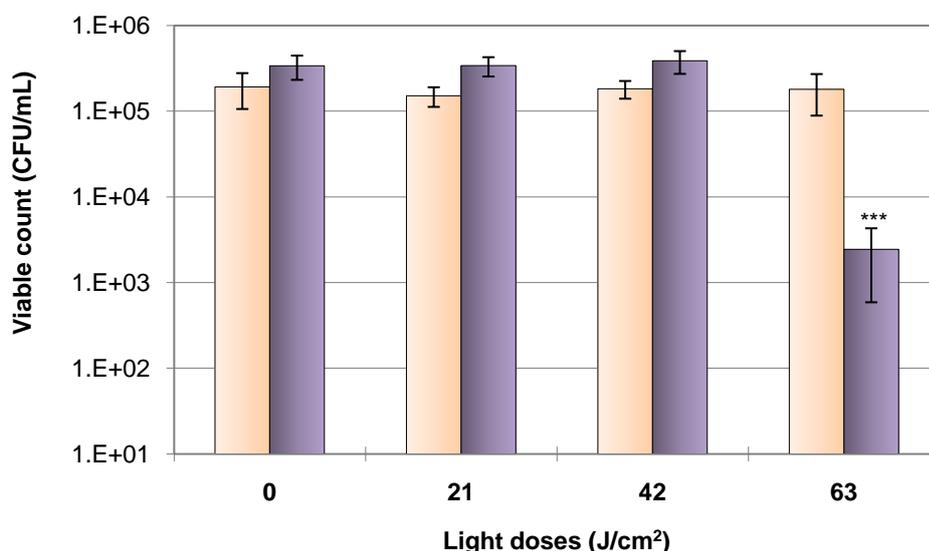


Figure 4-7: The effect of varying light energy on the viability of *P. aeruginosa*. Bacterial suspensions exposed to continuous light of 0, 21, 42 and 63 J/cm^2 in the presence of either 100 μL of PBS (□) or 100 $\mu\text{g}/\text{mL}$ ICG (■).

4.3.4 Measurement of temperature during bacterial irradiation

The temperature changes of bacterial suspensions during ICG photosensitization using continuous or pulsed light irradiation were measured to compare the thermal effect accompanying each mode of irradiation (Figure 4-8). The initial recorded temperature of all samples pre-irradiation was around 22 °C (RT). This was measured immediately before exposing the bacterial suspensions to a light dose of 63 J/cm^2 at fluence rate of 0.7 W/cm^2 in the presence of either 100 μL of PBS or 25, 100 and 200 $\mu\text{g}/\text{mL}$ ICG.

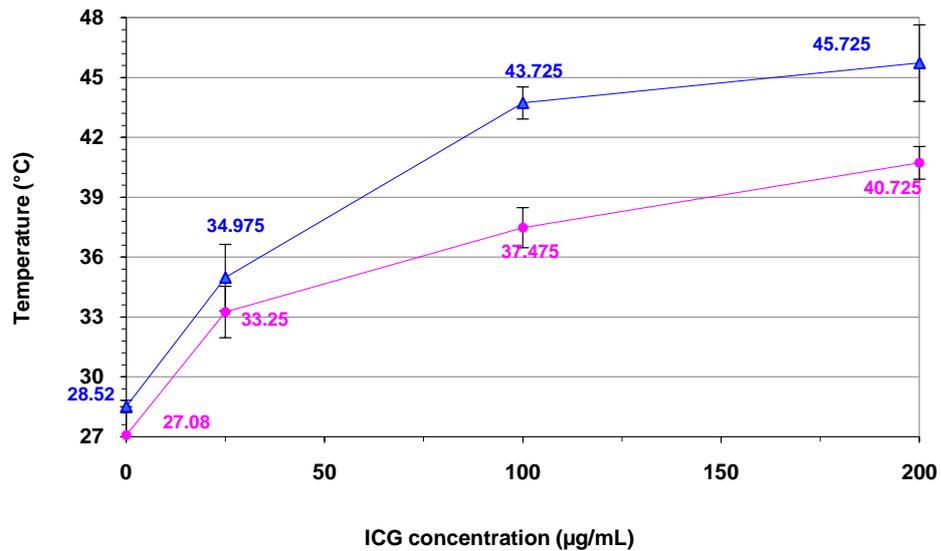


Figure 4-8: Comparison of the temperatures changes during continuous (—▲) and pulsed (—●) photosensitization of *Staph. aureus* treated with a range of ICG concentration of 0, 25, 100 and 200 µg/mL. Bacterial suspensions were exposed to 63 J/cm².

From the results shown in Figure 4-8, it is evident that the temperature rise during lethal photosensitization was dependent on both the ICG concentration and the irradiation mode (pulsed or continuous). Irradiation of bacterial aliquots in the absence of ICG was accompanied by a slight increase in the suspension temperature compared to the pre-irradiation temperature (~22 °C). However, the temperatures were almost the same 28.52 ± 0.3 °C and 27.08 ± 1.42 °C using continuous and pulsed modes of irradiation, respectively, in the absence of ICG. At the low concentration of 25 µg/mL ICG there was no difference in the heat generated by continuous and pulsed light (34.97 ± 1.66 °C and 33.25 ± 1.82 °C, respectively). Such temperatures do not affect the viability of the bacteria investigated. Under identical experimental conditions a significant reduction of 3.7 log₁₀ in the viable counts of *Staph. aureus* was attained (Figure 4-1b). The temperature increased to 43.7 ± 0.8 °C during treatment with the combination of continuous NIR laser light and 100 µg/mL ICG. The temperature recorded during irradiation at the same ICG concentration using pulsed light was slightly lower at 37.47 ± 1.0 °C. This temperature difference between both modes of irradiation was significant (P=0.000004).

4.4 Discussion

PDT has been demonstrated to be an effective cancer treatment with high specificity, minimal invasiveness and good cosmetic outcome. Nowadays PDT is considered a highly effective method for the treatment of a wide variety of diseases (Qiang *et al.*, 2006). Therefore, studies were directed to enhance the photo-cytotoxicity generated from this process with minimal collateral damage to the host tissues (Sawa *et al.*, 2004). This process requires the presence of 3 components: the PS, oxygen and light (Sibata *et al.*, 2000). Varying any of these three components may affect the photo-toxic effect. For example, the accumulation of appropriate concentrations of the PS in the target tissues after local or systemic administration may enhance the photosensitization process (Wilson *et al.*, 1997). Another factor that can affect the photosensitization process is the level of oxygen in the target tissues. It has been reported that hyperoxygenation can enhance the photo-cytotoxicity achieved during lethal photosensitization (Huang *et al.*, 2003). The last factor which can affect the photosensitization process is the light. Light can be delivered as continuous or pulsed waves, each has different tissue interactions (Mang, 2004) and this may affect the bactericidal effect elicited from the photosensitization process (Metcalf *et al.*, 2006). Pulsed light can be modulated via energy dosage, pulse duration and the frequency of irradiation (Miyamoto *et al.*, 1999; Ogura *et al.*, 2007). Therefore, in this *in vitro* work, the bactericidal effects produced by pulsed and continuous laser irradiation were investigated and compared.

Although continuous laser irradiation is the most common light source used in PDT, a large number of cancer studies have demonstrated that using pulsed light may improve the photosensitization process (Gibson *et al.*, 1990; Müller *et al.*, 1998). Yet its cytotoxic effect is still uncertain (Fisher *et al.*, 1995; Kawauchi *et al.*, 2004). In the current study, activation of ICG with continuous or pulsed light resulted in appreciable kills of the Gram-positive organisms investigated. In contrast, no significant kill was detected upon exposure of the Gram-negative bacterium *P. aeruginosa* to pulsed light.

It has been claimed that the pulsed laser light mediated cytotoxicity for cancerous cells would be different from those of the continuous laser. This difference could depend mainly on the photosensitizer concentration (Zamora-

Júarez *et al.*, 2005). In the current study, at a low concentration of 15.5 µg/mL ICG, pulsed NIR laser light resulted in slightly greater kills of the Gram-positive organisms *Staph. aureus* and *Strep. pyogenes*. However, these kills were not significantly different from kills induced by the continuous light. The difference in the kill achieved may be due to the slower rate of oxygen depletion and the photobleaching of ICG during pulsed light photosensitization (Kawauchi *et al.*, 2004).

Aveline *et al.*, (1998) demonstrated a great saturation of the photosensitizer triplet state when activated with a pulsed laser light. Also, it has been shown that pulsed light reduced the photo-bleaching rate of the PS molecules (Kawauchi *et al.*, 2004). However, the data presented herein showed that increasing the concentration of ICG from 15.5 to 25 µg/mL or more removed any difference in bacterial photo-toxicity attributed to the modulation of the light wave. Greater kills of *Strep. pyogenes* were achieved with continuous laser light compared to pulsed light. Activation of 100 µg/mL of ICG with continuous light energies of 21, 42 and 63 J/cm³ resulted in kills of *Strep. pyogenes* of 99.2, 99.99 and 99.999%, respectively. By comparison, pulsed light of the same light energies, achieved kills of *Strep. pyogenes* of 97.56, 99.2 and 99.99%, respectively. Pulsed laser light has been shown to be less cytotoxic against mammalian cells than continuous light (Miyamoto *et al.*, 1999; Kawauchi *et al.*, 2004); this is due to each mode of irradiation inducing cell death by a different mechanism. Pulsed laser light induces cell death via apoptosis while continuous wave laser light irradiation induces cell necrosis (Miyamoto *et al.*, 1999).

In the case of the Gram-negative bacterium *P. aeruginosa*, pulsed light did not result in any significant kill. This organism was not susceptible to photosensitization at all ICG concentrations in combination with pulsed light waves; only a maximum kill of 45% was achieved. In contrast, continuous light resulted in a significant reduction of 99% in the viable count of *P. aeruginosa* only at a light energy of 63 J/cm² and 100 µg/mL ICG.

The light energy used to photo-activate ICG played an important role in the bacterial killing achieved upon exposure to either continuous or pulsed light

modes. The higher the light dose used the greater the bacterial kill achieved. This effect was observed in both Gram-positive and Gram-negative organisms. Both *Staph. aureus* and *Strep. pyogenes* were easily killed, even using a low light dose of 21 J/cm². In contrast, *P. aeruginosa* was only killed in the presence of 100 µg/mL coupled with continuous light energy of 63 J/cm². This light dose was the minimal effective light energy necessary to photo-inactivate *P.aeruginosa* when delivered as continuous waves. The reduced susceptibility exhibited by this Gram-negative organism is likely to be due to the presence of the outer membrane containing LPS which acts as permeability barrier (Malik *et al.*, 1992).

After exposure of the PS to continuous or pulsed light of an appropriate wavelength, mild heat may be generated when the excited PS molecules decay back to the ground state (Sibata *et al.*, 2000). Pulsed light interacts mainly through a non-thermal mechanism. The energy emission in the very short pulses leads to diffusion of the generated heat into the surrounding tissues, causing photochemical effects with minimal thermal damage to the surrounding tissue (Cotton, 2004). Therefore, using pulsed light waves may reduce the side effects such as pain which may be associated with PDT using a continuous light source (Karrer *et al.*, 1999; Babilas *et al.*, 2007). In the current study, increased temperatures of bacterial cultures were observed especially with an ICG concentration of 100 µg/mL coupled with a continuous light dose of 63 J/cm². This increase was from 28.52°C to 43.7 °C, while pulsed light increased the temperature to 37.5°C. However, this increase in temperature would not affect the viability of the Gram-negative bacterium but may assist in the diffusion of the PS through the cell membrane to initiate the phototoxic effect (Leyko & Bartosz, 1986; Dougherty *et al.*, 1998). This may be a possible explanation for the photo-inactivation of *P. aeruginosa* at 100 µg/mL ICG and a continuous light energy of 63 J/cm².

MRSA is considered to be the most common cause of both community-acquired and hospital-acquired infections (Roghmann *et al.*, 2005; Klevens *et al.*, 2007). A number of MRSA infections may be life-threatening, especially in the case of immuno-compromised patients, causing bacteraemia, pneumonia, cellulitis, osteomyelitis, endocarditis and septic shock (Klevens *et al.*, 2007).

Considering the problems associated with their multi-resistance to antibiotics, lethal photosensitization may be a relevant and successful alternative to conventional antibiotic therapy. The results presented herein demonstrate that both *Staph aureus* strains, the MSSA strain NCTC 8325-4 and the MRSA EMRSA-16, were highly susceptible to lethal photosensitization using ICG and NIR laser light (>99.9% kills were achieved for both strains). The killing of both organisms was dependent mainly on the light dose but not on the mode of irradiation (continuous or pulsed light). A 99.99% reduction in the viable count of MSSA was achieved upon exposure to 100 µg/mL and 63 J/cm² delivered either as continuous or pulsed laser light. However, EMRSA-16 was less susceptible than the MSSA strain NCTC 8325-4 at similar ICG concentrations and light energy. Continuous irradiation resulted in a higher kill of MRSA of 99.97% than pulsed light which achieved a 99.8% kill. The photo-sensitivity of both *Staph. aureus* strains differed significantly when exposed to continuous light energy of 63 J/cm², 5.3 and 3.6 log₁₀ reductions in the viable count of MSSA and MRSA were observed, respectively. These experiments would have to be repeated in more strains of *Staph. aureus* to confirm this difference in sensitivity to lethal photosensitization between MSSA and MRSA strains. However, these results are supported by similar findings reported by Grinholc *et al.*, (2008) who showed that photo-inactivation of *Staph. aureus* using protoporphyrin diarginate was strain-dependent and ranged from 0 to 3 log₁₀-unit reduction in viable counts. The reduced susceptibility of clinical MRSA strains compared to MSSA strains to photosensitization may be due to the presence of capsular polysaccharides that may hinder the penetration of ICG or ROS. It has been found that MRSA are more likely to have type 5 microcapsule in comparison with MSSA strains (Roghmann *et al.*, 2005). Type 5 microcapsule is extracellular uronic acids containing polysaccharides formed by a trisaccharide repeat unit, having identical monosaccharide compositions, mainly *N*-acetyl mannuronic acids which are partially *O*-acetylated (Moreau *et al.*, 1990). This may play a key role in the reduced susceptibility of MRSA strains to photosensitization.

Currently, there are few reports comparing the effect of different modes of irradiation on microbial photosensitization. Metcalf *et al.*, (2006) carried out a study to investigate the effect of light fractionation on the viability of

Streptococcus mutans biofilms. The authors reported that continuous white light irradiation for 5 min resulted in a 2 log₁₀ bacterial kill. Fractionation of the light into 1 min X 5 doses separated by dark recovery periods of 5 min or 30 sec X 10 doses separated by 2 min recovery periods killed respectively 3 and 3.7 log₁₀. Other studies have shown that pulsed light can enhance the penetration of the PS deep into oral biofilms via the generation of mechanical shockwaves and so enhance the photosensitization process (Soukos *et al.*, 2000; Soukos *et al.*, 2003; Ogura *et al.*, 2007).

Finally, both continuous and pulsed NIR laser light combined with ICG are equally effective at photosensitizing the Gram-positive organisms (*Staph. aureus* and *Strep. pyogenes*). In contrast, only continuous NIR laser light combined with ICG was capable of photosensitizing the Gram-negative organism *P.aeruginosa*. Further research is required to examine the effectiveness of this light-activated antimicrobial agent coupled with each irradiation mode against these organisms *in vivo*.

Chapter 5
Enhancement of lethal
photosensitization of *Staph. aureus*

5.1 Introduction

Antibiotics are the conventional treatment for *Staph. aureus* infections. However, the increasing prevalence of antibiotic-resistant strains of this organism necessitates the development of new antimicrobial strategies (Taylor, 2008). An alternative approach for the treatment of localized *Staph. aureus* infections involves the use of light-activated antimicrobial agents (O’Riordan *et al.*, 2005; Maisch, 2007). The production of highly ROS, as an end product of the photosensitization process, can induce photo-oxidative damage to membrane lipids, essential proteins, DNA and other cellular components, usually leading to bacterial cell damage and death (Phoenix & Harris, 2003). Nowadays, there is a great scientific interest in improving and enhancing the outcome of the photosensitization process (Jori, 1996). These have been discussed widely in the literature (Konan *et al.*, 2002; Daniel & Astruc, 2004; Jakus & Farkas, 2005; Kramarenko *et al.*, 2006; Wieder *et al.*, 2006). In the previous chapter (Chapter 4), the effect of irradiation mode (pulsed and continuous NIR laser light) on the photo-bactericidal activity of ICG was investigated. Herein, the applications of three different strategies to enhance and improve the photosensitization process of ICG against *Staph. aureus* were studied.

5.1.1 Gold nanoparticles

At present, nanoscience is considered one of the major progressive scientific areas which should shortly result in advancements for the benefit of human health (Boisselier & Astruc, 2009). Nanomedicine covers medical diagnosis, monitoring, and treatment (Shaffer, 2005). Generally, Nanoparticles (NPs) are categorized as being either naturally occurring or synthetic. These are then sub-classified as being organic (carbon containing) or inorganic. Subsequent classification is based on their shape (sphere, rods, etc.) and the structure of the material they may contain (e.g. oxides, metals or salts) and are critical to function (Allison *et al.*, 2008). NPs can be used for cell targeting and destruction or as a vehicle for precise drug delivery (Brigger *et al.*, 2002). The application of metal nanoparticles is a promising approach to photothermally destruct cancer cells both *in vitro* and *in vivo* (Hirsch *et al.*, 2003) and also to enhance the photocytotoxicity of anticancer-PDT (Allémann *et al.*, 1996).

The biomedical applications of metal nanoparticles were started by Faulk and Taylor, (1971) with the use of nanobioconjugates after the discovery of immunogold labelling. Gold nanoparticles (AuNPs), also called gold colloids are the most stable metal nanoparticles (Daniel & Astruc, 2004). They exhibit many unique optical and physical properties in comparison to their bulk metal owing to their nanometer-order size that make them very attractive for diagnostic and therapeutic applications (Chen *et al.*, 2007). The surface plasmon resonance (SPR) absorption and scattering of AuNPs is a crucial property of these NPs and represent a key contribution in nanotechnology (Boisselier & Astruc, 2009). SPR is an optical phenomenon evolved from the collective oscillation of conduction electrons, which is also responsible for the brilliant colours of metal colloids (Skrabalak *et al.*, 2007). This property allows them to efficiently absorb laser light which is involved particularly in both the photodiagnostics and photothermal therapy of cancers and other main diseases (Pustovalov & Babenko, 2004). The AuNPs' particle size are available in the range from 1 to 100 nm (De Jong & Borm, 2008; Rao, 2008), and their SPR absorption property can be observed above 3 nm (McLean *et al.*, 2005). For example, AuNPs in the range of 5–20 nm has its SPR band around 530 nm, giving them their characteristic red colour (Westerlund & Bjørnholm, 2009). For a core diameter below 3 nm, quantum size effects lead to a sharp drop in the absorption with decreasing size i.e. for the 2-nm AuNPs, the SPR band is absent (McLean *et al.*, 2005).

PDT is an effective treatment for cancer and many other diseases but it is nonselective (Grant *et al.*, 1997). Nanotechnology has great potential to reshape the critical components of PDT to ultimately allow for clinical and scientific advances. AuNPs have recently been a focus of research because of their therapeutic potential as drug-delivery carriers (Han *et al.*, 2007; Park *et al.*, 2009). The applications of AuNPs to drugs such as a PS (Hone *et al.*, 2002; Ricci-Júnior & Marchetti, 2006; Wieder *et al.*, 2006; Khaing Oo *et al.*, 2008) or other anti-tumour drugs (Ganesh, 2007) promise a bright future by diminishing side effects due to toxicity, improving therapeutic efficiency, targeting, biodistribution, and overcoming the problems of solubility, stability related to quantum size, electronic, magnetic and optical properties (Wieder *et al.*, 2006; Ganesh, 2007). For example, nanoparticles have been used to

encapsulate a number of PSs to help their delivery for PDT (Gomes *et al.*, 2006; Ricci-Júnior & Marchetti, 2006). Also they can be conjugated to specific proteins and/or antibodies (El-Sayed *et al.*, 2005; Huang *et al.*, 2006; Pissuwan *et al.*, 2007). A AuNPs-antibody conjugate and visible laser light was able to induce a bactericidal effect at different light fluencies and nanoparticle sizes (Zharov *et al.*, 2006).

AuNPs are able to improve the bactericidal effect induced by TBO-photosensitization of *Staph. aureus* (Narband *et al.*, 2008; Gil-Tomás *et al.*, 2007). ICG is a NIR PS which has a large number of medical applications, but it becomes rapidly unstable and bleached quickly under intense illumination (Geddes *et al.*, 2003a). Therefore a number of researchers have investigated the ability of nanoparticles to enhance its photostability (Malicka *et al.*, 2003; Saxena *et al.*, 2004; Gomes *et al.*, 2006; Kim *et al.*, 2007; Altinoğlu *et al.*, 2008). It was reported that both noncovalent and covalent interactions of gold and silver nanoparticles with ICG provided stability and reduced its photobleaching rate (Geddes *et al.*, 2003a & b; Tam *et al.*, 2007). Therefore the effect resulting from the noncovalent interaction of 2 nm Gold colloids with ICG on the viability of *Staph. aureus* was investigated.

5.1.2 Antioxidants

PDT induces a highly complex series of permanent oxidative changes and damages in the target cells (e.g. bacteria, cancer) by generation of highly ROS (O'Riordan *et al.*, 2005). These ROS are produced mainly via two basic reactions (type I & type II reactions), both of which follow the excitation of the PS molecule with light photons. Type I and II reactions may occur simultaneously, and the ratio between the two processes mainly depends on the photosensitizer, substrate and oxygen concentration (Martins *et al.*, 2004). The efficacy of the PDT-triggered cytotoxic effect mainly depends on the amount of the ROS generated as a result of photoactivation of the photosensitizer. Thus, increasing the life span of $^1\text{O}_2$ and/or intensifying the oxidative activities of the ROS (i.e. prolonged oxidative chain reactions) during or immediately after the photosensitizer irradiation, may enhance the cytotoxic efficiency of PDT. Antioxidants, in contrast, are powerful reducing agents and have the function to scavenge free radicals (Burkitt, 2002). Subsequently,

antioxidants could compete with oxygen for quenching of the triplet photosensitizer (Jakus & Farkas, 2005) or can neutralise the generated reactive species by donating one of their own electrons, ending the "electron-stealing" reaction chain (Kelly, 2003). Thus it can counteract the effect of PDT. Several researchers demonstrated the protective effect of certain antioxidants against the oxidative stress induced by photosensitization (Chou & Khan, 1983; Perotti *et al.*, 2002). Although well-known for their free radical scavenging properties, some antioxidants can exhibit pro-oxidant activity, particularly in the presence of catalytic metals such as iron (Buettner & Jurkiewicz, 1996). During PDT, increasing antioxidant or transitional metal concentrations in cells shifts the reaction toward a Type I mechanism (Figure 5-1). Under hypoxic conditions production of $^1\text{O}_2$ decreases with the concurrent increase of the radical pathway (Type I) mediated by the antioxidant, producing photosensitizer anion (S^-) and antioxidant (Antiox^-) radicals.

Recently, antioxidant carrier photosensitizer (ACP) such as propyl gallate-substituted hematoporphyrins were used to photoinactivate both Gram-positive and Gram-negative bacteria. The ACP was more effective than free haematoporphyrins against MDR bacteria-*E. coli*, *A. baumannii* and *Staph. aureus* (Ashkenazi *et al.*, 2003a).

Both vitamins C and E are the most abundant antioxidants in the body and are thought to protect it against the oxidative stress caused by free radicals (Kelly, 2003). Yet both vitamins enhance lethal photosensitization (Girotti *et al.*, 1985; Melnikova *et al.*, 1999 & 2000). In this chapter, the photobactericidal effect of ICG was evaluated against *Staph. aureus* after the addition of either the water soluble Vit E-analog (TroloxTM) or ascorbic acid combined with ferrous sulphate.

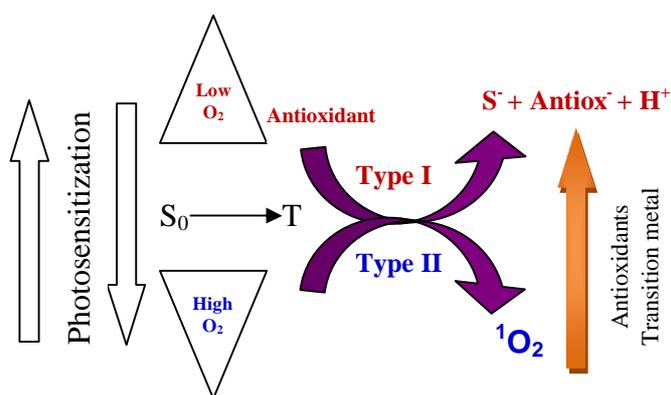


Figure 5-1: The possible mechanism of antioxidant-mediated free radical generation during photosensitization.

S_0 ground state photosensitizer, T triplet states, S^\bullet photosensitizer radical, Antiox $^\bullet$ Antioxidant radical
 Modified from Jakus & Farkas, (2005) Photochem. Photobiol. Sci . (4): 694 – 698

5.1.3 Thiocyanate salts

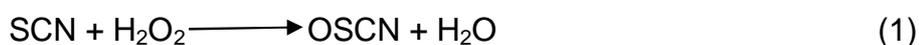
Sodium thiocyanate (NaSCN) is a pseudo-halide which can be oxidized by mammalian peroxidases forming products which exhibit powerful antibacterial activities (Ferrari *et al.*, 1997). This salt is one of the main sources of the thiocyanate anion. Thiocyanate salts are typically prepared by the reaction of cyanide with elemental sulphur. The pseudo-halide thiocyanate (SCN^-) has been shown to act as a substrate oxidized by myeloperoxidase (MPO) in the presence of H_2O_2 , consequently forming reactive species including hypothiocyanate (OSCN) and hypothiocyanous acid (HOSCN/ SCN^-) (Exner *et al.*, 2004). MPO is an enzyme secreted by activated phagocytes, which acts in cooperation with H_2O_2 during their respiratory burst (i.e. the rapid release of ROS). It is a crucial reaction that occurs in phagocytes to degrade internalized particles and bacteria (Senthilmohan & Kettle, 2006). Several studies have reported that SCN^- combined with H_2O_2 and catalyzed by lactoperoxidase or myeloperoxidase, form relatively stable chemical species with antimicrobial activity (Reiter *et al.*, 1976; Aune & Thomas, 1977; Marshall & Reiter, 1980). There are a number of likely cytotoxic products that may be formed. In studies of the chemical oxidation of thiocyanate by H_2O_2 , Wilson and Harris, (1960 & 1961) found sulphate, cyanate, carbonate, and ammonia as final products and postulated in the following equations:





During the photosensitization process, $^1\text{O}_2$ is typically generated by energy transfer from a photosensitizer in the relatively long-lived triplet excited state (T) to ground state oxygen (type II reaction). Reduced oxygen species (O_2^- , H_2O_2 , OH^-), on the other hand, are commonly generated by hydrogen or electron transfer from a substrate to the photosensitizer (type I reaction), followed by autoxidation of the latter (Girotti, 2001). Therefore H_2O_2 can be one of the free radicals produced during photosensitization of the bacteria. If NaSCN is present during irradiation of a photosensitizer, the thiocyanate ions (SCN^-) may be oxidized by the generated H_2O_2 , producing the oxidizing agent hypothiocyanite (OSCN^-). Carlsson *et al.*, (1984) demonstrated that lactoperoxidase in the presence of thiocyanate could protect bacteria and cultured mammalian cells from killing by H_2O_2 . However, thiocyanates potentiate the bactericidal effect in the absence of lactoperoxidase in a reaction between hypothiocyanite and H_2O_2 . In contrast, there are also several reports in which lactoperoxidase in the presence of thiocyanate had potentiated the bactericidal effect of hydrogen peroxide (Björck *et al.*, 1975; Reiter *et al.*, 1976; Marshall & Reiter, 1980; Thomas & Aune, 1978).

In biological systems, cyanide is immediately converted into SCN. SCN is found in almost all body fluids with levels, for instance, in blood plasma maximally amounting to 120 μM and up to 3-5 mM in saliva (Thomas & Fishman, 1986). Activated polymorphonuclear leukocytes (PMNs) are capable of converting SCN into the bactericidal OSCN which reacts with thiol groups according to (Hartmann *et al.*, 1996) (equations 1 and 2):



The reactions can be catalysed by MPO in blood plasma (Thomas & Fishman, 1986) or peroxidase in saliva (Thomas *et al.*, 1981), the products of this reaction have been found to contribute to the non-specific host defence mechanism against pathogens (Tenovuo *et al.*, 1982). Combination of SCN and H_2O_2 catalysed by peroxidase exhibited potent antimicrobial activities against a wide range of Gram-positive and negative bacteria (Oram & Reiter,

1966; Björck *et al.*, 1975; Reiter *et al.*, 1976; Marshall & Reiter, 1980). The antimicrobial activity of the peroxidase system is due to peroxidase-catalyzed oxidation of SCN⁻ either directly to hypothiocyanite ion (OSCN⁻) or to thiocyanogen (SCN₂) which hydrolyzes rapidly to yield hypothiocyanous acid (HOSCN) or OSCN⁻ (Thomas *et al.*, 1983). Such a reaction may be useful if applied in combination with lethal photosensitization. Thus, the effectiveness of NaSCN to enhance ICG-mediated photoinactivation of *Staph. aureus* was investigated.

5.2 Materials and methods

5.2.1 Target organisms and growth conditions

The organism used was: *Staph. aureus* NCTC 8325-4. The culture conditions have been described in Chapter 2, section 2.1.2.

5.2.2 Photosensitizer preparation and irradiation system

This was described in Chapter 2, section 2.1.4.

Irradiation was carried out using either the 0.5 W Ga-Al-As laser (Thor laser), or the 0.4 W diode laser (Ondine laser). Both lasers emit continuous wave laser light with a wavelength of 808 ± 5 nm.

5.2.3 Photosensitization of *Staph. aureus* with ICG-AuNPs mixture Gold Nanoparticles

An aqueous solution of 2 nm gold colloid was purchased from British Bio-cell International Ltd (Cardiff; UK); which contains 15×10^{13} particles/mL is approximately equivalent to 0.25 μ M. These were mixed with an equal volume of an aqueous solution of ICG (40 or 100 μ g/mL) and left at room temperature for 15 minutes.

5.2.3.1 Lethal photosensitization with ICG-AuNPs

50 μ L of the previously prepared ICG-AuNPs solution was added to 50 μ L of a suspension of *Staph. aureus* in PBS to give a final concentration of 10 or 25 μ g/mL for ICG and 0.0625 μ M for AuNPs. This was irradiated with NIR light from the Ondine laser for 5 minutes at a fluence rate of 0.3 W/cm² (L+S+G+) with a total light energy of 90 J/cm². Controls consisted of: (i) *Staph. aureus*

suspensions without ICG or AuNPs and kept in the dark (L-S-G-), (ii) *Staph. aureus* suspensions treated with AuNPs and kept in the dark (L-S-G+), (iii) *Staph. aureus* suspensions treated with ICG, without AuNPs (L-S+G-) or with AuNPs (L-S+G+) and kept in the dark, (vi) *Staph. aureus* suspensions without ICG but with AuNPs (L+S-G+) or without AuNPs (L+S-G-) and irradiated with NIR light, and (v) *Staph. aureus* suspensions treated with ICG but without AuNPs then irradiated with NIR light (L+S+G-). After irradiation, the number of surviving bacteria was determined by viable counting. The experiments were carried out in triplicate and on three separate occasions.

5.2.4 Enhancement of *Staph. aureus* photosensitization using antioxidants

The chosen antioxidants were Vitamin C (Vit C) combined with Ferrous Sulfate (FeSO_4) or TroloxTM, (a water-soluble vitamin E analog), both serve as standard antioxidants.

5.2.4.1 Vitamin C and Ferrous sulphate

Both L-Ascorbic acid sodium salt also known as Vit C sodium salt ($\text{C}_6\text{H}_7\text{NaO}_6$) (Figure 5-2) with a purity of $\geq 98\%$ and Ferrous Sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were purchased from Sigma-Aldrich, Inc (UK). A fresh aqueous solution of 200 μM Vit C sodium salt/40 μM FeSO_4 mixture was added to an equal volume of an aqueous solution of ICG (50 $\mu\text{g}/\text{mL}$) and kept in the dark at room temperature for 15 minutes.

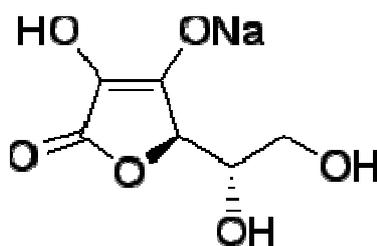


Figure 5-2: Chemical structure of Vit C sodium salt

5.2.4.2 Lethal photosensitization with ICG-Vit C and FeSO_4

In a 96-well micro-titre plate, 50 μL of the ICG-Vit C/ FeSO_4 solution was added to 50 μL of a suspension of *Staph. aureus* in PBS and this was irradiated with NIR light from the Ondine laser for 15 minutes at a fluence rate of $0.05 \text{ W}/\text{cm}^2$ and a total light energy of $45 \text{ J}/\text{cm}^2$ (L+S+VitC/ FeSO_4). Controls consisted of: (i) *Staph. aureus* suspensions without ICG-Vit C/ FeSO_4 kept in the dark (L-S-

VitC/FeSO₄-), (ii) *Staph. aureus* suspensions treated with Vit C/FeSO₄ solution and kept in the dark (L-S-VitC/FeSO₄+), (iii) *Staph. aureus* suspensions treated with ICG, without Vit C/FeSO₄ solution (L-S+ VitC/FeSO₄-) or with Vit C/FeSO₄ solution (L-S+ VitC/FeSO₄+ and kept in the dark, (vi) *Staph. aureus* suspensions without ICG but with Vit C/FeSO₄ solution (L+S-VitC/FeSO₄+ or without Vit C/FeSO₄ solution (L+S- VitC/FeSO₄-) and irradiated with NIR light, and (v) *Staph. aureus* suspensions treated with ICG but without Vit C/FeSO₄ solution and irradiated with NIR light (L+S+ VitC/FeSO₄-). After irradiation, the number of surviving bacteria was determined by viable counting. The experiments were carried out in quadruplicate and on two separate occasions.

5.2.4.3 Vitamin E analog Trolox™

6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid or Trolox™ (C₁₄H₁₈O₄) (Figure 5-3) was purchased at 97% purity from Sigma-Aldrich, Inc (UK). A fresh aqueous solution of Trolox™ was prepared immediately before each experiment.

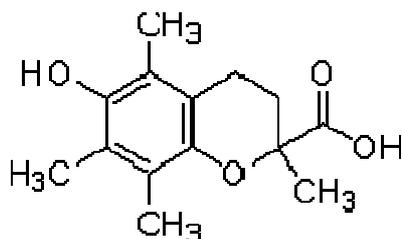


Figure 5-3: Chemical structure of Trolox™

5.2.4.4 Lethal photosensitization with ICG-Trolox™ mixture

Staph. aureus suspension was treated with a final concentration of 2 mM Trolox™ and 25 µg/mL ICG. Aliquots of 100 µL in at least triplicate wells were exposed to NIR light from the Thor laser for 1 minute at a fluence rate of 1.37 W/cm² and a total light energy of 82 J/cm² (L+S+Trolox™+), with stirring. A number of controls were included, these consisted of: (i) bacterial suspensions treated with ICG solution alone and irradiated with the same light energy (L+S+Trolox™-) (ii) bacterial suspensions treated with Trolox™ solution alone without ICG and kept in the dark (L-S-Trolox™+), (iii) bacterial suspensions neither received Trolox™ solution nor ICG and kept in the dark (L-S-Trolox™-), (iv) bacterial suspensions received both ICG and Trolox™ solutions and kept in the dark (L-S+Trolox™+), (v) bacterial suspensions received ICG without

TroloxTM solution and kept in the dark (L-S+TroloxTM-), (vi) bacterial suspensions irradiated in the presence of TroloxTM solution alone without ICG (L+S-TroloxTM+), and (vii) bacterial suspensions neither received TroloxTM solution nor ICG and irradiated with NIR light (L+S-TroloxTM-). After irradiation or incubation in the dark, samples were serially diluted 1 in 10 in PBS and plated in duplicate on blood agar and the number of surviving bacteria was determined by viable counting. The experiments were carried out in quadruplicate and on three separate occasions.

5.2.5 Enhancement of *Staph. aureus* photosensitization using thiocyanate

5.2.5.1 Thiocyanate salts

Sodium thiocyanate anhydrous (NaSCN) was purchased from Fisher Scientific UK Ltd (Loughborough, UK), with a purity of $\geq 98\%$. A fresh aqueous solution of 20 mM NaSCN was prepared at the time of each experiment.

5.2.5.2 Lethal photosensitization with ICG-NaSCN mixture

ICG was used at a final concentration of 25 $\mu\text{g}/\text{mL}$. 50 μL of a suspension of *Staph. aureus* in PBS was treated with an equal volume to give a final concentration of 25 $\mu\text{g}/\text{mL}$ ICG and 10 mM NaSCN. This was irradiated with NIR light from the Ondine laser for 3 minutes at a fluence rate of 0.3 W/cm^2 (L+S+NaSCN+). Controls consisted of: (i) *Staph. aureus* suspensions treated with ICG but without NaSCN then irradiated with NIR light (L+S+NaSCN -), (ii) *Staph. aureus* suspensions without ICG or NaSCN and kept in the dark (L-S-NaSCN -), (iii) *Staph. aureus* suspensions treated with NaSCN and kept in the dark (L-S-NaSCN+), (iv) *Staph. aureus* suspensions treated with ICG, in the absence of NaSCN (L-S+ NaSCN-) or in the presence of NaSCN (L-S+NaSCN+) and kept in the dark, (v) *Staph. aureus* suspensions neither receive ICG nor NaSCN but exposed to NIR light (L+S-NaSCN-) and *Staph. aureus* suspensions received NaSCN alone and irradiated with NIR light (L+S-NaSCN+). After irradiation, the number of surviving bacteria was determined by viable counting. The experiments were carried out in quadruplicate and on two separate occasions.

5.3 Statistical analysis

Comparisons between the experimental and the control groups were carried out using the Kruskal Wallis test followed by the Mann-Whitney *U* test to determine the variable that was different. Mainly, to determine the efficiency of each chemical (X) (AuNPs, antioxidants, or thiocyanate salts) added to ICG in enhancing its bactericidal effect, the Mann-Whitney *U* test was used to compare the number of survivors from the samples received both the enhancing chemical and ICG (L+S+X+) with the number of survivors from samples treated with ICG alone (L+S+X-). The number of survivors from all other samples was also compared to the number of survivors from control samples (L-S-). The level of significance was set at $P \leq 0.05$ throughout (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ***** $P < 0.00001$). All statistical analyses were carried out using the SPSS statistical package (version 12.0, SPSS Inc., Chicago, IL, USA).

5.4 Results

5.4.1 The effect of ICG-AuNPs on lethal photosensitization

The effect on the viability of *Staph. aureus* sensitized with 10 or 25 $\mu\text{g/mL}$ ICG, after exposure to NIR laser light for 5 min, at fluence rate of 0.3 W/cm^2 , in the presence and absence of 2 nm AuNPs is shown in Figure 5-4. In the absence of AuNPs, the ICG at a concentration of 10 $\mu\text{g/mL}$ achieved a $3.3 \log_{10}$ reduction in the viable count of a suspension of *Staph. aureus* containing 1.4×10^7 CFU/mL (mean=median). When a final concentration of 0.0625 μM AuNPs was combined with 10 $\mu\text{g/mL}$ ICG, there was a further $1.7 \log_{10}$ decrease in the viable count of the bacteria. This increased kill was found to be significant ($P=0.003$). A concentration of 25 $\mu\text{g/mL}$ ICG without AuNPs resulted in a kill of $4.4 \log_{10}$. As the ICG concentration increased to 25 $\mu\text{g/mL}$ in AuNPs-ICG mixture, the extent of the increased kill was only $0.5 \log_{10}$. However, this kill was not significantly different compared with that achieved when the same concentration of ICG was used in the absence of the AuNPs ($P=0.19$). The 2 nm AuNPs alone when irradiated did not achieve significant killing of the bacteria. Positive and negative controls showed no significant changes in cell numbers throughout the course of these experiments.

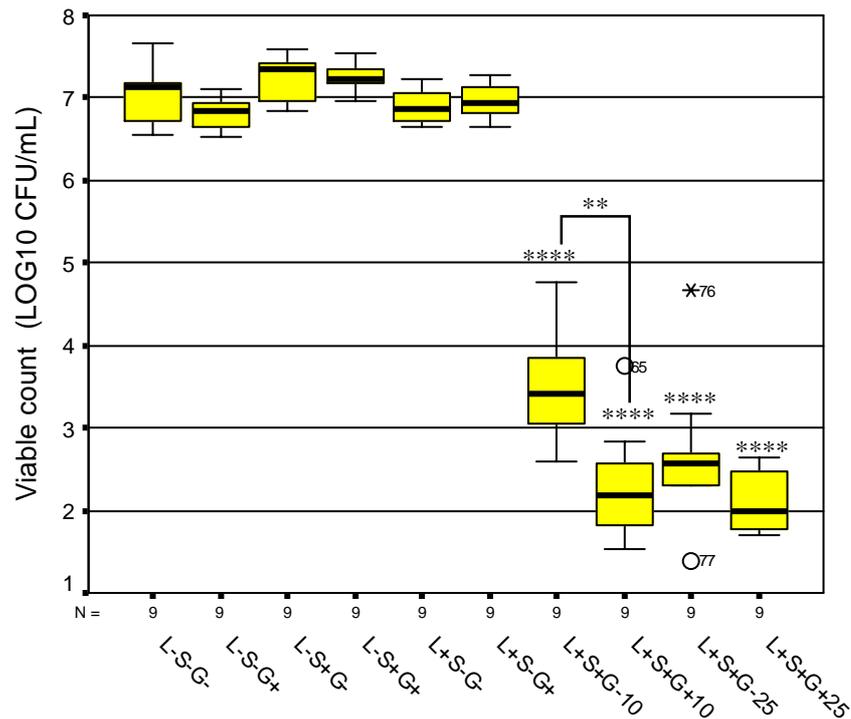


Figure 5-4: Lethal photosensitization of *Staph. aureus* by 10 and 25 µg/mL ICG in the absence (G-) and presence (G+) of 2 nm diameter AuNPs at a concentration of 0.0625 µM. The thick horizontal lines represent median values of the log₁₀ units of the viable counts. The bottom and top of the box are the 25% and 75% quartiles respectively. The error bars represent 1.5 times the interquartile range. Any outliers are marked with a circle and extreme cases with an asterisk.

5.4.2 The effect of antioxidants on lethal photosensitization

5.4.2.1 The effect of Vit C and FeSO₄ on lethal photosensitization

An enhanced kill of *Staph. aureus* was observed upon addition of a Vit C/FeSO₄ mixture to ICG during photosensitization process (Figure 5-5). In the absence of the Vit C/FeSO₄ mixture, a 15 min exposure of the organism, at a concentration of 3.4×10^7 CFU/mL, to a low fluence rate of NIR laser light in the presence of 25 µg/mL ICG resulted in a 4.0 log₁₀ reduction in the viable count. However, when Vit C/FeSO₄ mixture was added, the reduction in viable count amounted to 5.13 log₁₀ units, an enhanced kill of 1.13 log₁₀. Irradiation of *Staph. aureus* with NIR laser light alone at a fluence rate of 0.05 W/cm² and a light energy of 45 J/cm² resulted in a kill of around 0.3 log₁₀. This kill was significant ($P < 0.01$) when compared to the control in the dark which received neither ICG nor Vit C/FeSO₄ (L-S-PBS). None of the other controls showed

any significant change in cell numbers throughout the course of these experiments.

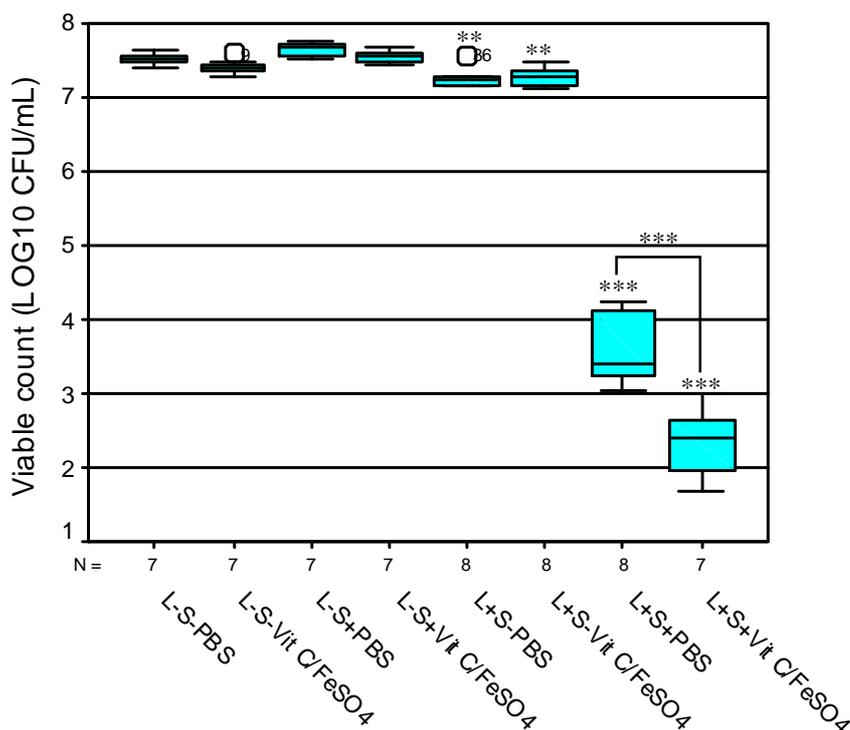


Figure 5-5: Lethal photosensitization of *Staph. aureus* by 25 $\mu\text{g/mL}$ ICG in PBS (control) or in the presence of a mixture of 100 μM vitamin C/20 μM FeSO_4 (Vit C/ FeSO_4). The thick horizontal lines represent median values of the \log_{10} units of the viable counts. The bottom and top of the box are the 25% and 75% quartiles respectively. The error bars represent 1.5 times the interquartile range. Any outliers are marked with a circle.

5.4.2.2 The effect of TroloxTM on lethal photosensitization

The effect of ICG-TroloxTM on photosensitization of *Staph. aureus* is shown in Figure 5-6. Neither 25 $\mu\text{g/mL}$ ICG nor TroloxTM solution alone was cytotoxic in the dark; addition of 2 mM TroloxTM to ICG induced dark cytotoxicity in *Staph. aureus*. Approximately one \log_{10} reduction in the viable count was observed. This reduction in the viable count was found to be significant ($P < 0.00001$). Yet, this enhancement in the killing was not as great as that observed after the irradiation with NIR laser for 1 minute at a fluence rate of 1.37 W/cm^2 and light energy of 82 J/cm^2 . As the combination of 25 $\mu\text{g/mL}$ and 2 mM TroloxTM resulted in a complete kill of a suspension of *Staph. aureus* containing approximately 2×10^6 CFU/mL, which amounted to a 6.3 \log_{10} reduction in the viable count. A reduced kill of 3.3 \log_{10} units was achieved with ICG alone.

The enhanced kill of 3.0 log₁₀ units was found to be significant (P = 0.00001). None of the other controls showed any significant change in cell numbers throughout the course of these experiments.

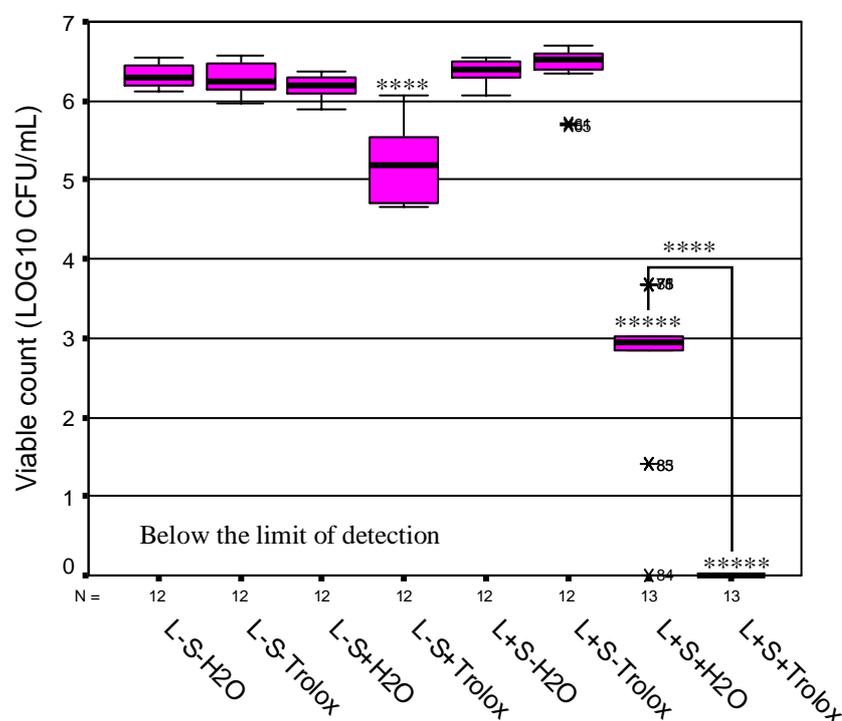


Figure 5-6: Lethal photosensitization of *Staph. aureus* by 25 µg/mL ICG in H₂O (control) or in the presence of 2 mM Trolox™. The thick horizontal lines represent median values of the log₁₀ units of the viable counts. The bottom and top of the box are the 25% and 75% quartiles respectively. The error bars represent 1.5 times the interquartile range. Any extremes are marked with an asterisk.

5.4.3 Sodium thiocyanate

Figure 5-7 illustrates the effect of ICG-NaSCN on the photosensitization of *Staph. aureus*. NaSCN alone or in combination with 25 µg/mL ICG had no dark cytotoxicity on *Staph. aureus*. Upon irradiation, however, there was little enhancement of the photosensitization, this amounted to a 0.6 log₁₀ kill compared to that achieved when ICG was used in the absence of the NaSCN solution. This difference was not significant (P= 0.33). Exposure of 25 µg/mL ICG alone to 3 minutes of NIR laser light with a total energy of 54 J/cm², achieved a kill of 3.7 log₁₀. While addition of NaSCN to the same concentration of ICG and exposure to an equal light dose resulted in a slightly higher kill of 4.3 log₁₀. The NIR laser light alone in the presence or absence of NaSCN resulted in a very small kill of 0.4 log₁₀. This kill was found to be significant (P=

0.0002 in both cases) compared to the control which was kept in the dark and neither received ICG nor NaSCN (L-S-NaSCN-). None of the other controls showed any significant change in cell numbers throughout the course of these experiments.

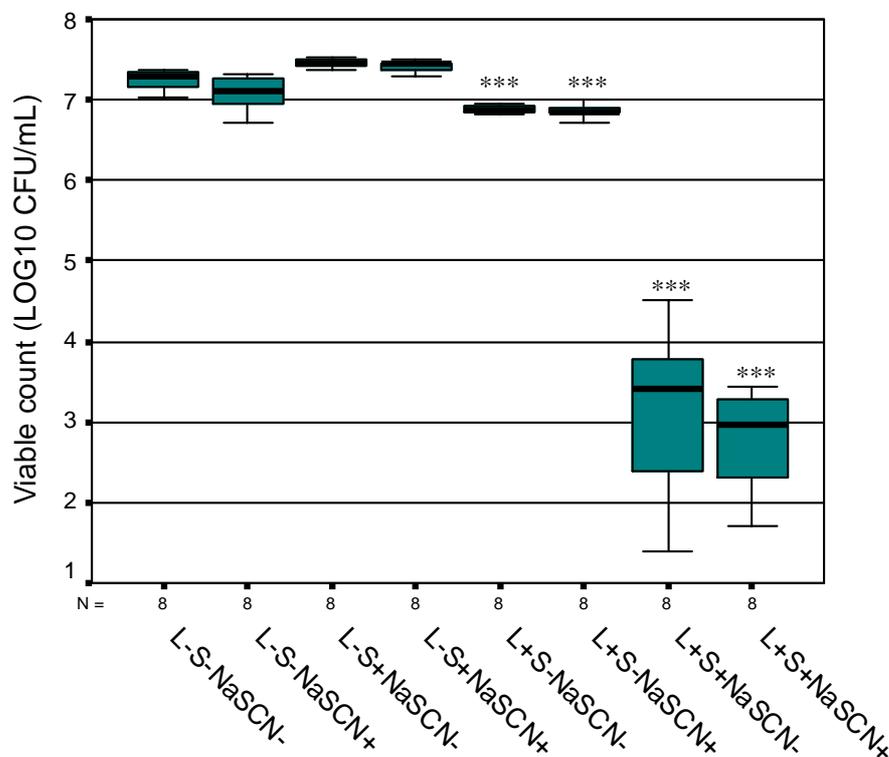


Figure 5-7: Lethal photosensitization of *Staph. aureus* by 25 μ g/mL ICG in the absence (NaSCN-) and presence (NaSCN+) of 10 mM NaSCN. The horizontal lines represent median values of the log₁₀ units of the viable counts. The thick horizontal lines represent median values of the log₁₀ units of the viable counts. The bottom and top of the box are the 25% and 75% quartiles respectively. The error bars represent 1.5 times the interquartile range.

5.5 Discussion

Despite the fact that ICG has a low quantum yields of singlet oxygen generation due to internal conversion (Kassab, 2002), the results presented in Chapters 3 and 4 have shown it to be an effective light-activated antimicrobial agent against a wide range of bacteria. Therefore the quantum yields of triplet formation for ICG in the range from 11% to 20% are sufficiently high to provide the singlet oxygen needed for cell destruction during lethal photosensitization (Reindl *et al.*, 1997).

Several researchers have reported the optical instability of ICG in physiologically relevant conditions in solutions such as water, salt solutions, plasma and blood (Gathje *et al.*, 1970; Landsman *et al.*, 1976; Simmons & Shephard, 1971). Under such conditions, oxidation and dimerization degrade the original molecule, resulting in decreased absorption, reduced fluorescence, and variability in the maximum absorption wavelength (Saxena *et al.*, 2003). High concentration of ICG in plasma and aqueous solutions are stable up to 8 h. Overtime, the optical density of ICG solutions reduces, even in the dark and within a week a new absorption maximum appears at $\lambda=900$ nm, possibly due to aggregate formation (Landsman *et al.*, 1976). Therefore, the novel applications of ICG in the PDT field are held back due to aqueous-instability, photo-degradation and thermal-degradation of the dye (Saxena *et al.*, 2004). However, by using carefully chosen macromolecular additives, the stability of these aqueous dye solutions may be enhanced significantly (Rajagopalan *et al.*, 2000). For example, the entrapment of ICG into nanoparticles reduced the extent of degradation of ICG in aqueous media. The extent of degradation was $60.5\pm 3.2\%$ for ICG loaded nanoparticles compared to $97.8\pm 0.8\%$ for free ICG solution over a period of four days. This clearly indicates the efficient stabilization of ICG provided by the nanoparticle preparation in the aqueous media (Saxena *et al.*, 2004). Thus, this chapter was concerned with improving the effectiveness of ICG in the lethal photosensitization of the important human pathogen—*Staph. aureus*—by means of AuNPs, antioxidants or thiocyanates.

5.5.1 AuNPs and lethal photosensitization

The results of the present study have demonstrated that the non-covalent interaction of the 2 nm AuNPs with ICG can significantly enhance the efficacy of ICG-mediated lethal photosensitization of *Staph. aureus*.

AuNPs have been covalently linked to PSs to increase their efficacy in lethal photosensitization of both mammalian (Wieder *et al.*, 2006) and bacterial cells (Gil-Tomás *et al.*, 2007). Wieder *et al.*, (2006) found that the photosensitization efficiency of HeLa cells (a cervical cancer cell line) by phthalocyanine-AuNPs conjugates was twice that obtained using the free phthalocyanine derivative. Gil-Tomás *et al.*, (2007) reported that *Staph. aureus* kills achieved by the TBO–tiopronin–gold conjugate were $2 \log_{10}$ units greater than those found using TBO

when each was used at a concentration of 2.0 mM. In this study, however, it was demonstrated that the light-dependent antibacterial activity of ICG can be enhanced by AuNPs even when these are not covalently linked. In agreement with these results, Narband *et al.*, (2008) showed that covalent linkage of the TBO and AuNPs was not essential to achieve an enhanced photosensitization effect on *Staph. aureus*. This was attributed to the association of the positively-charged TBO with the negatively-charged AuNPs. The surface of AuNPs can be derivatized using a variety of targeting molecules and ligands to achieve stability and specificity (Daniel & Astruc, 2004). Sulphur has a strong affinity for AuNPs. Any molecule with a sulphur atom can spontaneously assemble into stable and highly organized layers on the surfaces of AuNPs to form more stable compounds (Skrabalak *et al.*, 2007). In this study, the negatively charged dye ICG was used, each molecule of which possesses a sulphur trioxide (SO_3^-) group. Therefore ICG can assemble on the AuNPs surfaces through the SO_3^- group, consequently, increasing van der Waals attractive forces between ICG molecules and the AuNPs.

It was demonstrated that the increase in the NP size decreased photodynamic activity *in vivo* (Vargas *et al.*, 2008), thus herein 2 nm AuNPs were used. Such small size AuNPs have no effect on the absorbance of the PS because of the absence of SPR peak absorbance (Narband *et al.*, 2008). In the current study, the enhancement of lethal photosensitization was mainly dependent on the ICG concentration. At a low concentration of 10 $\mu\text{g}/\text{mL}$ ICG, enhancement of lethal photosensitization was evident. Such a low concentration of ICG- AuNPs achieved a maximum of 1.7 \log_{10} greater kill than the ICG alone. When a higher ICG concentration of 25 $\mu\text{g}/\text{mL}$ was used in combination with the AuNPs, only a 0.5 \log_{10} increase in the kill was observed compared to free ICG in the absence of AuNPs. These observations are consistent with a previous study which has shown that the enhancement of TBO-mediated lethal photosensitization by means of 2-3 nm AuNPs conjugate was mainly dependent on the concentration of the dye (Gil-Tomás *et al.*, 2007). In another study, the presence of 2 nm AuNPs in close proximity to TBO resulted in less than a one \log_{10} greater *Staph. aureus* kill than that found using TBO alone. Those kills were evident only at 20 μM TBO but not when either 10 μM or 50 μM TBO was used (Narband *et al.*, 2008).

ICG is one of the least toxic PS administered to humans (Frangioni, 2003), yet it is prone to photobleaching, solvatochromic effects, and nonspecific quenching, all of which limit its utility as a PS in PDT (Yaseen *et al.*, 2007; Yu *et al.*, 2007). It was reported that encapsulating ICG in biodegradable polymer nanoparticles provided efficient aqueous-stability, photo-stability and thermal stability to this dye (Saxena *et al.*, 2004; Rodriguez *et al.*, 2008) and enhanced its fluorescence ability (Gomes *et al.*, 2006; Kim *et al.*, 2007). Also, linking this dye to a metal nanoparticle such as silver (Geddes *et al.*, 2003b) or gold (Geddes *et al.*, 2003a) enhanced drastically its stability and its fluorescence (Tam *et al.*, 2007). Herein, the presence of the AuNPs in close proximity to ICG resulted in a 1.7 log₁₀ increase of the number of *Staph. aureus* killed following a short irradiation period of 5 minutes from a NIR laser. The enhanced kill may be attributed to the decreased degradation, the increased photostability and the slower photobleaching rate of ICG when ICG molecules present near gold colloids (Geddes *et al.*, 2003a) and an increase in the extinction coefficient when bound to nanoparticles (Malicka *et al.*, 2003; Gil-Tomás *et al.*, 2007). Malicka *et al.*, (2003) showed that ICG had a 20-fold increase in extinction coefficient when directly bound to nanoscale metallic islands of silver. This was attributed to the increased rates of radiative decay resulting from the interaction of the excited fluorophore with the freely mobile electrons on the metal.

No killing of the bacteria was observed upon irradiation of the AuNPs alone. These results are in keeping with those of previous studies demonstrating that the organism could not be killed upon irradiation of AuNPs (Gil-Tomás *et al.*, 2007; Narband *et al.*, 2008). In contrast, Zharov *et al.*, (2006) reported selective killing of *Staph. aureus* by targeting the bacterial surface using 10-, 20- and 40-nm AuNPs conjugated with anti-protein A antibodies. This effect was not observed using the unconjugated gold colloid.

In conclusion, non-covalent interaction between the 2 nm AuNPs and 10 µg/mL ICG resulted in a 47-fold increase in the number of *Staph. aureus* cells killed upon irradiation with NIR laser light compared to those achieved with free ICG.

5.5.2 Antioxidants and lethal photosensitization

Antioxidants are primarily reducing agents prone to scavenge and neutralise ROS in one way or another. These molecules under certain circumstances can protect or sensitise cells during the photosensitization process (Kramarenko *et al.*, 2006). The effect does not appear to depend on the nature of the photosensitizer, but rather on the structure of the antioxidant and essentially on the conditions of its action (Jakus & Farkas, 2005). Some antioxidants like ascorbic acid, α -tocopherol or butyl-4-hydroxyanisole, when added to the cells at adequate concentrations and appropriate timing, enhanced the photosensitization-induced cytotoxicity. The presence of transition metals and appropriate timing of antioxidant administration may also play an important role in increasing the efficacy of PDT (Jakus & Farkas, 2005). The results presented herein demonstrated that the tested antioxidants (Vit C/FeSO₄ or TroloxTM) enhanced the bactericidal effect of ICG against *Staph. aureus* after activation with NIR laser light.

5.5.2.1 Vitamin C and transition metals

Several research groups have reported the enhancing effect of the photosensitization process by certain antioxidant molecules, such as α -tocopherol, (Melnikova *et al.*, 1999), the water soluble Vit E-analog TroloxTM (Melnikova *et al.*, 2000), ascorbate (Girotti *et al.*, 1985; Rosenthal & Benhur, 1992; Buettner *et al.*, 1993; Kelley *et al.*, 1997) and 3(2)-tert-butyl-4-hydroxyanisole (BHA) (Shevchuk *et al.*, 1998). The effect was observed using different photosensitizers and different tumour models. Girotti and colleagues have shown that photodamage to lipids and erythrocyte membranes can be enhanced by the addition of ascorbate to uroporphyrin-mediated photosensitization (Girotti *et al.*, 1987). Rosenthal and Benhur, (1992) reported an increase in the rate of photohaemolysis of human red blood cells sensitised by chloroaluminium phthalocyanine sulfonate by ascorbate, with or without added iron salt. Therefore, the topical application of clinically approved antioxidants combined with transition metals may be a simple and cheap method to improve several PDT protocols. In the current study, when a Vit C/FeSO₄ solution was added to 25 μ g/mL ICG, enhanced kills of one log₁₀ of *Staph. aureus* were observed compared to those treated with ICG alone. Irradiation of ICG in the absence of the Vit C/FeSO₄, with 45 J/cm² at a low

fluence rate of 0.05 W/cm² resulted in a 4.0 log₁₀ reduction in the viable count. However, upon addition of Vit C/FeSO₄ solution to ICG immediately before irradiation, a 5.0 log₁₀ reduction in the viable count was observed. Neither Vit C/FeSO₄ solution alone nor mixed with ICG caused any dark toxicity to *Staph. aureus*.

It was postulated that the combination of the metal ion Fe (II) with 100 μM ascorbic acid increased the cytotoxic effect of photofrin-mediated photosensitization, on L1210 and SCC-25 tumour cells, through elevated lipid hydroperoxide formation (Buettner *et al.*, 1993). The author explained this by the reaction of ¹O₂ generated in the course of a Type II reaction with membrane lipids forming lipid hydroperoxides. Afterwards, the transition metals Fe⁺ catalyze lipid hydroperoxides producing highly oxidising cytotoxic free radicals (lipid alkoxy radicals). Simultaneously, ascorbate reduces ferric to ferrous iron, further augmenting lipid peroxidation (Buettner, 1986). Thus, the one log₁₀ enhanced kill achieved in this study may be attributed to the presence of ascorbic acid which reduces Fe (III) to Fe (II), by donating an electron to the formed peroxides during the course of photosensitization. Thereby, this helps initiating free radical chain reactions which in turn can enhance the photosensitization-induced cytotoxicity. Herein, ascorbic acid and Fe ions act as pro-oxidants. Kelley *et al.*, (1997) also, described that the increase in lipid peroxidation was two-fold when the photosensitizer was administered to cells before photosensitization and five-fold when administered after 5 min of illumination of SCC-25 cells. In the present study, the ascorbate and Fe (II) were applied to *Staph. aureus* cells immediately before irradiation. Over fourteen-fold enhancement in the number of *Staph. aureus* killed was observed. Lately, Kramarenko *et al.*, (2006) concluded that ascorbate increases hydrogen peroxide production by verteporfin and light. This hydrogen peroxide activates myeloperoxidase, producing toxic oxidants. This observations support the hypothesis that ascorbate assists the shift to a type I reaction during the course of the photosensitization process.

5.5.2.2 Trolox™

The water-soluble analog of Vit E, Trolox™ has been reported to augment the cytotoxic effect mediated by lethal photosensitization. Overall, compounds such as carotenoids, tocopherols or ascorbate derivatives can exhibit an anti-oxidant or pro-oxidant feature depending on the redox potential of the individual molecule, the inorganic chemistry of the cell and the cellular oxygen environment (Schwartz, 1996). Antioxidant molecules can act as substrates for photosensitizer-mediated reactions, producing anti-oxidant radicals in the process of either radical scavenging reaction (Bowry & Stocker, 1993) or impulsive auto-oxidation (Jakus & Farkas, 2005). Anti-oxidants can also act as pro-oxidants during PDT, especially under hypoxic conditions. The benefits of this enhancement approach will be invaluable and will improve the effectiveness of PDT if appropriate *in vivo*. The results herein, demonstrated that the ICG-Trolox™ complex enhanced remarkably the sensitization of *Staph. aureus* after a very short irradiation time of 1 minute.

Melnikova *et al.*, (1999) have shown in the colon carcinoma HT29 cell line that 0.33–1 mM of α -tocopherol can enhance the PDT activity of meta-tetra(hydroxyphenyl) chlorin (mTHPC) in cell culture, while lower concentrations of the antioxidant (0.001–0.1 mM) had no significant effect in the same system. Under the same conditions, α -tocopherol did not affect mTHPC-sensitised photo-killing of normal fibroblasts. A similar effect was observed *in vivo*, when Trolox™ was injected into nude mice bearing HT29 human adenocarcinoma xenografts before the administration of mTHPC-PDT (Melnikova *et al.*, 2000). Trolox™ had to be present in the photochemical stage to improve tumour response to PDT since its injection after irradiation was ineffective. The data presented herein showed that a complete eradication of *Staph. aureus* cells was achieved when 2 mM Trolox™ added to 25 μ g/mL ICG and irradiated with 82 J/cm². Trolox™ solution at the same concentration was not cytotoxic to the bacterial cells either in the dark or when irradiated with NIR laser light. However, addition of 2 mM Trolox™ to ICG triggered some dark toxicity, a one log₁₀ reduction in the viable count, albeit not as great as that achieved after NIR light irradiation (i.e. a kill of 6.3 log₁₀). On the other hand, photo-activated ICG alone resulted in a 3.3 log₁₀ reduction in the viable count. Jakus & Farkas, (2005) found that Trolox™ enhanced the efficacy of S180

murine cell photo-inactivation using pheophorbide a-photosensitization at concentrations as low as 1–100 μM . The authors suggested that the effect of the antioxidant on lethal photosensitization strongly depended on the nature of the added molecule.

TroloxTM enhanced *Staph. aureus* kills by 3.0 \log_{10} units, approximately over a thousand-fold enhancement of the cytotoxic effect. The effect can be better explained as a shift toward radical processes owing to oxygen depletion in the cells. Then, toxicity may result from the reaction of the ICG anion radical with residual oxygen, leading to formation of the superoxide anion radical, which in turn could produce cytotoxic H_2O_2 and $\cdot\text{OH}$ species, and/or from the TroloxTM radical formed concurrently. As a result, the TroloxTM-mediated radical pathway can work alongside with $^1\text{O}_2$ while the oxygen concentration is decreased in the course of photosensitization (Melnikova *et al.*, 2000). Laser flash photolysis measurements demonstrated that free radicals were formed in a deoxygenated methanolic solution of mTHPC in the presence of TroloxTM suggesting that a shift from a Type II reaction toward radical producing (Type I) processes was occurring, probably due to oxygen depletion in tumours. Then, the phototoxicity of mTHPC may be derived from the reaction of the photosensitizer radical ($\text{mTHPC}^{\cdot-}$) with oxygen, leading to generation of $^1\text{O}_2$, which could produce further ROS, and simultaneously form the TroloxTM radical ($\text{Trol}\cdot = \text{Rad}\cdot$) (Melnikova *et al.*, 2000).

Establishment of antioxidant carrier photosensitizers is a new idea showing promising results in combating bacterial infections. Recently, ACPs such as propyl gallate-substituted hematoporphyrins were tested for their bactericidal effect against a wide range of bacteria. This compound has an enhanced antioxidant capacity when compared to photofrin (Jakus & Farkas, 2005). It also proved to be an effective PS against MDR-bacteria, especially against the Gram-positive bacterium *Staph. aureus* (Ashkenazi *et al.*, 2003a). The enhanced bactericidal effect was attributed in this case to specific damage to different bacterial cell membrane ion pumps. Still, a more detailed mechanism of action of ACP molecules remains to be clarified (Ashkenazi *et al.*, 2003a).

In conclusion, the presence of TroloxTM at 2 mM concentration during ICG photo-activation had a strong enhancing effect on the lethal photosensitization of *Staph. aureus*. Upon adding TroloxTM, a 1000-fold enhancement in ICG-photosensitization efficiency was achieved. Such an enhancement, if applicable *in vivo*, would improve the efficacy of antimicrobial photodynamic therapy.

5.5.3 Sodium thiocyanate and lethal photosensitization

In the human body, SCN substrates are secreted in saliva, tears, and blood plasma (van Haeringen *et al.*, 1979; Tenovuo *et al.*, 1982; Thomas & Fishman, 1986). It was also found in the milk (Fweja *et al.*, 2007). The peroxidase present in exocrine secretions catalyzes oxidation of SCN⁻ producing the bactericidal agent OSCN⁻ (Thomas *et al.*, 1981). This is a naturally occurring antimicrobial system which plays a role in non-specific host defence against microbes (Hogg & Jago, 1970). Yet, a few reports demonstrated that the addition of lactoperoxidase to SCN protected different types of bacteria from the cytotoxic effect of H₂O₂ (Carlsson, 1980; Adamson & Carlsson, 1982; Carlsson *et al.*, 1984). The data shown herein, demonstrated that addition of 10 mM NaSCN to ICG prior to irradiation with NIR laser light (uncatalysed reaction) resulted in a 4-fold enhancement in the number of *Staph. aureus* killed.

Non-catalytic reaction of NaSCN-ICG combined with NIR laser light result in 4-orders more reduction in the viable count of *Staph. aureus*. Exposure of free ICG to 54 J/cm², achieved a kill of 3.7 log₁₀. While addition of NaSCN to the same concentration of ICG, exposed to an equal light dose resulted in a slightly higher kill of 4.3 log₁₀. Irradiation of ICG in the presence of NaSCN, achieved a small enhancement amounting to a 0.6 log₁₀ reduction in the viable count compared to that achieved when ICG was used in the absence of NaSCN, albeit this 4-fold difference was not significant. The enhancement of *Staph. aureus* killing may be attributed to the oxidization of SCN⁻ by the generated H₂O₂ during the photo-inactivation process, to yield the antimicrobial oxidizing radicals OSCN and HOSCN (Carlsson *et al.*, 1984). Yet the produced H₂O₂ via a Type I-reaction may be not enough to achieve a greater level of SCN

substrate oxidization. As a result, the OSCN produced resulted only in the observed 4-fold enhancement of ICG-mediated photosensitization.

The addition of NaSCN to ICG enhanced killing of *Staph. aureus* by 0.6 log₁₀, due to the formation of hypothiocyanite radicals. The antimicrobial activity of OSCN⁻ was attributed to its oxidative capability. Hence, OSCN⁻ oxidizes mainly the thiol groups present in bacterial membranes, with consequent disruption of their functions (Aune & Thomas, 1977 & 1978). Later on, Thomas & Aune, (1978) correlated thiol oxidation with an inhibition of respiration in *E. coli*. While Hoogendoorn *et al.*, (1977) also found that oxidation of thiol groups by OSCN resulted in the inhibition of respiration in *Streptococcus mutans*. Recently, Exner *et al.*, (2004) showed that the presence of SCN⁻ enhanced lipid peroxidation leading to oxidative damage to various bacterial components especially the cell membrane.

As only a limited enhancement of ICG-photosensitization in the presence of NaSCN was achieved, further research will be needed to optimize the enhancement of bacterial photosensitization using SCN ions.

5.6 Conclusion

To summarize, in this chapter, several approaches have been considered to enhance the efficiency of *Staph. aureus* lethal photosensitization. First, AuNPs enhance the photo-bactericidal capability of 10 µg/mL ICG by 47-fold more than the free ICG. Secondly, antioxidants such as Vits C and E which are clinically approved drugs can be a cheap approach to improve the efficiency of antimicrobial-PDT. Vit C combined with Fe (II) resulted in a 14-fold enhancement, while TroloxTM resulted in a 1000-fold increase in the number of *Staph. aureus* killed. Finally, however, the use of NaSCN resulted in only a small enhancement of *Staph. aureus* photo-inactivation (approximately a 4-fold enhancement). The first two approaches (AuNPs and antioxidants) were significantly effective in increasing photosensitization-mediated bacterial killing and show potential as antimicrobial-PDT enhancers. The latter approach needs further optimization to prove effective as an enhancer of the photo-inactivation of *Staph. aureus*.

Chapter 6

**The effect of light and the light-
activated antimicrobial agent on
biofilms**

6.1 Introduction

In the previous chapters it was established that ICG, in conjunction with NIR laser light, is an effective light activated-antimicrobial agent which acts against a wide range of planktonic bacterial cells responsible for wound infections. However, many bacteria responsible for diseases in humans exist in heterogeneous communities called biofilms. In fact, according to the National Institutes of Health (NIH), biofilms are responsible for more than 80% of infectious diseases in the body (Davies, 2003). As discussed in the introduction, the inability of wounds to heal is likely to be attributed to the presence of bacteria in the form of mixed communities in the wound bed. When present in a biofilm, the susceptibility of individual organisms to all types of antimicrobial agents is reduced. This applies to biocides such as iodine and hydrogen peroxide as well as to antibiotics, which act on specific targets. Several mechanisms are proposed to the reduced susceptibility of bacteria in biofilms to antimicrobial agents compared to planktonic cultures. These include incomplete penetration of the antimicrobial agent into the extracellular matrix, the slow growth of bacteria in biofilms due to the low nutrient environment, the environmental conditions of the biofilm itself, such as a low pH, which can affect the activity of antimicrobial agents; the expression of a biofilm specific phenotype and quorum sensing. Therefore it was very important to establish whether ICG would be effective in the photosensitization of *P. aeruginosa* and *Staph. aureus* when they were in the form of a biofilm.

Biofilms are microbial assemblages that are sheathed in a matrix of EPS (Hall-Stoodley *et al.*, 2004). This insoluble gelatinous matrix allows the growing biofilm to develop a complex, three-dimensional structure that secures long term survival of the bacteria and renders the individual bacterial cells less susceptible to antimicrobial agents (Bryers, 2008). As a result, biofilms are responsible for a large number of persistent and widespread human infections (Bryers, 2008). More importantly, the biofilm community can disseminate through detachment of small or large clumps of individual cells, known as "seeding dispersal", which allow bacteria to attach to a biofilm downstream of the original community (Cunningham *et al.*, 2008). This phenomenon may be responsible for the spread of an acute infection to neighbouring tissue or even

into the circulatory system. This kind of infection is very difficult to control even with intensive antimicrobial treatment (Davies, 2003). Chronic wounds are an example where such infections can flourish using necrotic tissue as a nidus for biofilm formation. Even when appropriate measures are used to control infections associated with chronic wounds colonized by bacteria, wounds still fail to heal due to the presence of biofilms (James *et al.*, 2008). The resistance of biofilm bacteria is most likely due to the sluggish metabolic and growth rates of the constituent bacteria, especially those deep within the biofilm. The biofilm EPS matrix may adsorb antimicrobial molecules or even prevent the penetration of such agents and also offers protective mechanisms (e.g. multidrug efflux pumps and stress response regulons) which are brought into play due to the specific phenotype of the bacteria within the biofilm (Drenkard, 2003). All of this helps to reduce the ability of the host immune system to combat biofilms. One promising solution to the problem of the reduced susceptibility of biofilms to antibiotics is PDT. Since the mechanism of bacterial killing is non-specific, with the cytotoxic species damaging many bacterial components, the development of resistance from repeated use is unlikely (Wainwright & Crossley, 2004).

So far, studies concerning bacterial biofilm photosensitization have mainly focused on oral biofilms and have employed PSs activated by visible light (Dobson & Wilson, 1992; Soukos *et al.*, 2000; O'Neill *et al.*, 2002; Zanin *et al.*, 2005 & 2006; Hope & Wilson, 2006; Wood *et al.*, 2006). Most of these studies reported that the number of bacterial cells killed within a biofilm was considerably lower than what can be achieved when treating their planktonic counterparts owing to the presence of EPS. The experiments in this chapter explored the capability of ICG to disrupt the slimy extracellular polymer matrix, in which the bacteria encase themselves within the biofilm, as well as to kill the bacterial cells while they are in such a highly organized population with an increased ability to resist any kind of environmental stress.

Numerous studies have shown that light can significantly kill diverse bacterial species in the absence of an exogenous PS (Ashkenazi *et al.*, 2003b; Guffey & Wilborn, 2006; Enwemeka *et al.*, 2009). The bactericidal effect of light seems to be dependent on its wavelength and the nature of the targeted organism

(Guffey & Wilborn, 2006). For example, *P. acnes* (Ashkenazi *et al.*, 2003b), *H. pylori* (Hamblin *et al.*, 2005), *Staph. aureus* (Maclean *et al.*, 2008; Enwemeka *et al.*, 2009; Lipovsky *et al.*, 2009) and *P. aeruginosa* (Nussbaum *et al.*, 2003; Guffey & Wilborn, 2006) are the most investigated species showing susceptibility to light. The phototoxic effect involves induction of ROS production by the bacteria on exposure to the light (Lipovsky *et al.*, 2008). The bactericidal effect in the case of *P. acnes* and *H. pylori* has been attributed to the presence of active endogenous porphyrins (Ashkenazi *et al.*, 2003b; Hamblin *et al.*, 2005). Guffey & Wilborn, (2006) demonstrated that combined blue and infrared laser lights (405 nm and 880 nm) exerted a bactericidal effect on *Staph. aureus* and *P. aeruginosa* - achieving 72% and 93.8% kills, respectively, after exposure to a light energy dose of 20 J/cm². The high intensities of visible light (400-800 nm) have caused inactivation of *Staph. aureus* in the absence of exogenous PS, whereas low intensities of light facilitated bacterial growth (Lipovsky *et al.*, 2009). *Staph. aureus* produces triterpenoid carotenoids (Marshall & Wilmoth, 1981), while *P. aeruginosa* releases a phenazine derivative, pyocyanin (Mavrodi *et al.*, 2006). These pigments may contribute to their killing by the light alone. In this study, as the investigated bacteria contain endogenous photosensitizers that absorb light throughout the visible and the NIR region (Hamblin *et al.*, 2005; Lipovsky *et al.*, 2008), the effect of NIR laser light on the survival of both *P. aeruginosa* and *Staph. aureus* biofilms was also studied.

6.2 Materials and Methods

6.2.1 Microtiter plate biofilm formation assay

One Gram-negative bacterium, *P. aeruginosa* strain PA01, and a Gram-positive bacterium, *Staph. aureus* NCTC 8325-4, were cultivated as mono-species biofilms in 96 well-flat bottom tissue culture plates. The plates were incubated stationary at 37°C for 18-22 h in air. First of all, biofilm formation in different growth media was tested for both species. The methods used were as described in section 2.2.1 but the bacterial cultures were diluted 1:100 in either nutrient broth (NB), Luria broth (LB), tryptic soy broth (TSB) or brain heart infusion broth (BHI). Aliquots of 200 µL of the same sterile growth medium served as the controls. Thereafter, biofilm formation in different growth media was assessed by staining with 0.1% crystal violet as described in section 2.2.3.

Once the culture medium which was able to form the thickest biofilm had been determined, the methods followed for further biofilm formation were exactly as described in section 2.2.1.

6.2.2 Photosensitizer formulation and illumination system

Fresh stock solutions of a 1.0 mg/mL solution of ICG were prepared immediately prior to each experiment in sterile distilled water (H₂O), then diluted to 200 µg/mL in PBS and kept in the dark at room temperature.

Illumination was provided with the 808 nm NIR diode laser (Ondine Biopharma Corporation., USA) coupled to a light delivery probe. The maximum power output from the laser probe was 0.4 W and the light was delivered at a fluence rate of 0.3 W/cm². The total energy dose to the sample (J/cm²) was varied from 0-180 J/cm² by varying the duration of light exposure during dose-response experiments.

6.2.3 Photodynamic inactivation of the biofilms

The method used was the same as that described in section 2.2.2. After photosensitization of the biofilms, the effect of the treatment was tested by two different methods, both of which are described below.

6.2.3.1 Crystal violet assay

In order to evaluate the disruption effect that ICG exerted on the extracellular polymeric substance in which the bacteria were encapsulated, monospecies biofilms of either *P. aeruginosa* or *Staph. aureus* were subjected to treatment with ICG photo-activated with NIR laser light. The biofilms were also treated using only the light in the absence of the dye as a negative control. Subsequently, the level of biofilm adherence to the surface of the well was analysed spectrophotometrically by reading the OD₅₉₀ values of crystal violet (CV)-stained adherent bacterial biofilms. The CV staining assay was used as described in section 2.2.3.

The OD₅₉₀ values of stained treated biofilms were compared to those of the controls which were incubated with PBS (L-S-) or ICG (L-S+) and kept in the dark.

6.2.3.2 Viable counting

After lethal photosensitization of the biofilm, a direct enumeration of the surviving bacteria was performed using the method described in section 2.2.4.

6.2.4 Measurements of the temperature during photodynamic inactivation of the biofilms

Two hundred microlitres of 200 µg/mL ICG were added to the biofilms in triplicate then exposed to a measured dose of NIR laser light for 5 minutes (L+S+) to determine the temperature elevation during the photosensitization process. Three additional wells containing the microbial suspension plus PBS instead of the PS were exposed to the same light doses to determine the rise in temperature in the absence of ICG (L+S-). The temperature of the solution was recorded immediately before and after irradiation for L+S- and L+S+ samples using an immersion thermocouple probe connected to a Fluke 179 digital multimeter (Fluke. USA).

6.2.5 CLSM of bacterial biofilms

The method was the same as that described in section 2.2.5.

6.3 Statistical analysis

The optical density data were analysed using the Univariate General Linear Model to determine if there was a difference between groups and between similar experiments performed on different occasions. A Post-Hoc Test in the form of a Bonferroni correction was applied to detect where the difference occurred. The survivor colony counts were transformed into logs to normalize the data then the same tests were applied. The mean difference (P) was significant at the level of 0.05.

6.4 Results

6.4.1 Quantitative assessment of the disruption of *Staph. aureus* and *P. aeruginosa* biofilms

First of all, biofilm formation in different media was evaluated. Figure 6-1 shows the data obtained for *P. aeruginosa* and Figure 6-2 shows the results for *Staph. aureus* biofilms.

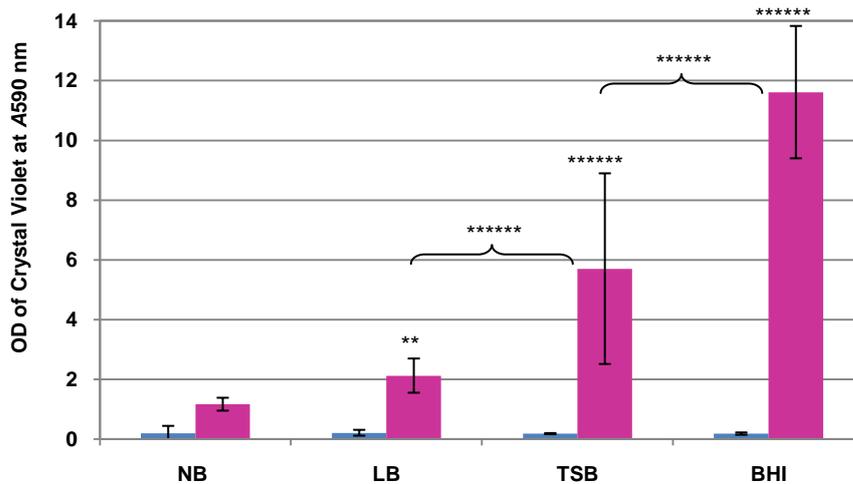


Figure 6-1: *P. aeruginosa* biofilms were grown in 96-well microtiter plates using different growth media: nutrient broth (NB); Luria broth (LB); tryptic soy broth (TSB); and brain heart infusion (BHI). The extent of biofilm formation was measured using a CV assay. *P. aeruginosa* biofilms (■) and negative control OD₅₉₀ values (■) were measured. The values displayed are the means of sixteen replicates performed in two experiments on two different occasions. Error bars represent the standard deviation from the mean. ** P < 0.01, ***** P < 0.000001.

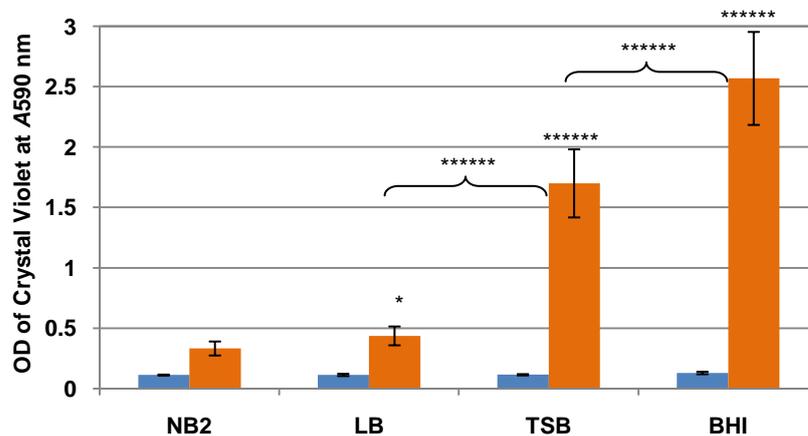


Figure 6-2: The formation of *Staph. aureus* biofilms in 96-well microtiter plates using different culture media: nutrient broth number 2 (NB2); Luria broth (LB); tryptic soy broth (TSB); and brain heart infusion (BHI). The extent of biofilm formation was measured by a crystal violet assay. *Staph. aureus* biofilms (■) and negative control OD₅₉₀ values (■) were measured. The values displayed are the means of eight replicates. Error bars represent the standard deviation from the mean. * P < 0.05, ***** P < 0.000001.

With the exception of nutrient broth, all of the culture media inoculated with either *P. aeruginosa* or *Staph. aureus* were found to produce significant biofilms when compared to the control sample composed of the identical sterile media. Figure 6-1 shows that BHI was superior to TSB and TSB was superior to LB for the growth of *P. aeruginosa* biofilms (LB $P=0.004$, TSB and BHI $P < 0.000001$). A similar pattern was observed for *Staph. aureus* biofilm formation, shown in Figure 6-2 (LB $P=0.017$, TSB and BHI $P < 0.000001$). Although TSB allowed significant biofilm formation, there was still a significant difference ($P < 0.000001$) between the ability of TSB and BHI to support biofilms formation in the case of both bacteria tested. Based on these results, BHI was the medium selected for the growth of bacterial biofilms in further studies.

Microtiter plate grown *P. aeruginosa* biofilms were disrupted when exposed to NIR laser light only and there was no significant difference when the light was combined with 200 $\mu\text{g/mL}$ ICG, as shown in Figure 6-3. Remarkably, the light alone reduced the biofilm by 59 % while in combination with ICG approximately 55 % disruption was detected ($P < 0.000001$ in each case).

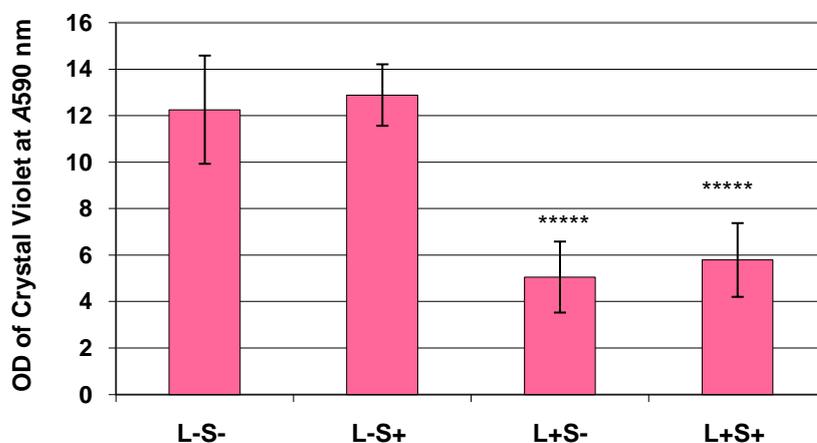


Figure 6-3: 22 hours-old *P. aeruginosa* biofilms exposed to a light dose of 90 J/cm^2 from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm^2 in the presence of either 200 μL PBS (L+S-) or 200 $\mu\text{g/mL}$ ICG (L+S+). The extent of disruption of the biofilm was determined by CV staining. The OD_{590} values of stained treated- biofilms were compared to the controls which were incubated with PBS (L-S-) or ICG (L-S+) only and kept in the dark. Bars represent mean values and error bars represent standard deviations ($n = 10$).

The results obtained encouraged further investigations on *P. aeruginosa* biofilms. Thus, the effect of light dose responses for both the light only and the combination of the light and the dye on younger, 18 and 20 hour-old, *P. aeruginosa* biofilms was carried out.

It was clear that the NIR laser light emitting at 808 ± 5 nm had a damaging effect on the integrity of the biofilm. This is verified by the data displayed in Figure 6-4 where a light dose of both 90 and 180 J/cm² resulted in significant biofilm disruption with P-values of 0.000002 and 0.000001 respectively. The combination of the dye with the light did not result in any extra disrupting capability, with a P-value of 0.000002 being obtained for biofilms treated with a combination of 90 J/cm² and 200 µg/mL ICG. Increasing the light dose to 180 J/cm² did not enhance the disruption effect (P=0.000001). There was no significant difference among the treatment groups of *P. aeruginosa* biofilms, with the percentage of biofilm disruption ranging between 40-45%.

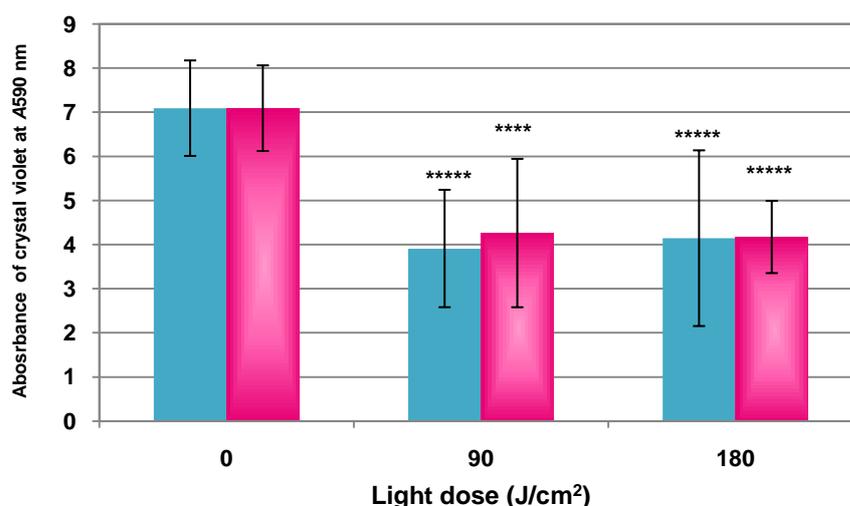


Figure 6-4: The effect of light dose response on 18 hours-old *P. aeruginosa* biofilms exposed to light doses of 0, 90 and 180 J/cm² from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm² in the presence of either 200 µL PBS (■) or 200 µg/mL ICG (■). Bars represent mean values and error bars represent standard deviations (n = 8).

Lower light doses from the NIR Ondine laser were also tested as shown in Figure 6-5. The minimal effective light dose was 54 J/cm². Figure 6-5 showed that a light dose of 18 J/cm² did not result in any significant disruption of *P. aeruginosa* biofilms, while both 54 and 90 J/cm² significantly (P= 0.00001 and P<0.000001) disrupted the biofilms when compared with the control biofilms

which were kept in the dark. The efficacy of 90 J/cm² was significantly more (P=0.002) than that of 54 J/cm² in damaging the biofilms, each resulting in 41% and 22% disruption respectively.

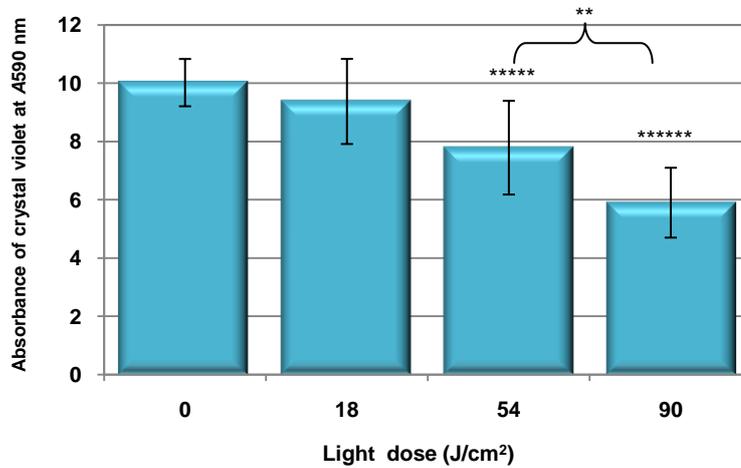


Figure 6-5: The disruptive effect of various light doses on 20 hours-old *P. aeruginosa* biofilms exposed to light doses of 0, 18, 54 and 90 J/cm² from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm². Bars represent mean values and error bars represent standard deviations (n = 8).

When *Staph. aureus* biofilms were subjected to the combination treatment of NIR laser light and 200 µg/mL ICG, significant (P=0.001) disruption of 38% was detected only at 90 J/cm² (Figure 6-6). No significant disruption was observed in *Staph. aureus* biofilms exposed to a light dose of 180 J/cm².

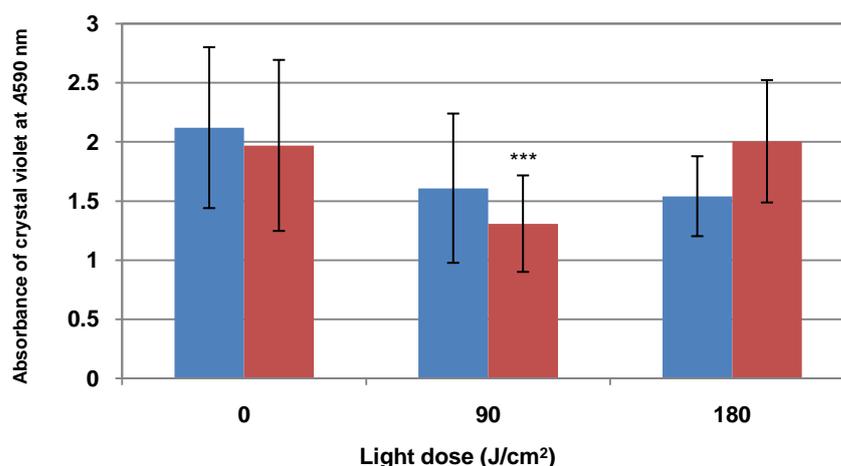


Figure 6-6: The effect of light dose on 18 hours-old *Staph. aureus* biofilms exposed to light doses of 0, 90 and 180 J/cm² from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm² in the presence of either 200 µL PBS (■) or 200 µg/mL ICG (■). Bars represent mean values and error bars represent standard deviations (n = 7).

6.4.2 Direct enumeration of *Staph. aureus* and *P. aeruginosa* biofilms using viable counting

In order to further investigate the effect of the treatments on the bacterial biofilms, direct enumeration of bacterial survivors after exposure to the NIR laser light alone or in combination with ICG was carried out. These experiments completed and verified the results obtained spectrophotometrically by the CV assay.

The data presented in Figure 6-7 demonstrates the susceptibility of both *P. aeruginosa* and *Staph. aureus* biofilms to 200 µg/mL ICG in combination with NIR laser light or to the light only.

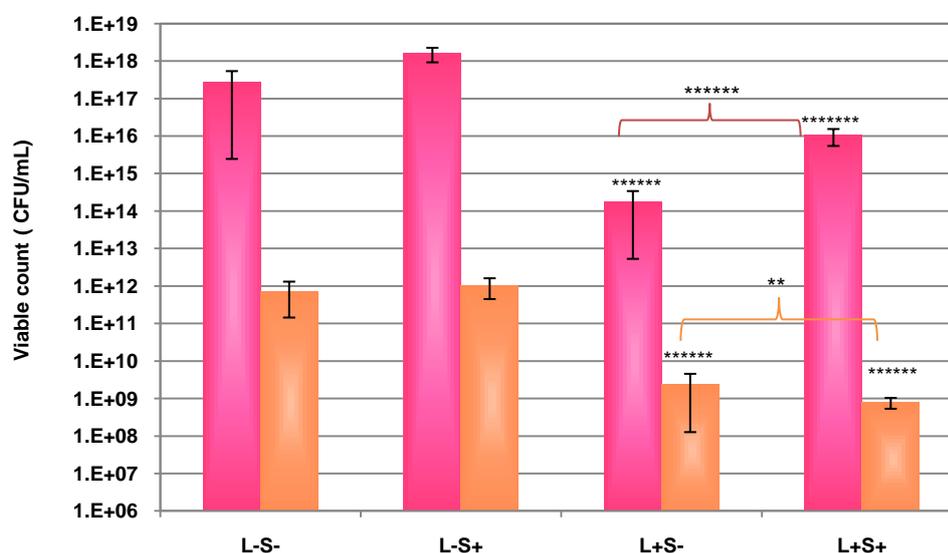


Figure 6-7: Viable counts of 18 hours-old *P. aeruginosa*-biofilms (■) and *Staph. aureus*-biofilms (■) exposed to a light dose of 90 J/cm² from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm² in the presence of either 200 µL PBS (L+S-) or 200 µg/mL ICG (L+S+). Bars represent mean values and error bars represent standard deviations (*P. aeruginosa* biofilms n = 8, *Staph. aureus* biofilms n = 10).

Looking at the *P. aeruginosa* biofilms, it is evident from Figure 6-7 that the use of NIR laser light alone or in conjunction with 200 µg/mL ICG resulted in a statistically significant ($P < 0.000001$) reduction in the viable count (L+S- & L+S+) when compared to the controls. There was a significant difference between the two treated biofilm groups ($P < 0.000001$) with the light alone resulting in a more effective kill. The NIR laser light killed approximately 99.9

% of the Gram-negative bacterial cells encased in biofilms, while a combination of both light and dye resulted in a 99.3% kill.

Exposure of *Staph. aureus* biofilms to 200 µg/mL ICG in combination with NIR laser light or to the NIR light alone (Figure 6-7) resulted in significant 99.9% and 99.7% reductions in the number of viable bacteria when compared to the control biofilms ($P < 0.000001$). ICG combined with the light significantly increased ($P=0.003$) the number of *Staph. aureus* cells killed when compared to the retrieved bacteria from the light treated-biofilms.

6.4.3 Measurement of temperature during biofilm irradiation

It was important to measure the temperature changes during biofilm photosensitization to exclude the possibility that the killing effect was attributed to thermal damage. Table 6-1 presents the recorded temperatures which were measured immediately before exposing the bacterial biofilms to a light dose of 90 J/cm² from the 808 nm NIR Ondine laser at fluence rate of 0.3 W/cm² in the presence of either 200 µL PBS (S-) or 200 µg/mL ICG (S+).

Table 6-1: The temperatures recorded during lethal photosensitization of biofilms

Biofilms	200 µg/mL ICG Absence (S-) or presence (S+) of ICG	Light dose of 90 J/cm ²	
		Pre-irradiation temperature (°C)	Post-irradiation temperature (°C)
<i>P.aeruginosa</i>	S-	21.5 ± 0.3	25.9 ± 0.9
	S+	20.8 ± 1	46.9 ± 1
<i>Staph. aureus</i>	S-	22.5 ± 1	26.6 ± 1
	S+	21.3 ± 1.4	47.9 ± 0.7

From the results shown in Table 6-1, it is evident that the temperature throughout the irradiation of the biofilms with NIR laser light alone did not exceed 27 °C. Such a temperature should not affect the viability of either *P. aeruginosa* or *Staph. aureus* biofilms. Under identical experimental conditions, the reduction in the viable counts of *P. aeruginosa* and *Staph. aureus* biofilms was found to be statistically significant. However, the temperature increased to 47 °C during treatment with the combination of the NIR laser light and 200 µg/mL ICG. Under the same conditions there was no enhancement of bacterial

kill, especially in the case of *P. aeruginosa* since more kill was observed in biofilms treated only with the light.

6.4.4 Confocal laser scanning microscopy

To better understand and confirm the effect of the NIR laser light alone or in conjunction with ICG on the viability of *P. aeruginosa* and *Staph. aureus* biofilms, a CLSM study was performed.

The CLSM images of the biofilms were taken under the same experimental conditions shown in Figure 6-7. Eighteen hour-old *P. aeruginosa* and *Staph. aureus* biofilms were exposed to a light dose of 90 J/cm² from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm² in the presence of either 200 µL PBS (L+S-) or 200 µg/mL ICG (L+S+). The control biofilms were incubated with 200 µL PBS in the dark (L-S-) and the pre-irradiation time equalled 15 minutes.

It is clear from Figure 6-8a, b, c that there was a dramatic difference between the untreated *P. aeruginosa* control biofilm (L-S-), shown in Figure 6-8a, and the light-exposed *P. aeruginosa* biofilms, shown in Figures 6-8b and c. In the control biofilm, incubated with PBS in the absence of light, CLSM images showed a thick cluster of viable bacteria most of which exhibited a green fluorescence. In Figure 6-8b, the NIR laser light-treated biofilm qualitatively appeared to have a greater number of non-viable bacteria (red) than viable ones and a combination of both (yellow). A similar pattern was observed in the ICG photosensitized biofilm in Figure 6-8c with high proportions of the non-viable cells compared to the control.

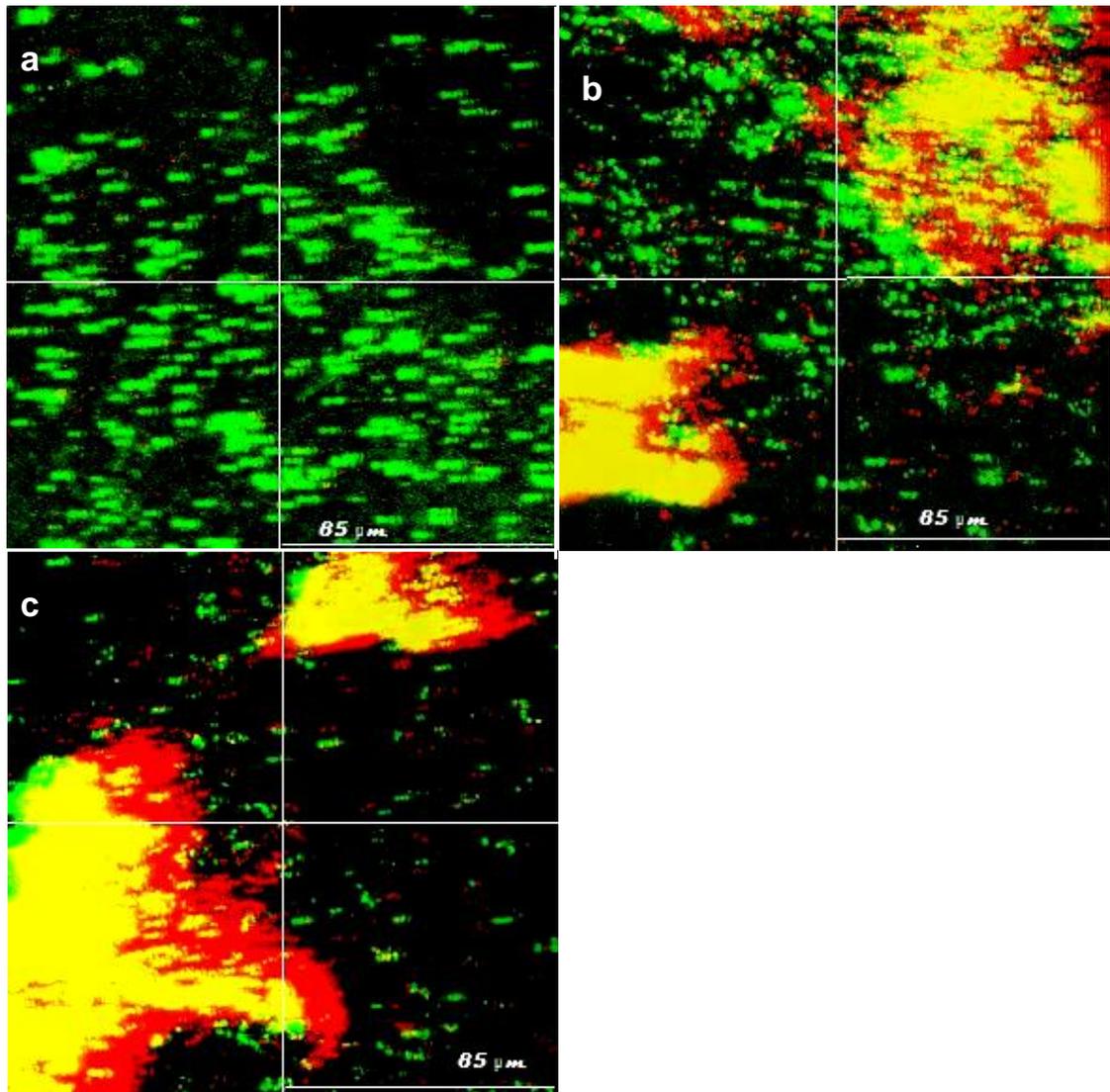


Figure 6-8: Confocal micrographs of a control (L-S-) 18 hours-old *P. aeruginosa* biofilm (a) and biofilms treated with 90 J/cm² of NIR laser light from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm² in the presence of 200 μL PBS (L+S-) (b) or 200 μg/mL ICG (L+S+) (c). The biofilms were stained with BacLight Live/Dead stain and viewed using CLSM.

CLSM images of *Staph. aureus* biofilms showed that *Staph. aureus* was less susceptible to NIR laser light alone or combined with ICG than *P. aeruginosa* biofilms, as can be seen in Figure 6-9a, b, c.

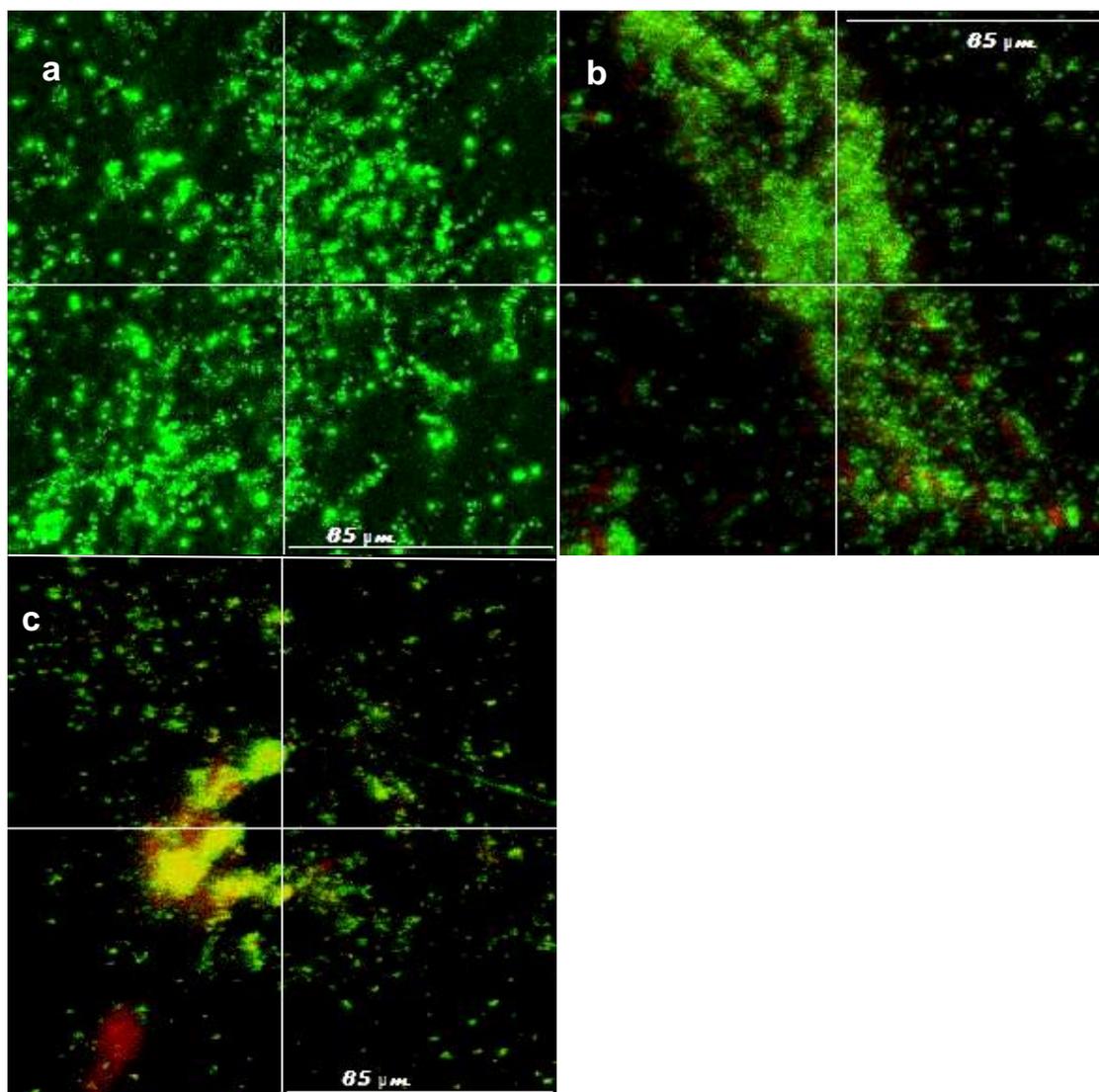


Figure 6-9: Confocal micrographs of a control (L-S-) 18 hours-old *Staph. aureus* biofilm (a) and biofilms exposed to 90 J/cm² of NIR laser light from the 808 nm NIR Ondine laser at a fluence rate of 0.3 W/cm² in the presence of 200 μL PBS (L+S-) (b) or 200 μg/mL ICG (L+S+) (c). The biofilms were then stained with BacLight Live/Dead stain and viewed using CLSM.

Figure 6-9a shows an intact 18 hour-old *Staph. aureus* biofilm which received 200 μL PBS and was kept in the dark (L-S-). After it was stained with BacLight Live/Dead stain, the control biofilm was completely viable (green) with no sign of any dead bacteria (red). Many microcolonies were visible. The light-treated biofilms shown in Figure 6-9b qualitatively exhibited a slightly higher proportion in the number of non-viable (red) cells in comparison to the control biofilm. *Staph. aureus* biofilms which were subjected to lethal photosensitization with ICG along with NIR laser light (Figure 6-9c) show far higher proportions of non-viable cells in ICG-photosensitized biofilms as compared with the control biofilm.

The CLSM data provides direct evidence for the disruption of biofilm structure and a decrease in cell numbers in NIR light/ICG-treated biofilms which fit with the data achieved from the viable counting and the CV assay.

6.5 Discussion

Staph. aureus and *P. aeruginosa* are multidrug-resistant (MDR) organisms which can cause emerging nosocomial and life-threatening infections especially in immunodeficient, cancer and burn patients. Densely aggregated microcolonies of both organisms were found attached to wound tissue often surrounded by an extracellular matrix which is the basic structures of biofilms (Bjarnsholt *et al.*, 2008; Davis *et al.*, 2008; James *et al.*, 2008). In injured tissues, the solid-liquid interface between the skin and an aqueous medium such as exudates or blood constitutes an ideal environment for the attachment and growth of microbial biofilms (Donlan, 2002). Thus, biofilm formation starts with the attachment of a number of planktonic bacterial cells to the exposed extracellular matrix on the surface of the wound. Thereafter they replicate and differentiate over time into microcolonies. These colonies then aggregate into larger groups known as biofilms. Within 10 hours, the bacteria are encased in an EPS which is the main characteristic component of biofilm (Widgerow, 2008). Water is the predominant constituent of EPS that account for 50% to 90% of the total matrix material of the biofilm with only 10-20% of embedded bacteria. The remainder consists of proteins, nucleic acids and polysaccharides. This matrix is perforated by tiny water channels in the form of what might be considered a primitive circulatory system (Smiley & Hassett, 2005). The results presented in this chapter show the effectiveness of NIR laser light alone or in combination with ICG at disrupting and killing *Staph. aureus* and *P. aeruginosa* when these organisms are in biofilms.

At least three exopolysaccharides contribute to biofilm formation in *P. aeruginosa*. These include alginate (a high molecular weight, acetylated polymer composed of nonrepetitive monomers of beta-1,4 linked L-guluronic and D-mannuronic acids), a polysaccharide synthesised by proteins coded for by the polysaccharide synthesis locus (Psl), and pellicles (Pel) (Ryder *et al.*,

2007). In the case of *Staph. aureus*, the matrix consists of the homoglycan polysaccharide intercellular adhesin (PIA) which is composed of beta-1,6-linked *N*-acetylglucosamine with partly deacetylated residues (Götz, 2002). The EPS provides a protective mechanism for micro-organisms allowing them to adapt to extreme temperature, radiation, or mechanical stress. However, a Q-switched Nd:YAG laser used at a wavelength of 1064 nm has been shown to effectively disrupt *P. aeruginosa* and *Staph. aureus* biofilms *in vitro* without causing damage to the underlying host tissue composition by generating powerful pressure shockwaves (Krespi *et al.*, 2008 & 2009). The results of the present study have shown that irradiation with 90 J/cm² of light from the 808 nm NIR laser can cause biofilm disruption - 41% in the case of *P. aeruginosa* but only 24% in the case of *Staph. aureus* biofilms. This disruption of *Staph. aureus* and *P. aeruginosa* biofilms upon exposure to the NIR laser light alone may be due to photo-oxidation of the endogenous pigments staphyloxanthin and pyocyanin, respectively. Lipovsky *et al.*, (2008) reported the generation of ROS upon exposure of *Staph. aureus* to a broadband light of 400-800 nm in the absence of any exogenous PS. The ROS may disrupt EPS via inducing oxidative damage of glucose and proteins, affecting the stability of the main components of the biofilm matrix (Wainwright *et al.*, 2002). In this study, significant disruptions of 38% and 55% have been achieved, respectively, for *Staph. aureus* and *P. aeruginosa* biofilms with a light dose of 90 J/cm² in combination with 200 µg/mL ICG. In support of these results, Baldursdóttir *et al.*, (2003a) have reported the degradation of alginate treated with the photosensitizer riboflavin (RF) irradiated with light of 310-800 nm. This photochemical degradation was attributed to the production of ROS and free radicals which can cause oxidative cleavage of glycosidic bonds, resulting in scission of polysaccharide chains (Akhlaq *et al.*, 1990; Baldursdóttir *et al.*, 2003b).

The photosensitization of pigment-containing bacteria is well documented in the literature. A known example is the photosensitization of *H. pylori* with blue light both *in vitro* (Hamblin *et al.*, 2005) and *in vivo* (Ganz *et al.*, 2005). Under the particular conditions of high cell density and nutrient limitation, *P. aeruginosa* produces the blue-green pigment pyocyanin (5-*N*-methyl-1-hydroxyphenazine) in high quantity (Price-Whelan *et al.*, 2006). This is a virulence factor, which is

released as an end product of a pathway regulated by the *P. aeruginosa* biofilm cell-to-cell communication process called quorum sensing (QS) (Lau *et al.*, 2004; Price-Whelan *et al.*, 2006; Dietrich *et al.*, 2006). This pigment is a compound belonging to the phenazine series, and is related to the known photosensitizer neutral red. It would be expected to cause photodamage upon irradiation with light of the appropriate wavelength (Wainwright *et al.*, 2002). The pigment's characteristic absorption bands are found at a number of wavelengths, including 250, 300, 550 and 690 nm (Reszka *et al.*, 2006). In the near-infrared region of the spectrum, a strong wide absorption band, covering the spectrum from 650 to 800 nm, is displayed by pyocyanin (Reszka *et al.*, 2006; Cheluvappa *et al.*, 2008). After its release from bacteria, pyocyanin has been shown to be rapidly and nonreversibly photo-inactivated by exposure to broad-spectrum light of 350-700 nm producing colourless photoproduct(s) with first-order kinetics (Propst & Lubin, 1979). Lipovsky *et al.*, (2008) have reported the photo-inactivation of *Staph. aureus* and *E. coli* irradiated with intense broadband visible light (400–800 nm) in the presence of pyocyanin. Thus, pyocyanin can be considered a photosensitizer which produces ROS (mainly hydroxyl and superoxide radicals) upon exposure to light (Lipovsky *et al.*, 2008). The lethal action of the *P. aeruginosa* pigments is due to the production of a reactive oxygen intermediate by the pyocyanin pigment that alters membrane permeability and causes chromosome breaks preventing DNA replication (Benathen & Saccardi, 2000). This explains the 3 log₁₀ fold kill of the 18 hour-old *P. aeruginosa* biofilms observed after irradiation with 90 J/cm² from the 808 nm NIR laser light. This is in agreement with earlier findings of a 0.75 log₁₀ reduction following treatment of 168 hour-old *P. aeruginosa* biofilms with 7.2 J/cm² from an incoherent light source of 600-700 nm (Wainwright *et al.*, 2002) and a 0.4 log₁₀ reduction upon irradiation of 24 hour-old *P. aeruginosa* biofilms with visible laser light of 670 nm (Street *et al.*, 2009). However, a reduced kill was reported in both studies in comparison to the substantial reduction of 3 log₁₀ in the current study. This may be due to the use of the near infrared optical energy, which can penetrate the bacterial biofilm to a greater extent than that of visible light, specifically as far as 8 mm (Detty *et al.*, 2004).

Surprisingly, the addition of ICG at a concentration of 200 µg/mL did not augment the killing effect of the NIR laser light; a decrease in the viable count of 2.2 log₁₀ was detected. These results may be attributable to the adsorption of ICG by, or reaction with, the EPS (Davies, 2003), which might reduce the amount of ICG penetrating deep into the biofilm. The quenching of the cytotoxic species by the EPS would thereby protect the bacteria from photosensitization (Soukos *et al.*, 2003). Another explanation for the results is that the photosensitizer itself acted as a competing or sheltering agent (Bhatti *et al.*, 1997), inhibiting light propagation deep into the biofilm and thereby failing to photo-activate the pyocyanin pigment, thus a lower kill was observed in ICG treated-biofilms.

Only three reports have looked into the lethal photosensitization of *P. aeruginosa* biofilms, each using a different biofilm model and a different light-activated antimicrobial agent which makes comparisons with the present study difficult (Wainwright *et al.*, 2002; Lee *et al.*, 2004; Street *et al.*, 2009). None of them studied all aspects of the biofilm (i.e. disruption, viability and microscopical analysis), in contrast to the current work. In the current study, a kill of 2.2 log₁₀ was observed after treatment of *P. aeruginosa* biofilms with photo-activated ICG. Wainwright *et al.*, (2002) reported a kill of 2.5 log₁₀ of *P. aeruginosa* biofilms upon treatment with NMB and red light of 7.2 J/cm², while others showed a reduction of 2.2 log₁₀ when biofilms were exposed to 13.2 J and MB (Street *et al.*, 2009). Another research group photosensitized 24 hour-old *P. aeruginosa* biofilms with 20-40 mM δ-ALA and 120 J/cm² from a 630 nm LED; no viable bacteria were detected under these conditions although re-growth was observed after 24 h. Biofilms appeared to re-form thereafter and reached 7.2 log₁₀ CFU/ cm² (Lee *et al.*, 2004).

The CLSM studies have provided a qualitative analysis of *P. aeruginosa* biofilms following irradiation with NIR laser light in the absence and presence of ICG. The results revealed that lethal photosensitization resulted in the loss of bacterial adhesion within the biofilm and subsequent loss of biofilm bulk (Wood *et al.*, 1999). The CLSM images also showed that the photosensitized bacteria appeared predominantly in the outer layers of the biofilm leaving the innermost bacteria alive (O'Neill *et al.*, 2002; Zanin *et al.*, 2005). The killing of bacterial

cells in the biofilms may be due to the generation of ROS which cause rapid, highly localised oxidative reactions and so exert bactericidal effects. This incomplete kill may be due to the inability of the photosensitizer to infiltrate these inner regions of the biofilm or the failure of light to penetrate into the biofilms (O'Neill *et al.*, 2002). Photosensitization may also disrupt EPS via inducing oxidative damage of glucose and proteins, affecting biofilm stability. Such photobactericidal/matrix-damaging activity is eminently desirable in the management of biofilm colonisation (Wainwright *et al.*, 2002).

In contrast to what was observed with *P. aeruginosa*, treatment of *Staph. aureus* biofilms using the combination of NIR laser light and ICG was more lethal in comparison to the light alone. Previous reports showed that *Staph. aureus* biofilms were sensitive to either merocyanine (MC) 540 activated with green light of 510 to 570 nm (Lin *et al.*, 2004) or TBO in conjunction with 640 nm laser light (Sharma *et al.*, 2008). In the current study, a light dose of only 90 J/cm² and 200 µg/mL ICG were used to photosensitize *Staph. aureus* biofilms with a subsequent kill of 3.12 log₁₀, while only a 2 log₁₀ kill was observed when biofilms were treated with 15 µg/ml MC 540 and a higher light dose of 300 J/cm² (Lin *et al.*, 2004). In this study, the irradiation of *Staph. aureus* biofilms with NIR laser light alone resulted in a 2.5 log₁₀ reduction in the number of viable bacteria. In accordance with this result, Maclean *et al.*, (2008) have shown that a 2.4 log₁₀ reduction of *Staph. aureus* including MRSA can be achieved using a white-light (400 to 500 nm) at 3.27mW/cm² irradiance and a light dose of 23.5 J/cm² in the absence of exogenous PSs. Lubart *et al.*, (2008) reported a reduction in the viability of clinical isolates of *Staph. aureus* when exposed to white light (400-800 nm) of 180 J/cm² at a fluence rate of 0.3 W/cm² in the absence of exogenous PS. This was attributed to the production of hydroxyl radicals confirmed by electron paramagnetic resonance (Lubart *et al.*, 2008). A possible explanation is that most strains of *Staph. aureus* are capable of producing staphyloxanthin - a C₃₀ carotenoid pigment (Horsburgh *et al.*, 2002). When *Staph. aureus* cells are grown in a biofilm, the corresponding genes involved in staphyloxanthin biosynthesis are expressed at higher levels than they are in cells grown planktonically (Resch *et al.*, 2005). In addition to the absorption maxima of staphyloxanthin at 463 and 490 nm (Pelz *et al.*, 2005), it has been observed that the carotenoid pigment also has a broad

photo-induced absorption band that extends from 600 to 950 nm (Cerullo *et al.*, 2002; Holt *et al.*, 2005). This is supported by the finding that a substantial kill of *Staph. aureus* can be achieved with a light dose of 90 J/cm² from a broadband light source of 400-800 nm in the absence of exogenous PS. This bactericidal effect was attributed to the presence of endogenous porphyrins and carotenoids which produce hydroxyl and superoxide radicals upon exposure to light, causing oxidative damage of the bacteria (Lipovsky *et al.*, 2009).

The CLSM investigation carried out by Sharma *et al.*, (2008) suggested that damage to bacterial cell membranes occurred when *Staph. aureus* biofilms were treated with photo-activated TBO. These data are consistent with the CLSM results of the current study, which showed bacterial cell killing that was denoted by staining with propidium iodide after irradiation with NIR light in the presence of ICG in comparison to the control. Less bacterial aggregation was also noted after photosensitization of *Staph. aureus*, biofilms with ICG. The killing of bacteria within the biofilms may result in cell detachment, and consequent disruption of the biofilm architecture (Di Poto *et al.*, 2009). In addition, the production of free radicals and ROS accelerate the decomposition of proteins, lipids and carbohydrates which may cause photo-oxidation of the main constituent of the biofilm matrix (Lyons & Jenkins, 1997).

The results presented in the current work show that NIR laser light is effective at killing both *P. aeruginosa* and *Staph. aureus* when these are in the form of biofilms. Furthermore, NIR light also induces the disruption of the structure of these biofilms. The presence of ICG does not enhance these effects in the case of biofilms of *P. aeruginosa* while, for *Staph. aureus* biofilms, there is a slight increase in the bactericidal effect. These combined bactericidal and biofilm-disruptive effects, if operative *in vivo*, would be of great benefit in the treatment of infections caused by bacterial biofilms.

Chapter 7

**The effect of physiological factors on the
lethal photosensitization of organisms
responsible for wound infections**

7.1 Introduction

The results described in previous chapters have shown that the most common organisms responsible for wound infections, when in the form of planktonic cells or biofilms, can be photo-inactivated using ICG combined with NIR laser light. However, *in vivo* there are several physiological and environmental factors which may affect the effectiveness of antimicrobial-PDT in the treatment of infected wounds. Furthermore, bacteria may react to antimicrobial therapy in different ways depending on the environment in which they exist. An overview of the wound environment may help to understand how antimicrobial-PDT may be affected *in vivo*.

In a wound the initial injury initiates inflammation which in turn increases capillary permeability. As a result of this increased permeability, white blood cells can escape and the blood vessels leak more fluid. Thus, the excess fluid enters the wound where it forms the basis of exudates which closely resemble blood plasma (Harding, 2007). Acute wound exudate contains molecules and cells that are vital to support the healing process. It has a high protein content (although lower than that found in serum), with a specific gravity greater than 1.020 (White & Cutting, 2006b). Its components include water, electrolytes, glucose, inflammatory mediators, white cells, protein-digesting enzymes (e.g. MMPs), growth factors, waste products and micro-organisms (Trengeve *et al.*, 1996). Also wound exudate may be contaminated with tissue debris. In the first 48 to 72 hours after wounding, platelets and fibrin may be present, but levels decrease as bleeding diminishes (White & Cutting, 2006b). In the case of healing acute wounds, exudate contains active growth factors. These are not found in chronic wounds (Baker & Leaper, 2000). Furthermore, the colour, consistency and amount of exudate may change according to the physiological status of the wound (Harding, 2007). For example, wounds will often respond to an increased microbial load with a sudden production of enormous amounts of exudate (Cutting, 2003). During infection a purulent thick exudate with malodour is found in the wound due to the presence of white blood cells and bacteria (high protein content) (White & Cutting, 2006b; Harding, 2007). The larger the wound surface area the higher the amount of exudate produced by a wound. However, some wound types are proposed to have high rates of exudate production such as burns and venous leg ulcers (Harding, 2007).

Disruption of the local vascular supply as a result of injury and thrombosis of vessels causes the wound microenvironment to be hypoxic. The oxygen tension at the wound bed is often less than 30 mm Hg (Greif *et al.*, 2000). Low levels of oxygen increase the risk of infection and chronicity in a wound (Tonnesen *et al.*, 2000). Growth of bacteria within a wound may lead to further hypoxia due to increased consumption of local oxygen by bacteria (Bowler, 2002).

Normally, the intact skin surface is an acidic milieu. This acidic pH varies between 4 and 6 according to the anatomical location and age of the person and is an important aspect of the skin's barrier function. This acidic pH also seems to be important for resistance to external chemicals and bacteria (Schneider *et al.*, 2007). In wounds, the skin's acidic pH is disturbed due to the injury, where the underlying tissue with the body's internal pH of 7.4 becomes exposed (Schneider *et al.*, 2007).

It is essential to evaluate the efficiency of lethal photosensitization of wound-infecting organisms under conditions that would exist in a wound environment. For example, to study the inactivation of wound-infecting organisms in the presence of serum which mimics the high protein level that might be found in an infected wound. In addition, understanding the effects of other biological factors such as hypoxia on lethal photosensitization may assist in optimization of the antimicrobial-PDT outcome to treat wound infections *in vivo*. In this part of the study, therefore, lethal photosensitization was performed in horse serum and in a low oxygen-containing environment. These are two important environmental characteristics to be found in a wound.

7.2 Materials and Methods

7.2.1 Kill experiments in horse serum

In order to investigate the lethal photosensitization of the bacteria in an environment similar to that which would exist in a wound, lethal photosensitization experiments were performed in the presence of either 50, 12.5 or 6.25 % HS. Control experiments were performed in PBS when appropriate.

7.2.2 Target organisms and growth conditions

The organisms used were: *Staph. aureus* NCTC 8325-4, MRSA-16, *Strep. pyogenes* ATCC 12202, *P. aeruginosa* strain PA01 and/or *E. coli* ATCC 25922. All organisms were grown as described previously in Chapter 2, section 2.1.2., and modified according to section 2.3.1.1. For the purpose of the comparison studies between pulsed and continuous modes of irradiation, the initial bacterial load was adjusted to approximately 10^5 - 10^6 CFU/mL for all targeted species except for the comparison of the susceptibility of *Staph. aureus* NCTC 8325-4 and EMRSA-16, an initial bacterial load of 10^7 CFU/mL was used.

7.2.3 Photosensitizer preparation and irradiation system

ICG preparation was described in Chapter 2, section 2.1.4.

Irradiation was carried out using the 0.5 W Ga-Al-As laser (Thor laser), or the 0.4 W diode laser (Ondine laser). Both lasers emit continuous wave laser light with a wavelength of 808 ± 5 nm.

For the comparison between pulsed and continuous mode of irradiation, the GaAlAs Velopex diode laser system (Medivance Instruments Ltd., UK) which emits light at a wavelength of 810 ± 10 nm was used. When the laser output power was set to 0.4 W the actual power output was found to be 0.525 W upon calibration using a thermopile TPM-300CE power meter (Genetic-eo, Québec, Canada). The light from this system was applied to the target specimens using an optical fiber of 400 μ m diameter, either in continuous or repeated pulse duration modes which was selected to switch on and off between 100 – 100 msec.

The characteristics of each laser were described in detail in Chapter 2, section 2.1.3.

7.2.4 The effect of ICG concentration and light fluence rate on lethal photosensitization

The effect of the light dose delivered at high or low fluence rate on the viability of *Staph. aureus*, *P. aeruginosa* and *E. coli* was studied in the presence of serum concentrations ranged between 6.25-50%. Final ICG concentrations of 25, 100 or 200 μ g/mL were used in these experiments. The light energies were delivered to each bacterial suspension either at a high fluence rate from both

the NIR Thor and Ondine lasers or at a low fluence rate from the Ondine laser. On the basis of the results achieved in Chapter 3, high intensity lethal photosensitization was carried out on both Gram-positive and -negative organisms. Low intensity photosensitization was tested only on the Gram-positive bacterium *Staph. aureus*. The light energies, irradiation times and the fluence rates used in this section are shown in Table 7-1. The procedure for bacterial photosensitization was followed as described in Chapter 2, section 2.3.1.2.

Table 7-1: The light dosimetry for the laser sources used

Laser used	Fluence rate (W/cm ²)	Irradiation time (sec)	Energy density (J/cm ²)
Thor Laser 808 nm	1.37	300	411
Ondine Laser808 nm	0.3	300	90
	0.05	1800	90

7.2.5 Comparison of the effect of pulsed versus continuous NIR light on lethal photosensitization

The effect of various light energies in combination with 100 µg/mL ICG, in the presence and absence of 12.5% HS, on bacterial viability (*Staph. aureus* NCTC 8325-4, *Strep. pyogenes* and *P. aeruginosa*) was studied. The continuous (CW) and pulsed (PW) light energies delivered were calculated as shown in Table 7-2. The procedure for bacterial photosensitization was followed as described in Chapter 2, section 2.3.1.2. Bacterial viability was determined by viable counting.

Table 7-2: The light dosimetric parameters for the 810 nm laser light.

Laser used	Fluence rate (W/cm ²)	Irradiation time (sec) CW	Irradiation time (sec) PW	Energy density (J/cm ²)
The Velopex diode laser system	0.7	30	60	21
		60	120	42
		90	180	63

7.2.6 Photosensitization of *Staph. aureus*: methicillin-sensitive strain versus methicillin-resistant strain in the presence of serum

The photo-susceptibility of a methicillin-sensitive strain (MSSA) (*Staph. aureus* NCTC 8325-4) was compared to the methicillin-resistant strain (MRSA) (EMRSA-16). An initial bacterial load of 10^7 CFU/mL of both *Staph. aureus* strains were photosensitized using ICG at a concentration of 100 $\mu\text{g/mL}$ in combination with light doses of 42 and 63 J/cm^2 at a fluence rate of 0.7 W/cm^2 either in 12.5% HS or in PBS to serve as control to detect any inhibition of the killing.

7.2.7 Kill experiments under anaerobic conditions

7.2.7.1 Target organisms

Two Gram-positive organisms were used in these experiments: *Staph. aureus* NCTC 8325-4 and *Strep. pyogenes* ATCC 12202. The culture conditions used are described in Chapter 2, section 2.1.2, and modified according to section 2.3.2.1.

7.2.7.2 Lethal photosensitization in an anaerobic pouch incubation system

For lethal photosensitization of bacterial suspensions in the BBL™GasPak™ Pouch system, before and after anaerobic conditions were achieved, the method described in Chapter 2, section 2.3.2.2 was followed. In this system, the bacterial suspensions were exposed to light energy of 90 J/cm^2 at a fluence rate of 0.3 W/cm^2 from the 808 nm NIR Ondine laser.

7.3 Results

7.3.1 The effect of serum on the lethal photosensitization of bacteria

7.3.1.1 Photosensitization at a high fluence rate

7.3.1.1.1 Thor laser

In the presence of 50% HS, the lethal photosensitization of *Staph. aureus* using a high fluence rate of 1.37 W/cm^2 was significantly inhibited (Figure 7-1). It was shown in Chapter 3 (Figure 3-2a) that 25 $\mu\text{g/mL}$ of ICG was the optimum concentration to kill *Staph. aureus*, achieving a 5 \log_{10} reduction in the

absence of serum. However, only a 0.3 log₁₀ (P= 0.3) reduction in the viable count was observed using the same ICG concentration in 50% HS. However, increasing the concentration of ICG to 200 µg/mL and exposure to the same light dose of 411 J/cm² achieved a 4.61 log₁₀ reduction in the viable count in the presence of 50% HS (P = 0.00004). The difference of 4.31 log₁₀ in *Staph aureus* killing resulting from the increased ICG concentration was significant (P = 0.00004).

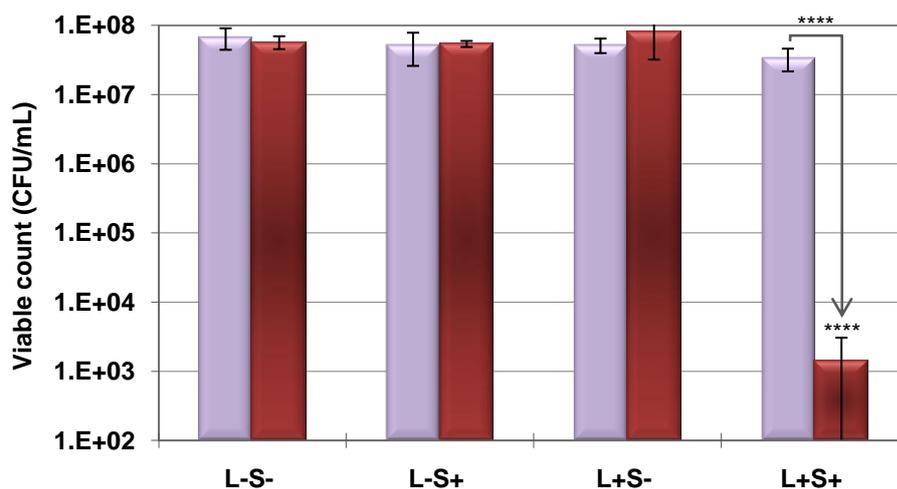


Figure 7-1: Lethal photosensitization of *Staph. aureus* in 50% serum by ICG of concentrations of 25 (■) or 200 (■) µg/mL. Samples were irradiated with a light dose of 411 J/cm² from the NIR 808 nm Thor laser at fluence rate of 1.37 W/cm². Control suspensions were kept in the dark without (L-S-) or with ICG (L-S+). Error bars represent the standard deviation from the mean.

7.3.1.1.2 Ondine laser

7.3.1.1.2.1 High intensity photosensitization of *Staph. aureus* in 50% serum

Treatment of bacterial suspensions in 50% HS with 25 µg/mL ICG in combination with exposure to 90 J/cm² from the 808 nm Ondine laser, at a high fluence rate of 0.3 W/cm², did not result in significant killing of *Staph. aureus* (Figure 7-2). The photo-activated 25 µg/mL ICG achieved approximately a 4 log₁₀ greater kill in PBS than in 50% HS. This difference in kill was significant (P<0.000001). At a higher concentration of 100 µg/mL ICG, when exposed to the same light dose, significant reductions of 5.1 log₁₀ and 5.4 log₁₀ (P < 0.05) in the viable count were achieved in 50% HS and in the absence of serum, respectively. This kill amounted to >99.999% in each case and the difference of 0.3 log₁₀ was not significant. A slight growth (ranging from 0.6-0.9 log₁₀) of *Staph. aureus* was observed in the control samples suspended in 50% HS compared to those in PBS (P>0.01). However, none of the positive controls (L-

S+ or L+S-) showed significant changes in cell numbers compared to their own controls that did not receive either light or ICG (L-S-).

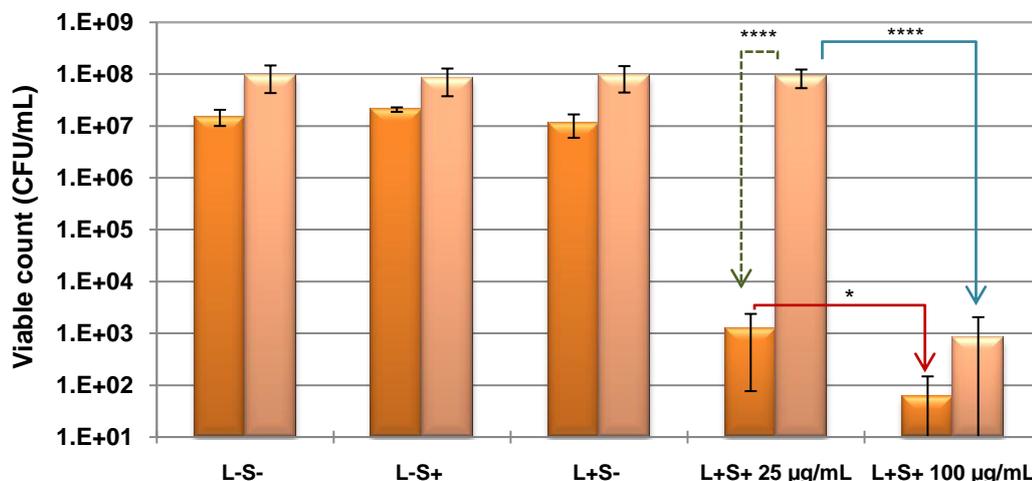


Figure 7-2: High-intensity photosensitization of *Staph. aureus* in PBS (■) or 50% serum (□) using ICG concentrations of 25 and 100 µg/mL. Samples were irradiated with a light dose of 90 J/cm² from the NIR 808 nm Ondine laser at a fluence rate of 0.3 W/cm². Control suspensions were kept in the dark without (L-S-) or with ICG (L-S+). Error bars represent the standard deviation from the mean.

7.3.1.1.2.2 High intensity photosensitization of *P. aeruginosa* and *E. coli* in 50% HS

In contrast, the photosensitization of the Gram-negative bacterium *P. aeruginosa* was not significantly affected by the presence of serum (Figure 7-3a). A viable count reduction of 4.2 log₁₀ (P=0.0002) by 200 µg/mL ICG was observed in the presence of 50% HS compared to 4.8 log₁₀ reduction (P<0.000001) in the absence of serum. This difference of 0.6 log₁₀ was not significant (P=1). In the absence of ICG, significant kills of 0.7 log₁₀ (p = 0.0003) and 1.2 log₁₀ (0.00004) were also observed, upon exposure of *P. aeruginosa* to the NIR light alone, both in the absence and presence of serum, respectively.

Lethal photosensitization of *E. coli* was slightly inhibited by the presence of serum (Figure 7-3b). *E. coli* photosensitization by 100 µg/mL was affected by the presence of serum, achieving a 2.5 log₁₀ reduction (P < 0.0000001) compared with a 4.3 log₁₀ reduction in the absence of serum (P = 0.00002). For *E. coli*, the kill difference of 1.8 log₁₀ when the bacteria were

photosensitized with ICG in PBS or in serum suspensions was significant ($P = 0.005$).

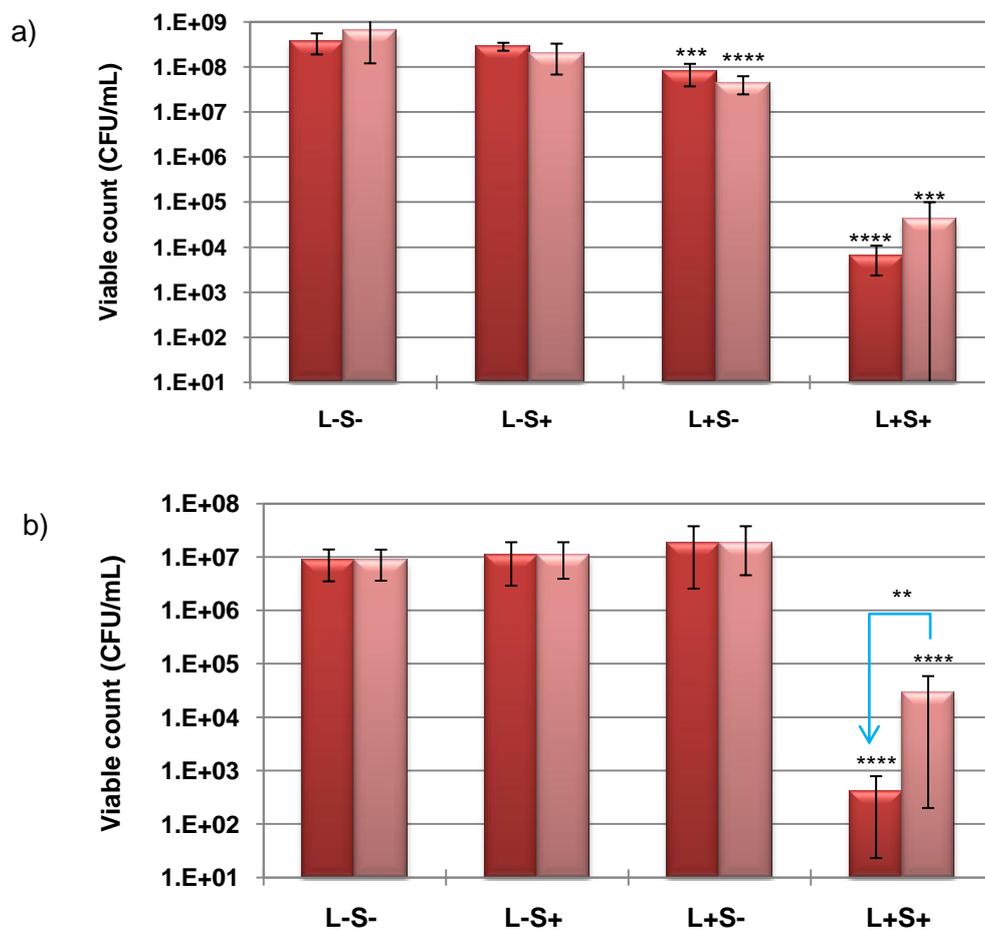


Figure 7-3: High-intensity photosensitization of (a) *P. aeruginosa* with 200 µg/mL ICG and (b) *E. coli* with 100 µg/mL ICG. Bacterial suspensions in PBS (■) or in 50% serum (■) were irradiated with a light dose of 90 J/cm² from the NIR 808 nm Ondine laser at a fluence rate of 0.3 W/cm². Control suspensions were kept in the dark without (L-S-) or with ICG (L-S+). Error bars represent the standard deviation from the mean.

7.3.1.2 Photosensitization of *Staph. aureus* at a low fluence rate

Figure 7-4a and b shows the lethal photosensitization of *Staph. aureus* suspended in a low concentration of HS of 6.25% by 25 and 100 µg/mL ICG photo-activated at a low fluence rate. Irradiation of *Staph. aureus* in 6.25% HS using 25 µg/mL ICG, activated with light energy of 90 J/cm² delivered at a fluence rate of 0.05 W/cm² resulted in a kill of 0.24 log₁₀ (Figure 7-4a). However, the same ICG concentration in the absence of the serum achieved a 4.34 log₁₀ reduction in the viable count. Even such a low concentration of serum inhibited bacterial kill with a low ICG concentration of 25 µg/mL, the

difference of 4.1 log₁₀ was found to be significant (P<0.000001). Increasing the ICG concentration to 100 µg/mL, enhanced the kill significantly: 2.7 log₁₀ kills (P=0.001) were achieved in serum compared to 5.2 log₁₀ (P=0.00003) in the absence of serum (Figure 7-4b). Again, the kill difference of 2.5 log₁₀ in serum presence and absence was significant (P=0.01).

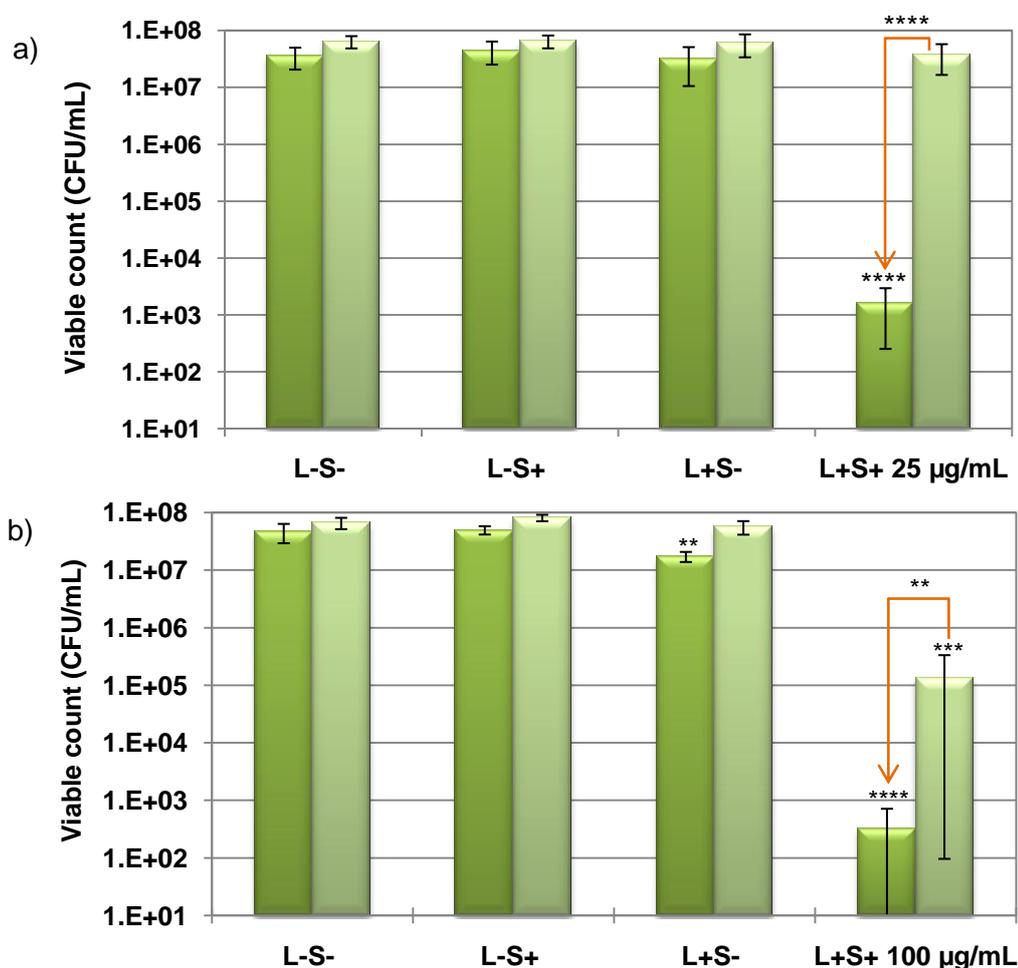


Figure 7-4: Low-intensity photosensitization of *Staph. aureus* in PBS (■) or 6.25% serum (■) by ICG concentrations of (a) 25 µg/mL and (b) 100 µg/mL. Samples were irradiated with a light dose of 90 J/cm² from the NIR 808 nm Ondine laser at a fluence rate of 0.05 W/cm². Control suspensions were kept in the dark without (L-S-) or with ICG (L-S+). Error bars represent the standard deviation from the mean.

Under the same experimental conditions, at a higher serum concentration of 12.5%, no difference in the kill efficiency was observed, achieving a significant (P=0.001) reduction of 2.9 log₁₀ in the viable count using 100 µg/mL ICG (Figure 7-5). In the absence of serum, at the same ICG concentration, a kill of

4.7 log₁₀ was achieved. The kill difference of 1.8 log₁₀ was significant (P=0.003).

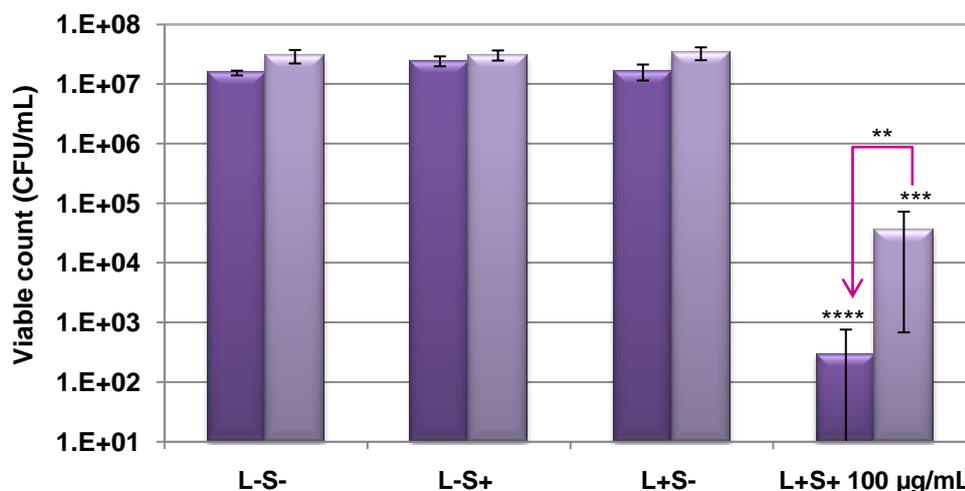


Figure 7-5: Low-intensity photosensitization of *Staph. aureus* in PBS (■) or in 12.5% serum (□) by an ICG concentration of 100 µg/mL. Samples were irradiated with a light dose of 90 J/cm² from the NIR 808 nm Ondine laser at a fluence rate of 0.05 W/cm². Control suspensions were kept in the dark without (L-S-) or with ICG (L-S+). Error bars represent the standard deviation from the mean.

7.3.1.3 Comparison of the effect of pulsed versus continuous NIR light on lethal photosensitization in the presence of serum

Figure 7-6 shows the photo-susceptibility of *Staph. aureus* and *Strep. pyogenes* when treated with 100 µg/mL ICG then activated with different light energies transmitted as continuous or pulsed waves in the presence or absence of serum.

Figure 7-6a demonstrates that both continuous and pulsed light modes, used in the presence of serum, resulted in a statistically significant (P ≤ 0.001) reduction in *Staph. aureus* viable counts at all light doses. The lowest light energy of 21 J/cm² delivered as pulsed or continuous waves, resulted in significant kills (P < 0.001) of approximately 1.5 log₁₀ and 0.2 log₁₀ in the absence and presence of serum, respectively. The difference of 1.3 log₁₀ was significant when using either pulsed (P = 0.00001) or continuous (P = 0.001) irradiation mode. At this light dose, the presence of serum reduced the proportion of *Staph. aureus* killed from 97% to 40% only. Increasing the light energy dose to 42 J/cm² delivered as continuous waves, achieved significant 3.8 log₁₀ kills in both the

absence ($P= 0.00006$) and presence of serum ($P= 0.006$). However, when the same light energy was transmitted in a pulsed mode, significant kills of $3.8 \log_{10}$ ($P= 0.001$) and $1.9 \log_{10}$ ($P= 0.003$) were observed in the absence or presence of serum, respectively. Still, there was a significant ($P= 0.01$) difference between the number of *Staph. aureus* killed in the presence and absence of serum when 42 J/cm^2 was transmitted in a pulsed mode. When a light dose of 63 J/cm^2 was applied in a continuous mode, a significant kill of $5.69 \log_{10}$ ($P < 0.000001$) was observed in the absence of serum compared to $5.33 \log_{10}$ ($P= 0.0001$) in the presence of serum. Only at a light energy of 63 J/cm^2 was a pulsed mode of irradiation equally effective in the absence and presence of serum, achieving $> 5 \log_{10}$ reduction in the viable count in each case.

In the case of *Strep. pyogenes* (Figure 7-6b), a continuous light energy of 21 J/cm^2 produced $0.4 \log_{10}$ kills in the presence of serum compared to a significant ($P= 0.0001$) kill of $2.1 \log_{10}$ in the absence of serum. This $1.7 \log_{10}$ difference was significant ($P= 0.0001$). When the same light energy was transmitted in a pulsed mode, significant kills of $1.6 \log_{10}$ and $0.34 \log_{10}$ were achieved in the absence ($P= 0.01$) and presence ($P= 0.02$) of serum, respectively. This difference of $1.26 \log_{10}$ was significant ($P= 0.015$). In the presence of serum, both continuous and pulsed light modes resulted in statistically significant reductions of $2.2 \log_{10}$ ($P= 0.004$) and $1.9 \log_{10}$ ($P= 0.01$), respectively, in *Strep. pyogenes* viable counts at a light dose of 42 J/cm^2 . In the absence of serum the same light dose achieved $4.2 \log_{10}$ ($P= 0.0001$) and $3.1 \log_{10}$ ($P= 0.0002$) reductions in *Strep. pyogenes* viable counts when transmitted in continuous and pulsed modes, respectively. These differences of $2 \log_{10}$ and $1.2 \log_{10}$ were significant when using continuous ($P= 0.005$) or pulsed ($P= 0.03$) irradiation modes, respectively. At the highest light energy of 63 J/cm^2 , both continuous and pulsed light were equally effective, achieving approximately $5 \log_{10}$ kills in the absence of serum and $4 \log_{10}$ kills in the presence of serum. This difference was not significant ($P= 0.9$ for pulsed and continuous irradiation modes).

For both bacteria, no difference in the efficiency was detected between pulsed and continuous mode of irradiation at all identical light energies tested either in the presence or absence of serum.

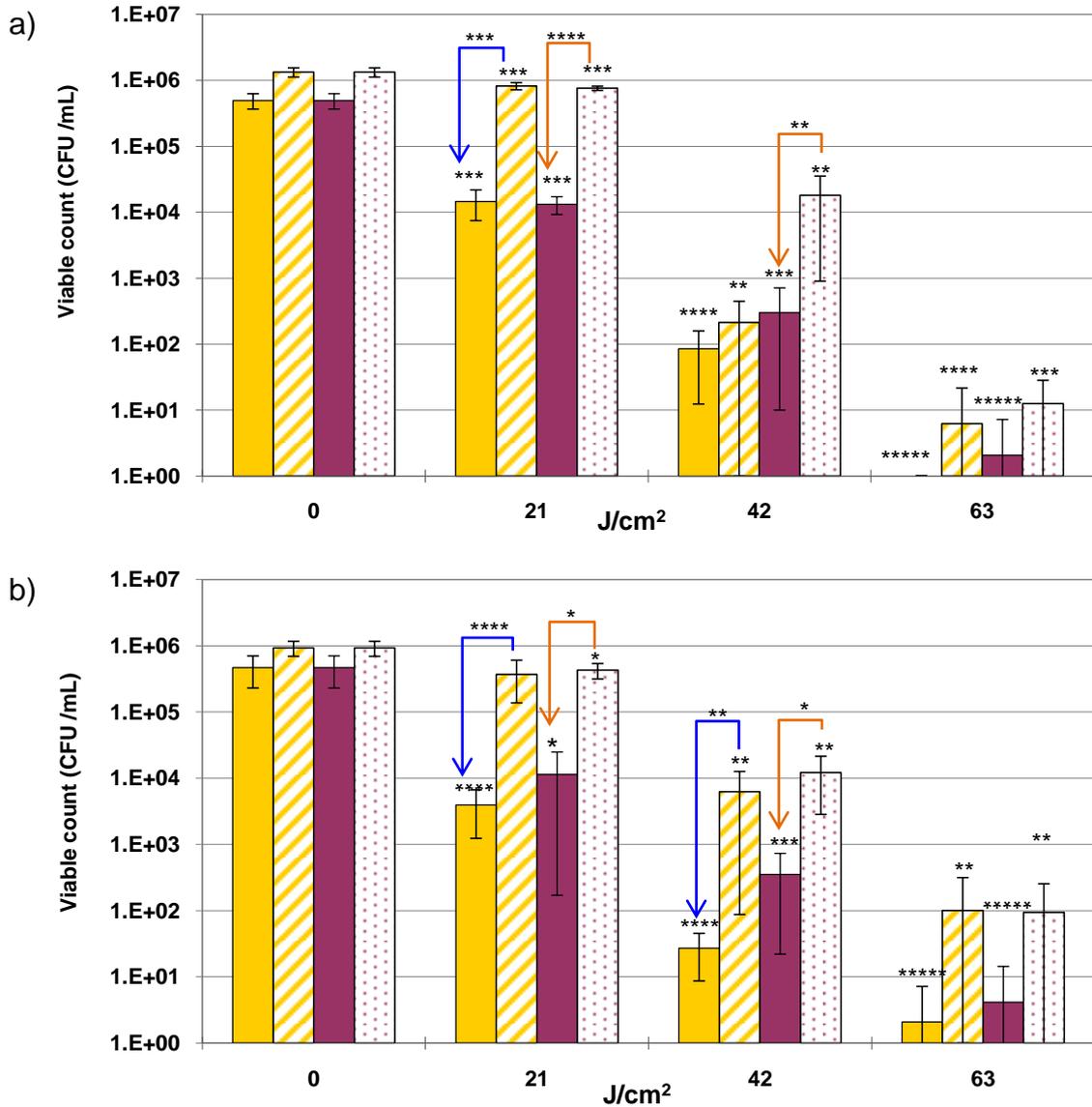


Figure 7-6: Lethal photosensitization of (a) *Staph. aureus* and (b) *Strep. pyogenes* with 100 µg/mL ICG. Bacterial suspensions in PBS were exposed to 21, 42 and 63 J/cm² transmitted either in the continuous mode (■) or in the pulsed mode (■). Identical light energies were delivered either in a continuous mode (▨) or in a pulsed mode (▨) to bacterial suspensions in 12.5% serum. Error bars represent the standard deviation from the mean.

7.3.1.3.1 Photo-sensitivity of methicillin-resistant *Staph. aureus* compared to methicillin-sensitive *Staph. aureus* when present in serum

The difference between the photosensitivity of MSSA and MRSA to 100 µg/mL ICG, when suspended in 12.5% serum, is illustrated in Figure 7-7a and b. The kills of *Staph. aureus* were dependent on the strain, the light dose and the light delivery mode employed. In the presence of serum, greater reductions of 1.8

and 0.9 log₁₀ in the viable count were observed in the case of MSSA, compared to 1.1 and 0.6 log₁₀ reductions for MRSA when exposed to ICG and 42 J/cm² delivered as continuous or pulsed waves, respectively. The presence of serum significantly reduced the number of MRSA killed by 1.1 (P= 0.01) and 1.4 log₁₀ (P= 0.005) upon exposure to 42 J/cm² delivered in continuous and pulsed modes, respectively. Similar patterns of killing inhibitions of 0.8 log₁₀ (P= 0.049) and 1.5 log₁₀ (P= 0.001), due to the presence of serum, were observed in the case of MSSA exposed to 42 J/cm² delivered in continuous or pulsed modes, respectively. In the presence of serum, a continuous light dose of 63 J/cm² resulted in a 5.3 and 3.3 log₁₀ reduction in the viable counts of MSSA and MRSA, respectively. Pulsed light at the same light dose, in the presence of serum, achieved 4.1 and 1.5 log₁₀ reductions in the viable counts of MSSA and MRSA, respectively. Greater kills of MSSA were achieved compared to those of MRSA although these differences were not significant. Both strains were equally susceptible (P=0.5) to ICG-photosensitization (Figure 7-7).

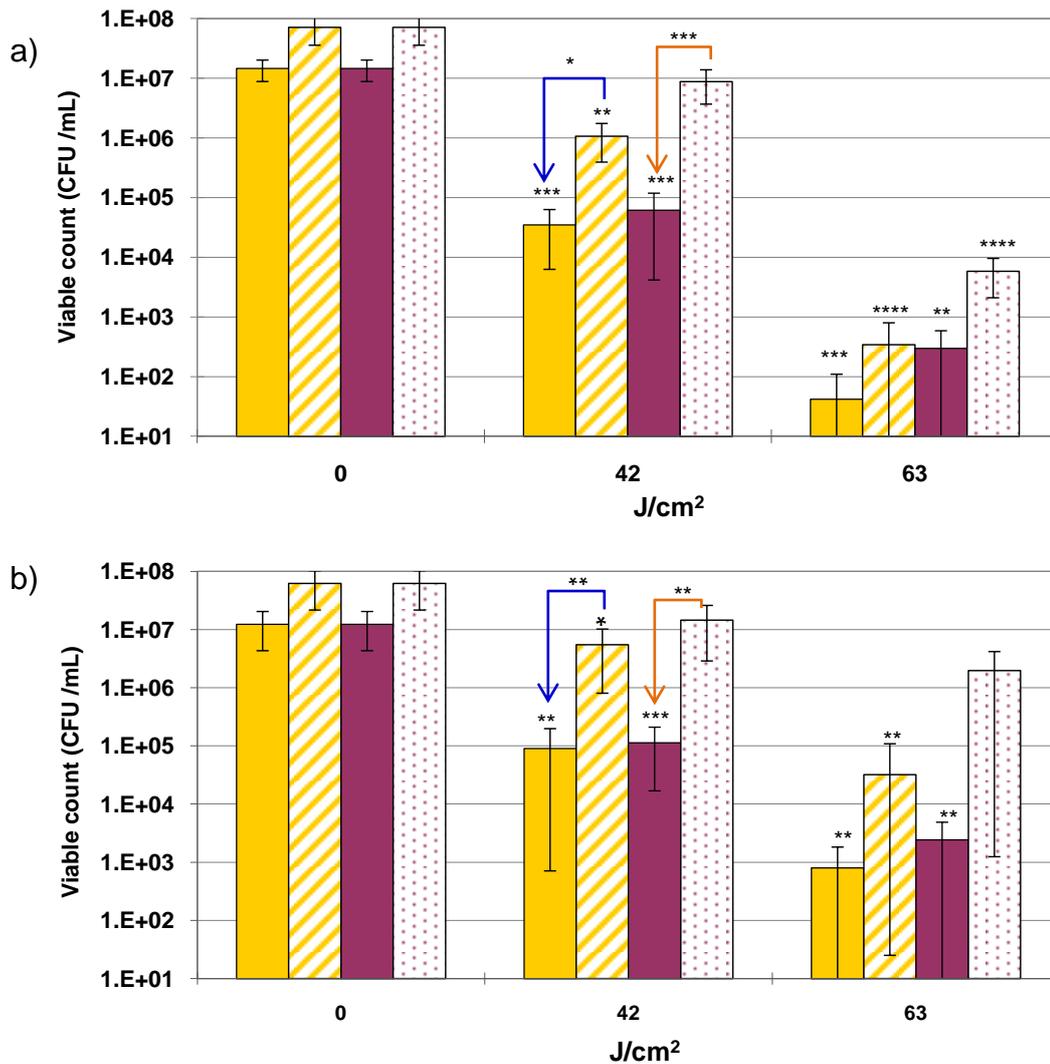


Figure 7-7: Comparison between the susceptibility of (a) MSSA and (b) MRSA to lethal photosensitization using 100 µg/mL ICG combined with the 810 nm NIR laser light. Bacterial suspensions in PBS were exposed to 0, 42 and 63 J/cm² transmitted either in a continuous mode (■) or in a pulsed mode (▨). Identical light energies were delivered either in a continuous mode (▨) or in a pulsed mode (□) to bacterial suspensions in 12.5% serum. Error bars represent the standard deviation from the mean.

7.3.1.3.2 Photosensitization of the Gram-negative organism: *P. aeruginosa* in serum

Continuous NIR-light coupled with 100 µg/mL ICG was capable of photosensitizing 99% of *P. aeruginosa* cells (Figure 7-8). When *P. aeruginosa* cells were treated with 100 µg/mL ICG and exposed to a continuous light dose of 63 J/cm², a significant kill of 1.88 log₁₀ (P= 0.004) was detected in the absence of serum compared to 2.57 log₁₀ (P= 0.001) in its presence. In

contrast, *P. aeruginosa* did not exhibit lethal photosensitization to ICG using pulsed light; kills of 0.2 and 0.5 log₁₀ (P= 0.45 and P= 0.17) were achieved in the absence and presence of serum, respectively (Figure 7-8). Delivering the light continuously was significantly (P= 0.004) more effective than pulsed light at killing *P. aeruginosa* suspended in serum; the reductions in the viable count were 2.57 and 0.5 log₁₀, respectively. The same was observed in the absence of serum, achieving kills of 1.88 log₁₀ by continuous light and 0.2 log₁₀ by pulsed light.

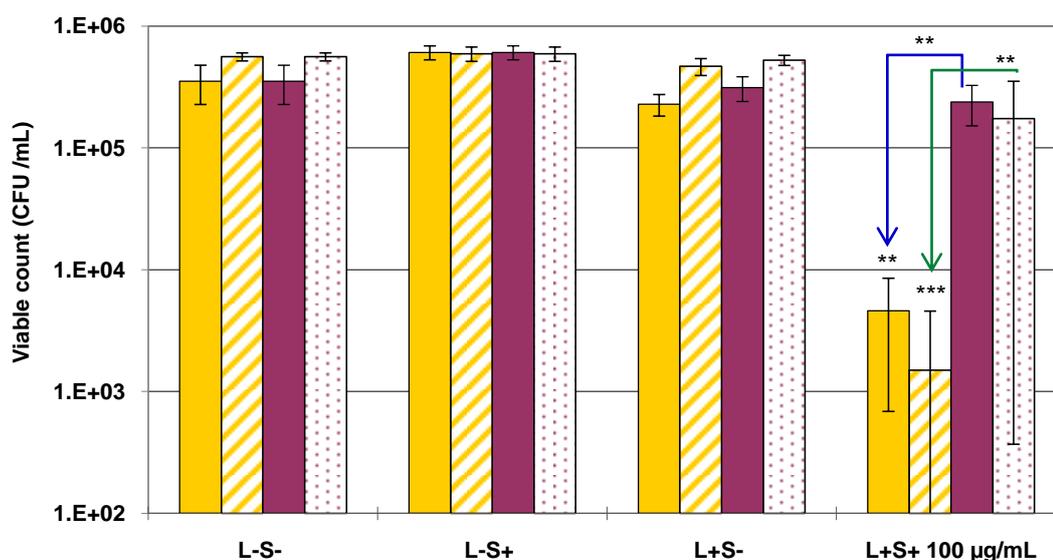


Figure 7-8: Lethal photosensitization of *P. aeruginosa* using an ICG concentration of 100 µg/mL. *P. aeruginosa* suspensions in PBS were exposed to 63 J/cm² delivered either in a continuous mode (■) or in a pulsed mode (▨). The same light energy was delivered either in a continuous mode (▨) or in a pulsed mode (□) to bacterial suspensions in 12.5% serum. Error bars represent the standard deviation from the mean.

7.3.2 The effect of low oxygen concentration on lethal photosensitization

Numerous factors affect the antimicrobial effectiveness of lethal photosensitization, one of these factors is the concentration of the oxygen in the tissue which is directly related to the ¹O₂ yield. Photosensitization of *Staph. aureus* (Figure 7-9a) with 25 µg/mL ICG and a light dose of 90 J/cm² in an anaerobic GasPak™ Pouch system while the system was completely reduced (i.e., an anaerobic atmosphere of less than 1% oxygen and approximately 5% carbon dioxide), resulted in a 1.5 log₁₀ (P= 0.000001) decrease in the viable

count compared with a 2.36 log₁₀ (P= 0.000001) decrease in an unreduced GasPak™ Pouch system. This difference of 0.86 log₁₀ was significant (P= 0.004).

For *Strep. pyogenes* (Figure 7-9b), irradiation of bacterial suspensions under anaerobic conditions significantly inhibited (P= 0.00004) the effectiveness of bacterial killing. Only a 0.6 log₁₀ reduction was achieved in the reduced GasPak™ Pouch system in comparison to a 3.5 log₁₀ reduction in the unreduced GasPak™ Pouch system. For both bacteria, exposure of the suspensions to either light or photosensitizer alone (L+S-, L-S+) induced no significant reductions in the viable counts.

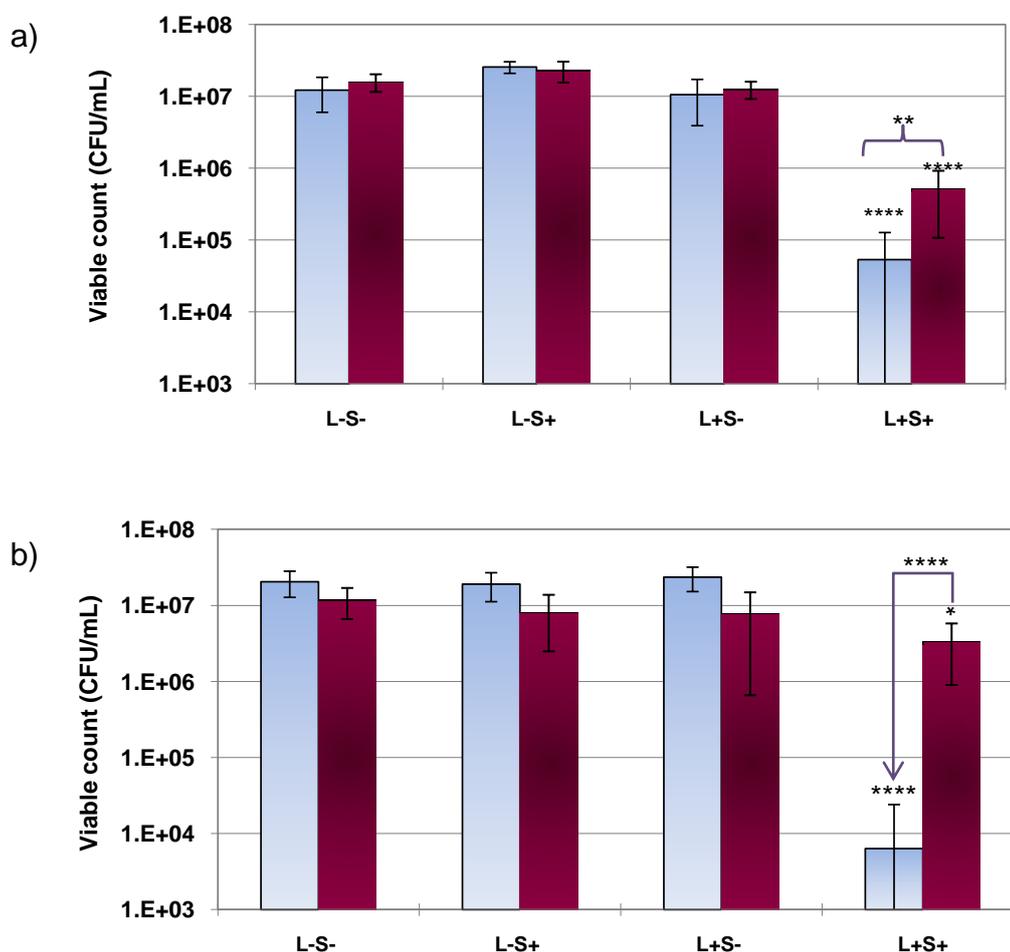


Figure 7-9: Lethal photosensitization of (a) *Staph. aureus* and (b) *Strep. pyogenes* using an ICG concentration of 25 µg/mL. Bacterial suspensions in unreduced (□) or in reduced-anaerobic GasPak™ Pouch system (■) were irradiated with a light dose of 90 J/cm² from the NIR 808 nm Ondine laser at a fluence rate of 0.3 W/cm². Control suspensions were kept in the dark without (L-S-) or with ICG (L-S+). Error bars represent the standard deviation from the mean.

7.4 Discussion

In this part of the study, the effectiveness of lethal photosensitization by ICG on common wound-infecting organisms was accomplished under conditions emulating those found in the wound environment *in vivo*.

A prerequisite for successful antimicrobial PDT to treat wound infections is its effectiveness in the presence of wound fluid. To more closely mimic the conditions likely to be experienced *in vivo*, the effect of serum on the lethal photosensitization of a range of organisms causing wound infections was investigated. The results presented herein showed that substantial kills of wound-infecting bacteria are possible and depend mainly on ICG concentration and the fluence rate of the light.

Lambrechts *et al.*, (2005a) showed that albumin inhibited the photo-inactivation of *Staph. aureus* and exerted a protective effect against the photo-inactivation of the organism. In this study, it was found that a low concentration of ICG (25 µg/mL) was able to photosensitize up to 99.99% of *Staph. aureus* suspensions in saline but only 49.9% of the organism in serum. This inhibition of killing was observed at both low and high fluence irradiation rates. For example, a high fluence rate of 1.37 and 0.3 W/cm² resulted in 49.9% and 7.2% kills, respectively, in 50% serum. Irradiation of *Staph. aureus*, in 6.25% serum, at a low fluence of 0.05 W/cm² reduced the number of viable bacteria by 42.13%. This inhibition of killing may be attributed to the fact that ICG shows high affinity binding to beta-lipoprotein which is a protein present in horse serum (Saito *et al.*, 1996). Serum was found to reduce the effectiveness of lethal photosensitization with ICG possibly because serum proteins in the environment bind to the ICG thereby preventing its uptake, or binding, by the organism (Nitzan *et al.*, 1998). Alternatively, serum proteins may act as quenchers of the singlet oxygen produced thereby protecting the bacterial cells from their lethal effects (Lu & Atkins, 2004). A further possible mechanism is the shielding effect of protein molecules that may decrease light penetration through the suspension (Wilson & Pratten, 1995).

Although serum has an inhibitory effect on the lethal photosensitization of bacteria, increasing the light dose can counteract this effect (Komerik & Wilson,

2002). In the current study, increasing the concentration of ICG overcame the inhibitory effect of serum suggesting that killing of *Staph. aureus* may be achievable *in vivo*. When ICG concentrations of 100-200 µg/mL were activated at high fluence rates by light energies of 411 or 90 J/cm², >99.99% reductions in the viable counts of *Staph. aureus* were achieved in 50% serum. In fact at this point ICG-mediated killing was equally effective both in the absence and presence of serum. When 90 J/cm² was delivered at a low fluence rate of 0.05 W/cm², 100 µg/mL ICG inactivated nearly 99.8% of *Staph. aureus* suspended in 6.25 or 12.5% serum. The fluence rates at which the bacteria were irradiated influenced the bactericidal effect of ICG. Irradiation of *Staph. aureus* with 90 J/cm² at a high fluence rate of 0.3 W/cm² achieved greater kills of 5 log₁₀ compared to 3 log₁₀ at a low fluence irradiation of 0.05 W/cm². Similar results were reported by Urbanska *et al.*, (2002), since a greater photocytotoxic effect was exerted by ICG on SKMEL 188 melanoma cells when a light fluence of 0.2 W/cm² was used instead of 0.1 W/cm². Also, Zeina *et al.*, (2001) showed that the photosensitization of *Staph. epidermidis* using 100 µg/mL MB was dependent on the light intensity. The kill rate increased in proportion to the light intensity.

In a previous study, 2 log₁₀ reductions in the viable count of *P. aeruginosa* and *Klebsiella pneumonia* were achieved using 74.4 J/cm² and 25 µg/mL of the photosensitizer TBO (Koemerik & Wilson, 2002). Herein, in the presence of 50% serum, high fluence irradiation combined with ICG photosensitized approximately 99.99% and 99.67% of *P. aeruginosa* and *E. coli*, respectively. 200 µg/mL ICG activated with 90 J/cm² delivered at 0.3 W/cm² was effective at killing *P. aeruginosa* in saline as well as in serum, achieving 99.99% kills in each case. Serum slightly inhibited *E. coli* killing from 99.99% to 99.67%. 100 µg/mL ICG activated with 90 J/cm² delivered at 0.3 W/cm² resulted in 2.5 log₁₀ kills of *E. coli* in serum compared to 4.3 log₁₀ kill in saline. In contrast, Koemerik & Wilson, (2002) reported that *E. coli* was not susceptible to photosensitization using TBO when suspended in horse serum. This difference may be due to the high concentration of 100% serum used in the latter study.

Interestingly, high fluence 808 nm light alone exerted a cidal effect of 0.7-1.2 log₁₀ against *P. aeruginosa*, achieving 79.3% and 93.2% kills in saline and

serum, respectively. This killing effect was not heat-mediated as the temperature of the bacterial suspensions did not exceed 33°C during irradiation. This finding is supported by the results of a previous study in which irradiation of the organism with 1–80 J/cm² laser light at a wavelength of 810 nm and using an irradiance rate of 0.03 W/cm² resulted in a significant inhibition of bacterial growth (Nussbaum *et al.*, 2003). A possible explanation for this observation is that *P. aeruginosa* has endogenous pigments (pyoverdinin and pyocyanin) that may absorb the light and result in the production of bactericidal species (Reszka *et al.*, 2006). This is supported by the finding that pyocyanin has been successfully used to photosensitize both *Staph. aureus* and *E. coli* (Lipovsky *et al.*, 2008). The present findings suggest that NIR laser light irradiation by itself would also inhibit growth of *P. aeruginosa* in infected wounds.

Further comparisons of the effect of the pulsed versus the continuous mode of irradiation on the lethal photosensitization of wound-infecting organisms in serum were conducted. The results revealed that the pulsed-mode of irradiation was as effective as the continuous-mode for inactivating *Staph. aureus* and *Strep. pyogenes*. However, only the continuous-mode of irradiation was capable of killing *P. aeruginosa*.

For both modes of irradiation, the bactericidal effect was light energy-dose dependent. At the highest light energy of 63 J/cm² and 100 µg/mL ICG, pulsed and continuous modes of irradiation were equally effective in the absence and presence of serum, achieving > 5 log₁₀ reductions in the viable count of *Staph. aureus*. The same pattern of kills was observed in the case of *Strep. pyogenes*, achieving approximately 5 log₁₀ and 4 log₁₀ kills, respectively, in saline and in serum for both modes of irradiation. However, *P. aeruginosa* was photosensitized only using continuous light; it was effective in serum as well as in saline, achieving over 99.99% kills in each case. The photosensitization of the bacteria in serum may be attributed to the ability of ICG to capture the highest proportion of the radiant energy emitted from the NIR laser of 810 nm when bound with the macromolecules in serum. Landsman *et al.*, (1976) showed that the peak absorption of ICG was shifted from 775 nm in H₂O to 805 nm in plasma.

Slightly greater kills of MSSA were achieved than MRSA; both strains were equally susceptible to ICG-photosensitization using pulsed or continuous mode of irradiation. The greater kills observed in the case of MSSA compared to MRSA may be due to the difference in the cell wall structure of both strains. Most MRSA isolates have type 5 polysaccharide microcapsules (Lowy, 1998). Moreover, MRSA strains with reduced vancomycin susceptibility have been proposed to have thickened cell walls (Hiramatsu *et al.*, 1997). This could be the result of up-regulation of cell wall synthesis (Pienaar *et al.*, 2009), or down-regulation of autolysis (Gustafson & Wilkinson, 1989).

Trengove *et al.*, (1996) showed that the total protein concentration in healing wounds is in the range of 36-51 g/L, while for non-healing wounds it lies between 26-46 g/L. Another study (James *et al.*, 2000) reported that the total protein found in wound fluid ranged between 30-49 g/L, while albumin ranged between 25-29 g/L. A third study (Moseley *et al.*, 2004) demonstrated that acute wound fluids had a protein concentration of 1.476 ± 0.123 mg/mL, whereas the chronic wound fluids had a mean protein concentration of 0.644 ± 0.153 mg/mL. The kills described in these studies were conducted in horse serum which has a higher protein content of 60–75 g/L compared with that of wound fluid (30-49 g/L). This suggests that the use of ICG activated with NIR laser light of 810 nm may be clinically applicable *in vivo* to treat wound infections.

Oxygen concentration is one of the factors known to affect the effectiveness of lethal photosensitization. During lethal photosensitization, the formation of cytotoxic ROS occurs through two pathways: the Type I and Type II reactions (Luksiene, 2003, Hamblin & Hasan, 2004). Type II reactions are very dependent on oxygen concentration as the energy is transferred directly to molecular oxygen producing $^1\text{O}_2$. In type I reactions, however, the energy can be transferred to a substrate other than oxygen (e.g. H_2O) yielding free radicals. Thus, under local hypoxia, the mechanism of action may change from a type II to a type I process as a result of the low oxygen concentration (Ochsner, 1997).

Local tissue oxygen tension values of less than 30 mm Hg have been recorded in non-healing wounds and infected wounds (Bowler, 2002). In the current study, in an anaerobic atmosphere of less than 7.6 mm Hg (1% oxygen), the bactericidal effect of ICG activated with 90 J/cm² of light decreased from 99.97% to 71.62% in the case of *Strep. pyogenes*. However, this effect was less pronounced in the case of *Staph. aureus* and only decreased from 99.56% to 96.77%. With decreasing oxygen concentration, the extent of deactivation of the photosensitizer triplet state by oxygen decreases and most of the photosensitizer molecules return to their own ground state. This leads to a reduction in the yield of ¹O₂ which is the main antimicrobial cytotoxic species (Maisch *et al.*, 2007).

In summary, in the presence of serum, high and low light intensities were able to achieve killing of *Staph. aureus* at high concentrations of ICG. ICG-mediated photo-cytotoxicity was slightly inhibited in the case of *E. coli*, but was unaffected for *P. aeruginosa*. Furthermore, pulsed and continuous modes of irradiation resulted in substantial kills of *Staph. aureus* and *Strep. pyogenes* - even in the presence of serum the kills achieved were light-energy dose-dependent. Only continuous irradiation was capable of photosensitizing *P. aeruginosa*, both in the absence and presence of serum, achieving substantial kills of this organism. If these kills are achievable *in vivo*, ICG in combination with NIR light may be an effective means of eradicating bacteria from wounds and burns. The decrease in the effectiveness of lethal photosensitization of bacteria under anaerobic conditions confirms that the greater the oxygen concentration present in the environment, the greater the photolethal effect of ICG. These findings imply that the level of tissue oxygenation is an important factor to consider during the attempted eradication of bacteria from wounds.

Chapter 8

The underlying mechanism of lethal photosensitization

8.1 Introduction

The results discussed in the previous chapters show that wound-infecting organisms can be killed by ICG photosensitization, yet the mechanism by which ICG causes bacterial cell death has not been established. It may occur by a type I or type II mechanism. This chapter focuses on studies to investigate the underlying mechanisms involved in the lethal photosensitization of the most common wound-infecting organisms.

Type I and type II reactions are two mechanisms by which the triplet state PS can react with oxygen, water or biomolecules in the tissues to produce ROS and free radicals. The cytotoxic effects of lethal photosensitization are due to photo-damage to subcellular organelles and biomolecules by these ROS and free radicals (Macdonald & Dougherty, 2001). Type I reactions produce free radicals which then react promptly, usually with oxygen, producing highly ROS (e.g. the superoxide and the peroxide anions). Type II reactions produce singlet oxygen which is an electronically excited and highly reactive state of oxygen (Gomer *et al.*, 1989). During the course of the photosensitization process, it is difficult to distinguish between the two reactions. Although the type II process is considered the more important reaction mechanism in photosensitization, cytotoxic species generated by the type I reaction process can also play a part. There is probably a contribution from both type I and II processes depending mainly on oxygen tension (Tanielian *et al.*, 2000).

Since $^1\text{O}_2$ is the main ROS that is responsible for the photo-damage of bacteria, it was important to detect its production during the excitation of ICG by NIR laser light. Methods for $^1\text{O}_2$ detection include spin trapping Electron Paramagnetic Resonance (EPR) spectroscopy (Hideg *et al.*, 1994), chemical trapping (Telfer *et al.*, 1994) and luminescence signal at 1270 nm (Maisch *et al.*, 2007). The current spread of fluorescence imaging techniques has led to the development of a number of $^1\text{O}_2$ fluorescent probes. A new fluorescent probe, singlet oxygen sensor green reagent (SOSGR), has been successfully used to detect $^1\text{O}_2$ formation in various fields such as light-activated plant defence (Ramel *et al.*, 2009), and plasmonic engineering of $^1\text{O}_2$ production (Zhang *et al.*, 2008). Normally, this reagent emits weak blue fluorescence peaks at 395 and 416 nm under excitation of 372 and 393 nm. In the presence

of $^1\text{O}_2$, it emits a green fluorescence similar to that of fluorescein with excitation/emission of 504/525 nm respectively (Molecular Probes Product Information, 2004). This green fluorescence emission is produced due to an endoperoxide generated by the interaction of $^1\text{O}_2$ with the anthracene component of SOSG, as observed for other fluorescein–anthracene probes (Tanaka *et al.*, 2001). In this part of the study, the photodynamic activity of the dye ICG was assessed by determining its ability to generate singlet oxygen using the SOSG reagent. The study of photosensitization of *Staph. aureus* in the presence of a $^1\text{O}_2$ enhancer or quencher may elucidate some of the mechanisms involved in ICG-mediated photo-killing.

An activated PS can induce photo-damage when it is in close proximity to bacterial cells. When the PS is taken up by bacterial cell the sites of photo-damage depend on its subcellular localisation (Minnock *et al.*, 1996). A variety of cellular components may be targeted including amino acids (mainly cysteine, histidine, tryptophan, tyrosine and methionine), nucleosides (primarily guanine) and unsaturated lipids which can react with $^1\text{O}_2$ (Girotti, 2001). The diffusion distance of $^1\text{O}_2$ is relatively short up to 75 nm (Moan, 1990; Ouedraogo & Redmond, 2003), therefore, the binding of PS molecules with the substrate may lead to more efficient photosensitization. Determination of the uptake of ICG by different bacterial species was also an additional aim in this study.

The lethal photosensitization process may be accompanied by heat production due to the decay of the excited PS molecules back to the ground state (Green *et al.*, 1988). The use of light fluence rates greater than 300 mW/cm² during PDT can also induce localized heating in the exposed area; therefore, adjunctive hyperthermia may be in progress during certain PDT procedures (Gomer *et al.*, 1988). In the current study, it was very important to record the temperature increase, especially during high fluence irradiation, and to establish whether or not kills were partially mediated by the thermal effect.

8.2 Materials and Methods

8.2.1 Photosensitizer preparation and irradiation system

ICG preparation was described in section 2.1.4.

Irradiation was carried out using the 0.5 W Ga-Al-As laser (Thor laser), or the 0.4 W diode laser (Ondine laser). Both lasers emit continuous wave laser light with a wavelength of 808 ± 5 nm. The characteristics of each laser were described in detail in section 2.1.3.

8.2.2 The evaluation of the role of ROS in ICG-photosensitization

8.2.2.1 Detection of $^1\text{O}_2$ formation by ICG using the SOSGR Assay

The procedures for the SOSGR assay are described in Chapter 2, section 2.4.1.

Irradiation was carried out using the 808 nm NIR Ondine laser. The light was delivered at a fluence rate of 0.3 W/cm^2 . The total energy dose applied to the samples was in the range of $0\text{-}90 \text{ J/cm}^2$, by varying the irradiation time. The measurements were then expressed as the relative change in the fluorescence over time for solutions containing: $5 \mu\text{M}$ SOSGR and $25 \mu\text{g/mL}$ ICG or $5 \mu\text{M}$ SOSGR on its own, as a control.

8.2.2.2 Evaluation of lethal photosensitization of *Staph. aureus* in the presence of a singlet oxygen scavenger or enhancer

The extent of *Staph. aureus* killing in the presence of the $^1\text{O}_2$ quencher L-Tryptophan or in the presence of the $^1\text{O}_2$ enhancer D_2O was investigated.

8.2.2.2.1 Target organisms and growth conditions

The organism used in this series of experiments was: *Staph. aureus* NCTC 8325-4. The culture conditions have been described in section 2.1.2. The procedures were modified according to section 2.4.2.1 for the purpose of L-tryptophan experiments or according to section 2.4.3.1 for D_2O experiments.

8.2.2.2.2 Preparation of L-Tryptophan and detection of its minimal toxic concentration

Preparation of the scavenger L-tryptophan was carried out as described in section 2.4.2.2. Detection of the minimum toxic concentration of L-tryptophan for *Staph. aureus* was carried out as described in section 2.4.2.3.

8.2.2.2.3 Lethal photosensitization in the presence of L-Tryptophan

To determine if singlet oxygen or free radicals were involved in the lethal photosensitization process, 10 or 12 mM L-tryptophan was used to quench any ROS generated by ICG photosensitization. The procedures are described in detail in section 2.4.2.4. In 10 mM L-tryptophan, bacterial suspensions were treated with 25 µg/mL ICG and irradiated at a fluence rate of 1.37 W/cm² and a light dose of 82 J/cm² from the 808 nm Thor laser. While in 12 mM L-tryptophan, bacterial suspensions were treated with an identical ICG concentration and irradiated at a fluence rate of 0.3 W/cm² and a light dose of 54 J/cm² from the 808 nm Ondine laser.

8.2.2.2.4 Lethal photosensitization in D₂O

To determine if singlet oxygen was involved in the lethal photosensitization process, D₂O was used to extend the life span of any singlet oxygen generated by exposure of ICG to NIR laser light. The procedures were carried out as described in Chapter 2, section 2.4.3.2. *Staph. aureus* cells suspended in D₂O and a final ICG concentration of 25 µg/mL were irradiated at a fluence rate of 1.37 W/cm² and light dose of 82 J/cm² from the 808 nm Thor laser. Also bacterial suspensions exposed to light energies of 18 or 54 J/cm² delivered at a fluence rate of 0.3 W/cm² from the 808 nm Ondine laser.

8.2.3 Uptake of ICG by bacterial cells

8.2.3.1 Organisms investigated

The organisms used in these experiments were: *Staph. aureus* NCTC 8325-4, *Strep. pyogenes* ATCC 12202 and *P. aeruginosa* PA01. The culture conditions have been described in section 2.1.2. The bacterial suspensions contained 10⁷ CFU/mL for *Staph. aureus* and, *Strep. pyogenes*, and 10⁸ CFU/mL for *P. aeruginosa*

8.2.3.2 Lethal photosensitization of bacteria after removal of unbound

ICG

1.7 mL of the bacterial suspension was treated with an equal volume of ICG to give a final ICG concentration of 25 µg/mL for *Staph. aureus*, *Strep. pyogenes* and 200 µg/mL for *P. aeruginosa*. These suspensions were incubated with ICG for 30 minutes in the dark at RT. Aliquots of 100 µL of these cultures were

placed in four replicate wells of a sterile, flat-bottomed, untreated 96-well plate (Nunc, Roskilde, Denmark) and irradiated with a light energy of 90 J/cm^2 at a fluence rate of 0.3 W/cm^2 from the NIR Ondine laser, with stirring. Four additional wells containing similar 100 μL aliquots were kept in the dark to serve as a control. Unbound ICG was washed out twice (by 1 mL PBS) from 1 mL of the dye/bacterial suspension by centrifugation for 10 min at 14,000 rpm. Bacterial pellets were resuspended in 1 mL PBS. Aliquots (100 μL) of these washed bacterial suspensions were irradiated with an identical light energy of 90 J/cm^2 . Four additional 100 μL aliquots of the washed suspensions were incubated in the dark to serve as a control. Following irradiation/dark incubation, each sample was serially diluted 1 in 10 in PBS. 20 μL of each dilution was plated in duplicate either on blood agar (*Staph. aureus* and *Strep. pyogenes*) or nutrient agar (*P. aeruginosa*) plates and the plates incubated for 48 hours at 37°C . The surviving organisms were enumerated by colony counts.

8.2.3.3 Extraction of ICG from bacterial cells and quantification of its uptake

Bacterial cells (10 mL) were incubated with 25 $\mu\text{g/mL}$ ICG for 30 minutes in the dark at RT. After the incubation, bacterial cells were washed twice, as described in the previous section, then treated with 10 mL of 2% sodium dodecyl sulfate (SDS) solution. These were placed on a slow orbital shaker for 2 hours in the dark at room temperature in order to extract the cell-bound ICG. The supernatant solution was taken for ICG quantification. Quantification of ICG was done spectrophotometrically in six-replicates by scanning of the absorbance spectrum of the dissolved cells in the range of 500 – 850 nm using a UNICAM UV 500 UV/Visible spectrophotometer (ThermoSpectronic, Rochester, NY, USA). Blanks were constructed for each bacterial culture incubated with ICG for 0 minute, washed then lysed in 2% SDS.

8.2.4 Measurements of the temperature during bacterial photosensitization

The temperatures of 100 μL bacterial aliquots, treated with different concentrations of ICG (0-200 $\mu\text{g/mL}$), were recorded immediately before and after irradiation of the samples using an immersion thermocouple probe

connected to a Fluke 179 digital multimeter (Fluke. USA). The temperatures were recorded for (1) bacterial suspensions treated with 25 µg/mL and irradiated at fluence rate of 1.37 W/cm² with various light energies from 0-411 J/cm²; (2) bacterial suspension treated with various ICG concentrations and irradiated with 90 J/cm² at a fluence rate of 0.3 W/cm²; and (3) bacterial suspensions irradiated with a pulsed or continuous light energy of 63 J/cm² at a fluence rate of 0.7 W/cm² at various ICG concentrations

8.2.5 Experiments to determine the effect of elevated temperatures on bacterial viability

8.2.5.1 Organisms investigated

The organisms used in these experiments were: *Staph. aureus* NCTC 8325-4, *P. aeruginosa* PA01 and *E. coli* ATCC 25922. The culture conditions are described in section 2.1.2. For the purpose of these experiments, bacterial cells were then harvested by centrifugation and were resuspended in an equal volume of PBS or 100% HS (for *Staph. aureus* only). All bacteria were diluted in PBS except for *Staph. aureus* which were diluted in either PBS or 100% HS to an optical density of 0.05.

8.2.5.2 Effect of elevated temperatures on bacterial viability

In the case of *Staph. aureus*, 63 µL of bacterial suspensions either in PBS or in 100% HS were added to an equal volume of ICG to give a final concentration of 25 or 200 µg/mL. Aliquots (63 µL) of *P. aeruginosa* or *E. coli* in PBS were added to equal volumes of ICG to give a final concentration of 200 µg/mL for *P. aeruginosa* or 100 µg/mL for *E. coli*. Controls were prepared by adding an equal volume of PBS instead of ICG. The *Staph. aureus* suspensions were incubated either at 40 °C (suspension in PBS) or 50 °C (suspension in 50% HS) whereas *P. aeruginosa* was incubated at 50 °C and *E. coli* was incubated at 42 °C. All aliquots were incubated for 10 minutes in the dark. The survivors were enumerated by viable counting. Each experiment was performed at least twice in four replicates.

8.3 Results

8.3.1 The role of reactive oxygen species in lethal photosensitization

8.3.1.1 Imaging the production of singlet oxygen, using a new fluorescent sensor, singlet oxygen sensor green

In order to quickly image the production of $^1\text{O}_2$, the fluorescence of the SOSGR was measured using a spectrofluorometer using excitation and emission wavelengths of 485 nm and 538 nm, respectively, for solutions containing: 5 μM SOSGR and 25 $\mu\text{g/mL}$ ICG or 5 μM SOSGR in 50% methanol after 0, 1, 3 and 5 minutes of irradiation with light from the 808 nm NIR Ondine Laser at a fluence rate of 0.3 W/cm^2 (Figure 8-1). The fluorescence intensity of 5 μM SOSGR containing 25 $\mu\text{g/mL}$ ICG solutions increased linearly as the irradiation time increased. The aqueous solution of SOSGR alone showed no increase in fluorescence with increasing irradiation time. At a light dose of 18 J/cm^2 , the fluorescence increased significantly ($P < 0.000001$) compared to the control kept in the dark. This fluorescence was significantly lower ($P < 0.000001$) than that observed at higher light doses of 54 and 90 J/cm^2 . Increasing the light dose to 54 J/cm^2 , increased the fluorescence value significantly from 425 to 1773. The greatest fluorescence ($P < 0.000001$) was observed at the highest light dose of 90 J/cm^2 . This differed significantly ($P = 0.001$) from those observed at a light dose 54 J/cm^2 . These results suggest that the greater the light energy absorbed by the dye, the greater the extent of $^1\text{O}_2$ generation.

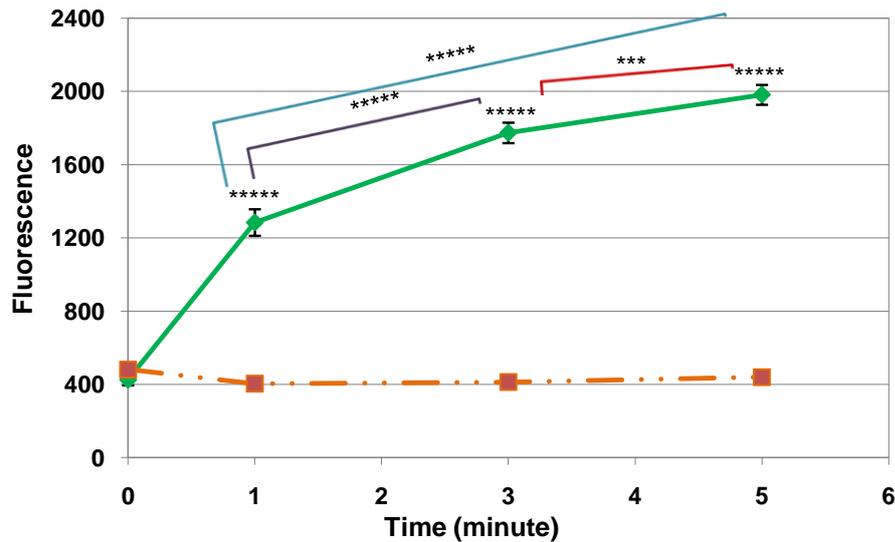


Figure 8-1: Fluorescence response of singlet oxygen sensor green reagent to different light exposure times in the presence of ICG using a spectrofluorometer and excitation and emission wavelengths of 485 nm and 538 nm, respectively. Solutions contained: 5 μM SOSGR and 25 $\mu\text{g/mL}$ ICG (\blacklozenge) or 5 μM SOSGR in 50% methanol (\blacksquare) after 0, 1, 3 and 5 minutes irradiation. Error bars represent the standard deviation from the mean.

8.3.2 Lethal photosensitization of *Staph. aureus* by ICG in the presence of a singlet oxygen scavenger or enhancer

8.3.2.1 Thor laser

The singlet oxygen scavenger, L-tryptophan, had no bactericidal effect on *Staph. aureus* (Figure 8-2). Furthermore, L-tryptophan significantly reduced the lethal photosensitization of *Staph. aureus* by ICG ($P = 0.00002$) upon exposure to a light dose of 82 J/cm^2 delivered at a fluence rate of 1.37 W/cm^2 . One \log_{10} reduction in the number of *Staph. aureus* killed was found in the absence of 10 mM L-tryptophan over that in the presence of this scavenger (Figure 8-3a). Conversely, D_2O considerably enhanced the killing of *Staph. aureus* by ICG with a 1.62 \log_{10} greater reduction in viable counts compared to those achieved in the absence of D_2O ($P = 0.007$) (Figure 8-3b).

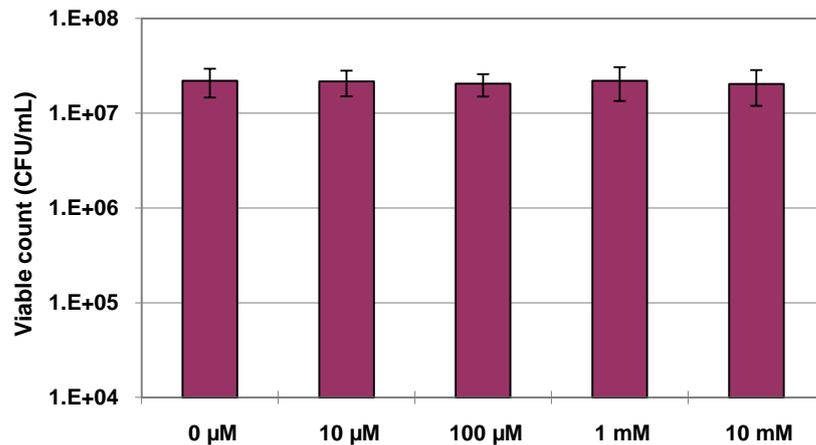


Figure 8-2: Viability of *Staph. aureus* in different concentrations of L-tryptophan. Error bars represent the standard deviation from the mean.

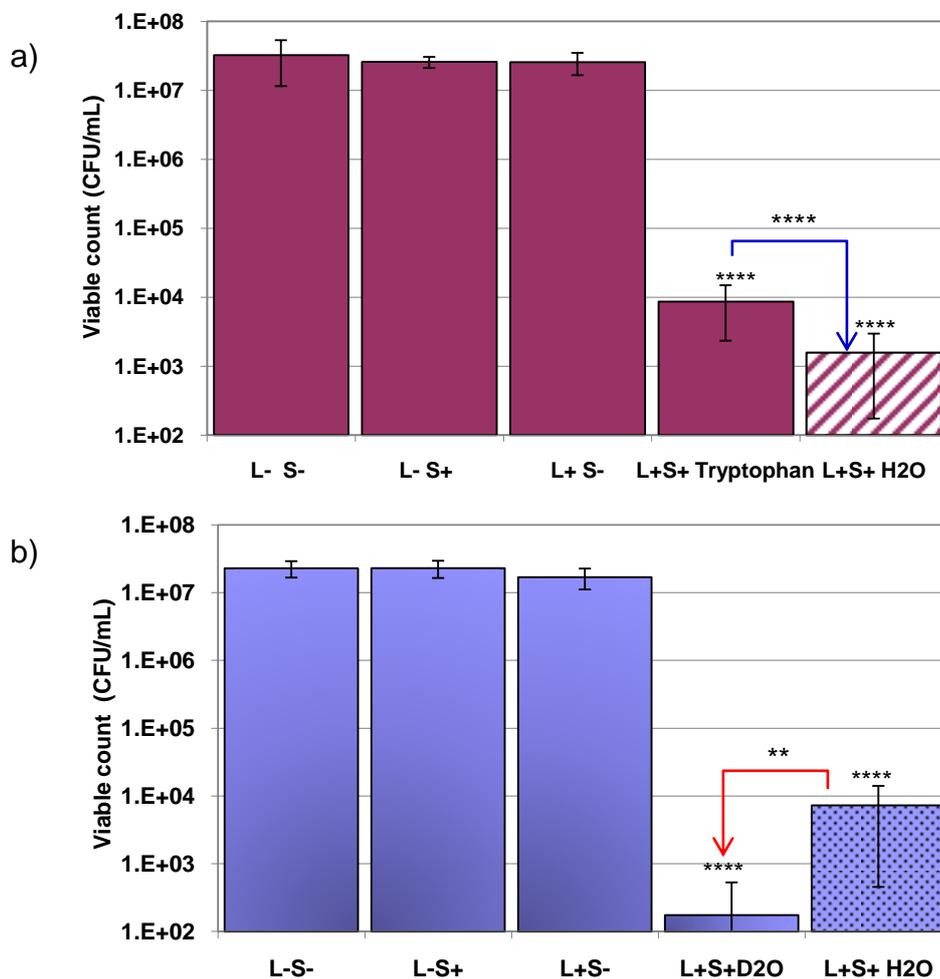


Figure 8-3: Lethal photosensitization of *Staph. aureus* (a) suspended in 10 mM L-tryptophan (■) or suspended in H₂O (▨) and (b) suspended in D₂O (■) or suspended in H₂O (▨), using 25 μg/mL ICG and irradiated at a fluence rate of 1.37 W/cm² and a light dose of 82 J/cm² from the 808 nm Thor laser. Error bars represent the standard deviation from the mean.

8.3.2.2 Ondine laser

Increasing the concentration of L-tryptophan to 12 mM partially protected *Staph. aureus* from ICG-photosensitization upon exposure to a light dose of 54 J/cm², delivered at a fluence rate of 0.3 W/cm² from the Ondine laser (Figure 8-4). By using a higher concentration of 12 mM L-tryptophan, a greater protection was provided, achieving 2.3 log₁₀ reduction in *Staph. aureus* viable counts compared to 4.5 log₁₀ in the absence of this scavenger. This reduction of 2.2 log₁₀ in the number of *Staph. aureus* killed was significant (P = 0.007).

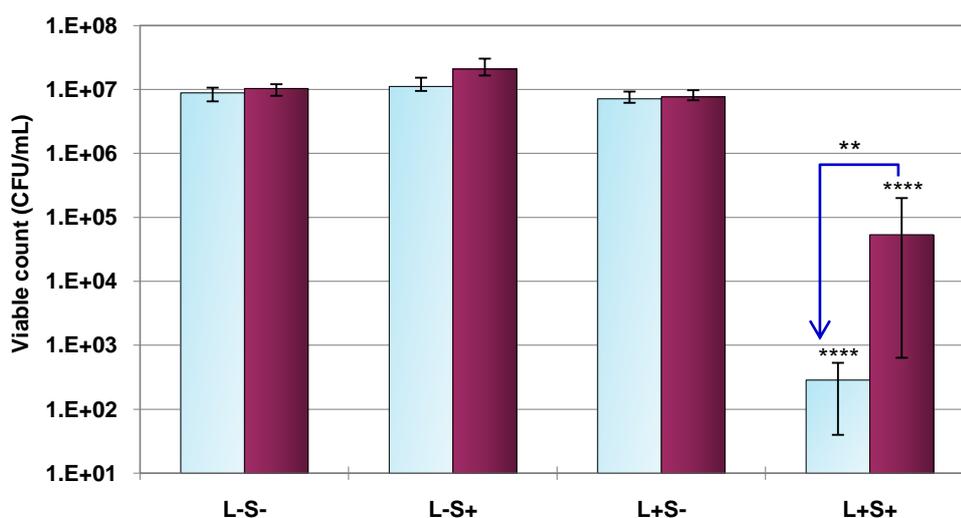


Figure 8-4: Lethal photosensitization of *Staph. aureus* suspended in H₂O (□) or suspended in 12 mM L-tryptophan (■) by 25 µg/mL ICG irradiated at a fluence rate of 0.3 W/cm² and a light dose of 54 J/cm² from the 808 nm Ondine laser. Error bars represent the standard deviation from the mean.

Figure 8.5 shows the enhancement caused by D₂O in the photosensitization of *Staph. aureus* using 25 µg/mL ICG and light energies of 18 or 54 J/cm² delivered at a fluence rate of 0.3 W/cm². At a light dose of 18 J/cm², D₂O considerably enhanced (P = 0.001) the reduction of *Staph. aureus* viable counts by 0.6 log₁₀ (Figure 8-5a). At a higher light dose of 54 J/cm², a further reduction in *Staph. aureus* viable counts of 1.5 log₁₀ (P = 0.0003) was achieved in the presence of D₂O (Figure 8-5b).

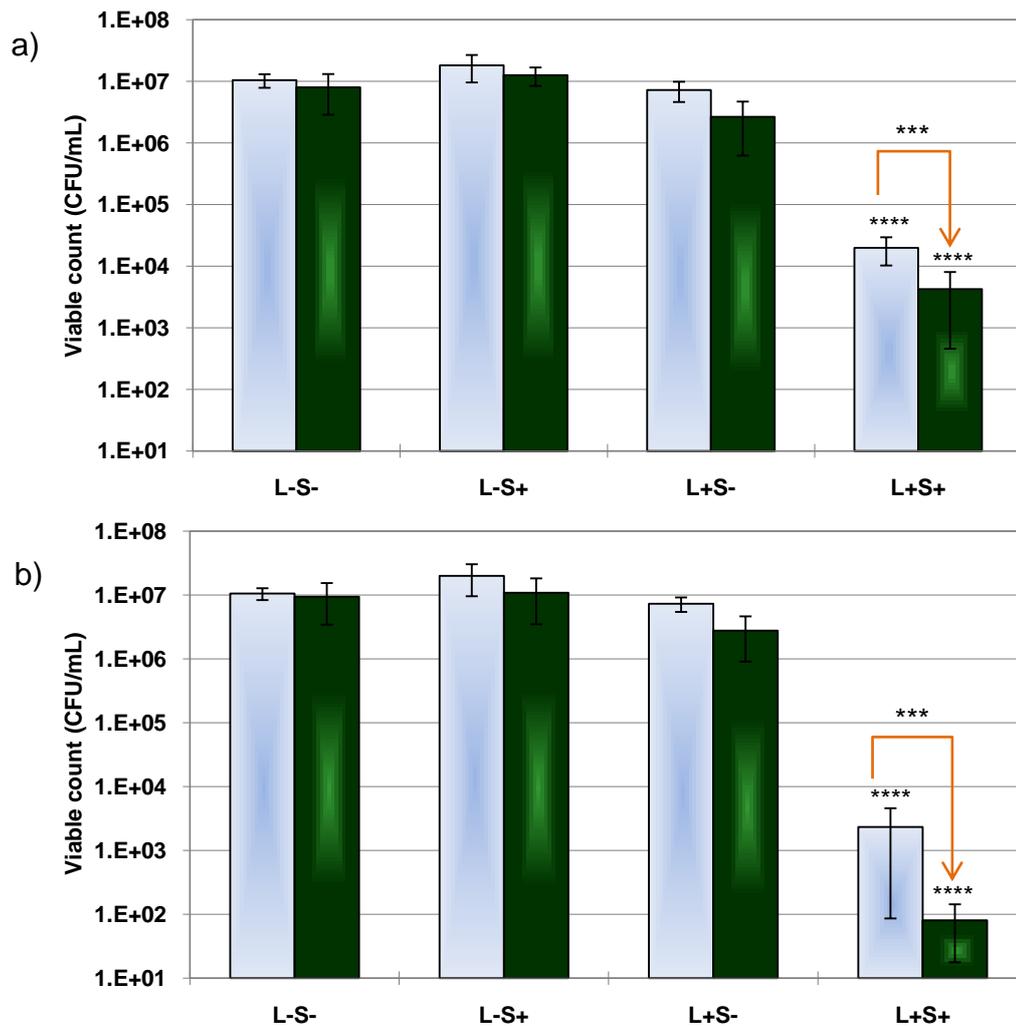


Figure 8-5: Lethal photosensitization of *Staph. aureus* suspended in H₂O (□) or suspended in D₂O (■) by 25 µg/mL ICG irradiated at a fluence rate of 0.3 W/cm² and light energies of (a) 18 J/cm² and (b) 54 J/cm² from the 808 nm Ondine laser. Error bars represent the standard deviation from the mean.

8.3.3 Effect of washing ICG from cell suspension on photosensitization of bacteria

The effect that washing ICG from the cell suspension had on lethal photosensitization was dependent on the target species (Figure 8-6). The number of *Staph. aureus* killed with ICG washed from the cell suspension was significantly lower ($P = 0.01$) than when ICG was still present (Figure 8-6a). However, the 2.9 log₁₀ reduction in *Staph. aureus* viable counts, when ICG was washed from the cell suspension, was still significant ($P = 0.00001$). A similar kill pattern was observed for *Strep. pyogenes*: the number of *Strep. pyogenes* killed with ICG washed from the cell suspension was significantly lower ($P = 0.0001$) than when ICG was still present (Figure 8-6b). Yet, the 2.7 log₁₀

reduction in *Strep. pyogenes* viable counts, when ICG was washed from the cell suspension, was still significant ($P = 0.00001$).

In contrast, the effectiveness of lethal photosensitization was dramatically inhibited when ICG was washed out from *P. aeruginosa* cell suspensions (Figure 8-6c). Washing ICG from the suspension resulted in a $0.02 \log_{10}$ reduction in the viable counts of *P. aeruginosa* compared to the significant $6.77 \log_{10}$ ($P < 0.000001$) reduction achieved without washing the PS. These amounted to 4% and 99.999% kills with and without washing ICG from the bacterial suspensions, respectively. This kill difference of $6.75 \log_{10}$ was significant ($P < 0.000001$).

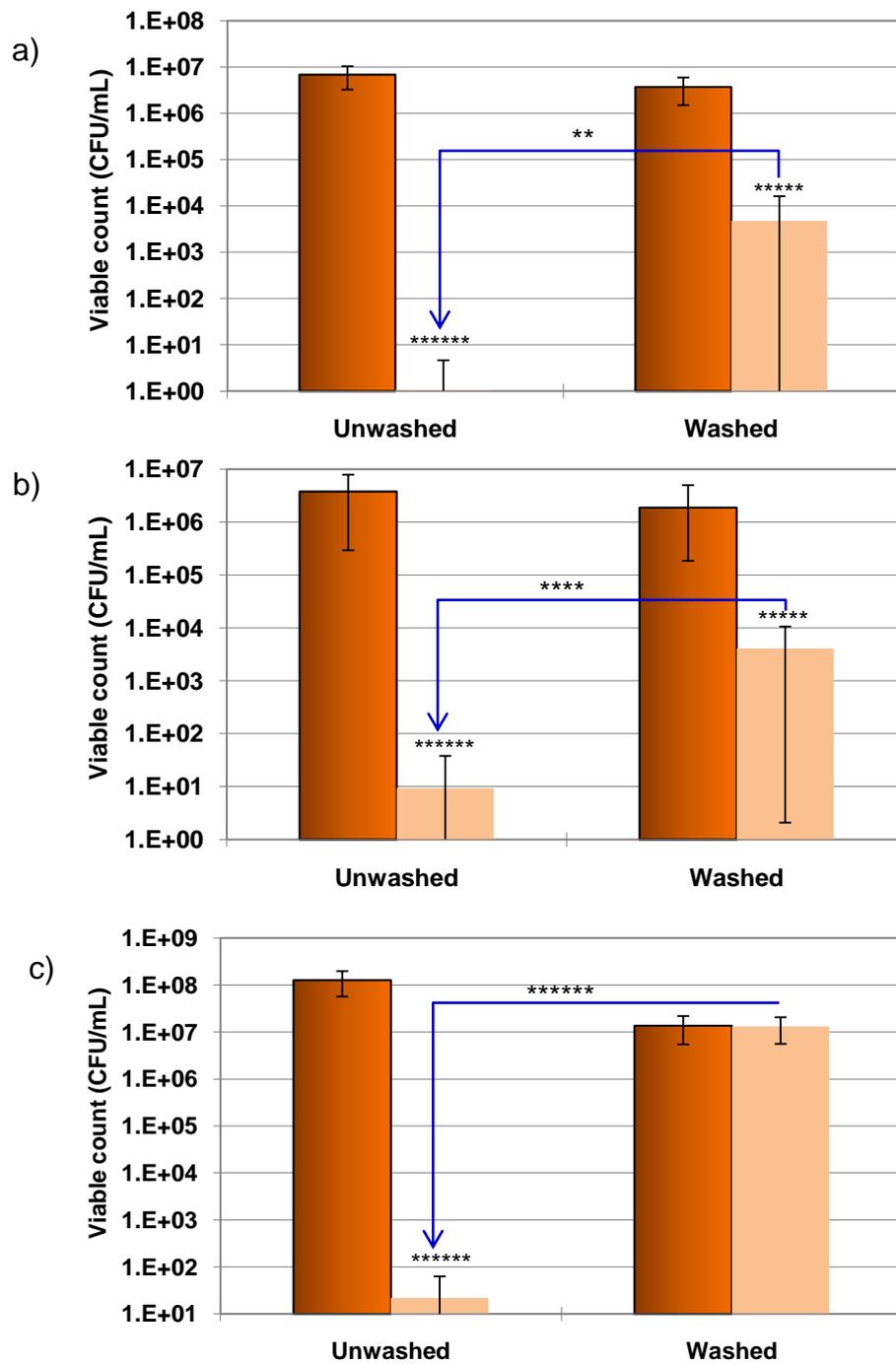


Figure 8-6: Lethal photosensitization of (a) *Staph. aureus*, (b) *Strep. pyogenes* by 25 $\mu\text{g/mL}$ ICG and (c) *P. aeruginosa* by 200 $\mu\text{g/mL}$ ICG. Bacterial cells were irradiated at a fluence rate of 0.3 W/cm^2 and a light energy of 90 J/cm^2 (□) from the 808 nm Ondine laser either while ICG left in cell suspension during illumination or washed from the cells before illumination. Control cultures of washed and unwashed bacteria were kept in the dark (■). Error bars represent the standard deviation from the mean.

Figure 8-7 shows the absorption spectrum of bacterial cells which were pre-incubated with 25 $\mu\text{g/mL}$ ICG for 30 minutes then washed out from the PS and lysed by 2% SDS solution. The scan revealed that even after washing ICG

from *Staph. aureus* and *Strep. pyogenes* cultures, the lysed cells in 2% SDS showed a peak absorbance at 795 nm. Conversely, scanning of lysed *P. aeruginosa* cells which were washed from the same concentration of ICG showed no peak absorbance compared to the Gram-positive bacteria. These results imply that ICG may be able to bind to the Gram-positive bacteria but not to the Gram-negative bacteria.

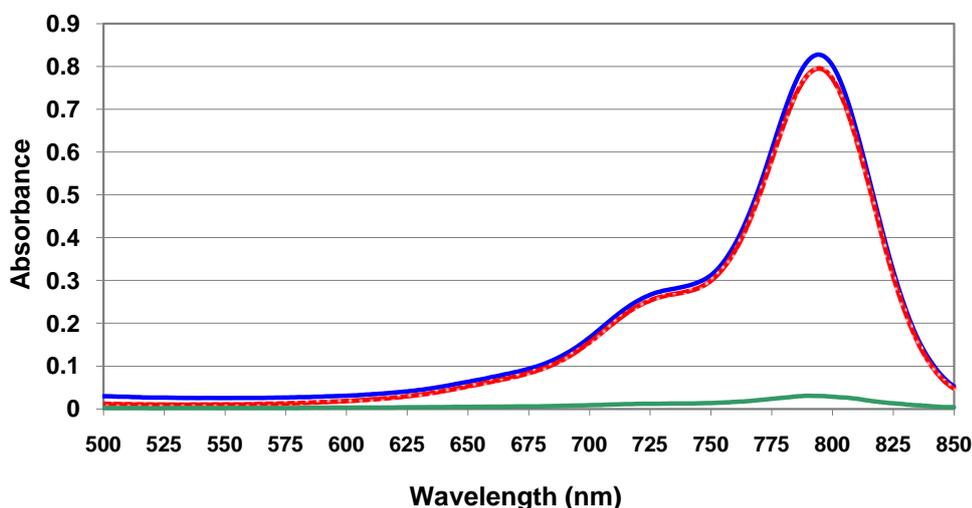


Figure 8-7: Absorbance scan of *Staph. aureus* (—), *Strep. pyogenes* (—) and *P. aeruginosa* cells (—) lysed in 2% SDS after being washed out from 25 µg/ml ICG (incubation time with ICG= 30 minutes), demonstrating amount of ICG taken up by cells.

8.3.4 Temperature elevation during lethal photosensitization and its effect on the viability of bacteria

8.3.4.1 Temperature changes during high intensity lethal photosensitization

The temperature of the bacterial suspensions was elevated during high intensity lethal photosensitization with ICG (Tables 8-1, 8-2 and 8-3). The rises in temperature depended on the light dose delivered and the concentration of the photosensitizer. The temperature of the bacterial suspensions increased from 22°C to 35.63 ± 1.62°C during irradiation with the highest light dose and a dye concentration of 25 µg/mL, whereas using 200 µg/mL of ICG in either PBS or 50% HS, the temperature increased to a maximum of 47°C (Table 8-1). Table 8-1 shows that the temperature of bacterial suspensions in the presence of 25 µg/mL ICG increased to 25, 30.4, 33.5, 36 °C upon exposure to light energies of 0, 82, 247 and 411 J/cm², respectively, delivered at a high fluence

rate of 1.37 W/cm², whereas in the absence of ICG the temperature was 24.4, 28, 31 and 33 °C, respectively. Not only increasing the light energy absorbed by the dye, but also increasing the concentration of the dye itself resulted in an increased temperature. Irradiating the suspensions with a light energy of 90 J/cm², delivered at a fluence rate of 0.3 W/cm², with varying ICG concentrations resulted in a linear temperature increase (Table 8-2). However, these increases were less than those attained with the highest light dose (411 J/cm²), resulting in temperatures of 27.5, 30.8, 40, 43.5 °C at concentrations of 0, 25, 100 and 200 µg/mL ICG and a light dose of 90 J/cm². The same linear temperature increases were also observed when the light energy of 63 J/cm² was pulsed and delivered at a fluence rate of 0.7 W/cm² (Table 8-3).

Table 8-1: The temperature of the bacterial suspension upon exposure to different light energies, at a fluence rate of 1.37 W/cm², in the presence or absence of ICG solutions

Light energies (J/cm ²)	0	82	247	411
0 µg/mL ICG (PBS)	24.42 ± 0.97 °C	27.9 ± 2.25°C	30.92 ± 2.79°C	32.97 ± 1.39°C
25 µg/mL ICG (in PBS)	25.05 ± 0.65 °C	30.35 ± 1.67°C	33.48 ± 1.35°C	35.63 ± 1.62°C
200 µg/mL ICG	In PBS	-	-	47.13 ± 1.38°C
	In 50% HS	-	-	44.63 ± 3.01°C

Table 8-2: The temperature of the bacterial suspension upon irradiation with a light energy of 90 J/cm² at a fluence rate of 0.3 W/cm² at various ICG concentrations

ICG concentrations (µg/mL)	0	25	100	200
Temperature (°C)	27.52 ± 0.86	30.8 ± 0.26	40.13 ± 1.22	43.51 ± 1.91

Table 8-3: The temperature of the bacterial suspension upon irradiation with a pulsed or continuous light energy of 63 J/cm² at a fluence rate of 0.7 W/cm² at various ICG concentrations

ICG concentrations (µg/mL)	0	25	100	200
Continuous irradiation	28.52 ± 0.3 °C	34.97 ± 1.66 °C	43.7 ± 0.81 °C	45.7 ± 1.92 °C
Pulsed irradiation	27.08 ± 1.42 °C	33.25 ± 1.29 °C	37.5 ± 1.0 °C	40.7 ± 0.82 °C

8.3.4.2 Effect of elevated temperatures on bacterial viability

No significant change in the viable count was observed after incubation of *Staph. aureus* suspended in PBS, in the absence or presence of 25 µg/mL ICG, and incubated for 10 minutes at 40°C in the dark (Figure 8-8). Horse serum provided a protective effect for *Staph. aureus*; no change in the viable count was noted after *Staph. aureus* was suspended in PBS containing 50%

HS in the absence or presence of 200 µg/mL ICG and incubation for 10 minutes at 50°C as seen in Figure 8-8. A statistically non-significant reduction was observed in the viable count of *P. aeruginosa* suspended in PBS, in the absence or presence of 200 µg/mL ICG, and incubated for 10 minutes at 50°C (Figure 8-9). The viable count reduction was always less than 0.4 log₁₀. *E. coli* also showed no significant change in the viable count after 10 minutes incubation at 42 °C in the presence and absence of 100 µg/mL ICG (Figure 8-9).

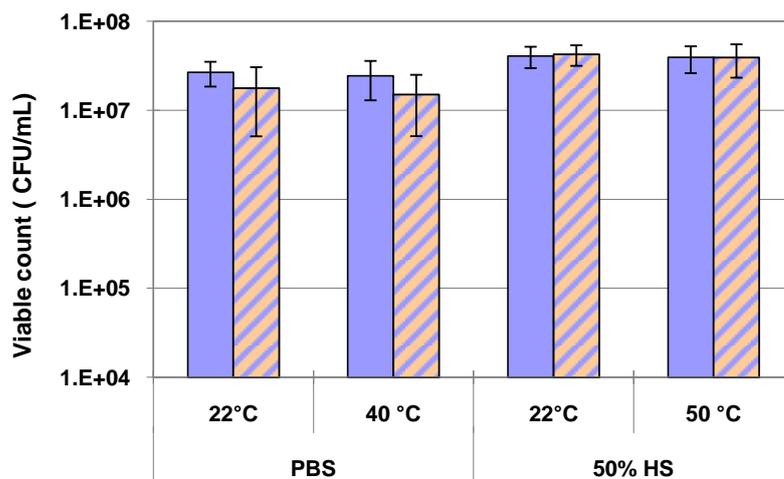


Figure 8-8: Viability of *Staph. aureus* suspended in PBS or in 50% HS after 10 minutes incubation in the absence (■) and presence (▨) of ICG, with 25 µg/mL at 40 °C for suspensions in PBS or with 200 µg/mL at 50 °C for suspensions in 50% HS. Error bars represent the standard deviation from the mean.

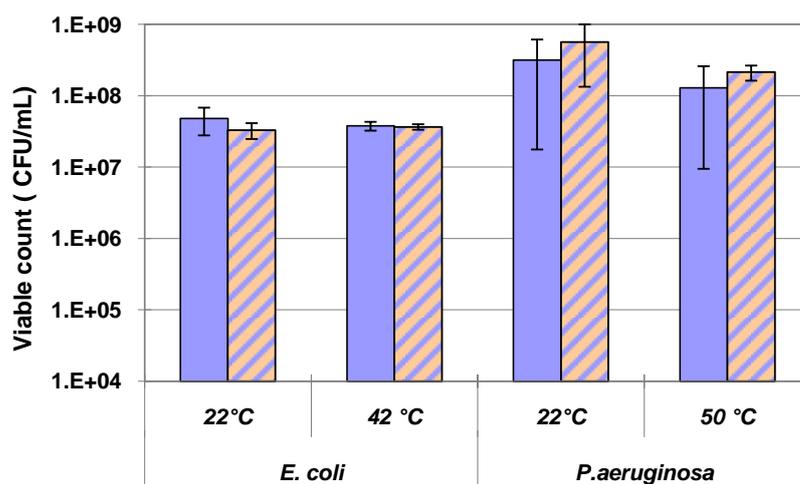


Figure 8-9: Viability of *P. aeruginosa* and *E. coli* in PBS after 10 minutes incubation in the absence (■) and presence (▨) of ICG, with 200 µg/mL at 50 °C for *P. aeruginosa* or with 100 µg/mL at 42 °C for *E. coli*. Error bars represent the standard deviation from the mean.

8.4 Discussion

In this chapter, some of the possible underlying mechanisms involved in ICG-mediated photo-inactivation of wound-infecting bacteria were explored. The results showed that ICG was able to produce $^1\text{O}_2$. The ICG-mediated photo-cidal effect was enhanced by D_2O and inhibited by the scavenger L-tryptophan. Moreover, the photo-activated ICG was able to kill Gram-positive bacteria, even after unbound molecules had been washed from the suspensions.

The production of $^1\text{O}_2$ upon activation of ICG with NIR laser light was determined by imaging the fluorescence intensity of the SOSGR. This reagent is highly selective for $^1\text{O}_2$ and does not show any appreciable response to hydroxyl radicals or superoxide. SOSG exhibits weak blue fluorescence with excitation peaks at 372 nm and 393 nm and emission peaks at 395 nm and 416 nm. In the presence of $^1\text{O}_2$, SOSG emits a green fluorescence with excitation and emission peaks at 504 nm and 525 nm, respectively (Molecular Probes Product Information, 2004). Flors *et al.*, (2006) reported that the increase in SOSG fluorescence is a good indicator of the increase in $^1\text{O}_2$ production in an *in vivo* leaf model. The SOSGR was an excellent detector for the generation of $^1\text{O}_2$ during the photosensitization of amyloid fibrils using thioflavin and 442 nm-laser light. The laser-irradiated samples showed high fluorescence of the SOSG in the presence of thioflavin (Ozawa *et al.*, 2009). Herein, the fluorescence intensity of 5 μM SOSGR containing 25 $\mu\text{g}/\text{mL}$ ICG solutions increased linearly as the light energy increased from 18 to 90 J/cm^2 , while the aqueous solution of SOSGR alone showed no increase in the fluorescence when irradiated with similar increasing light energies. Thus, the greater the light energy absorbed by ICG molecules, the greater the extent of $^1\text{O}_2$ formation. Similar increased fluorescent intensities of SOSG were observed during 370–450 nm light irradiation in presence of uroporphyrin (Tam *et al.*, 2009). The authors postulated that the photo-degradation of uroporphyrin proceeds via a type II photosensitization reaction, producing $^1\text{O}_2$. $^1\text{O}_2$ is a highly ROS that can damage cell components such as lipids, proteins and nucleic acids (Maisch *et al.*, 2007; Luksiene & Zukauskas, 2009). The photo-cidal effect of ICG is, therefore, likely to be due mainly to the generation of $^1\text{O}_2$

and other ROS, which oxidize lipids and proteins in several bacterial components leading to bacterial cell death.

To reveal some of the underlying mechanisms involved in the killing of wound-infecting organisms by ICG, lethal photosensitization experiments were carried out in the presence of an enhancer of $^1\text{O}_2$ life span (D_2O) or a quencher of $^1\text{O}_2$ (L-tryptophan). In the current study, the 1.5-1.6 \log_{10} augmentation of *Staph. aureus* killing in the presence of D_2O demonstrated that enhancement of the life span of $^1\text{O}_2$ increases the bactericidal effect thereby suggesting the involvement of this cytotoxic species in the lethal photosensitization process. Further evidence of the involvement of $^1\text{O}_2$ is provided by the protective effect of the singlet oxygen scavenger, L-tryptophan. The data presented here demonstrate that in the presence of increasing concentrations of L-tryptophan, decreased numbers of *Staph. aureus* were killed. The kill of *Staph. aureus* was reduced by 1 and 2.2 \log_{10} in the presence of 10 and 12 mM L-tryptophan, respectively. These results suggest the involvement of ROS in cell killing and were in agreement with previous findings for TBO-photosensitization of *P. gingivalis* (Bhatti *et al.*, 1998) and *Vibrio vulnificus* (Wong *et al.*, 2005). Both studies showed that there was increased bacterial killing in the presence of D_2O and decreased killing in the presence of L-tryptophan. In contrast, Sabbahi *et al.*, (2008), reported that the presence of tryptophan failed to protect *Staph. aureus* from the photo-cytotoxic effect of MB. The authors claimed that the bactericidal activity of MB was mediated in part, via a type I reaction in which $\cdot\text{OH}$ generation is dominant.

The penetration of a PS through the bacterial membrane is dependent on the chemical structure of the PS, the solvent and the nature of the bacteria (Tardivo *et al.*, 2005). Herein, uptake studies showed that when the excess unbound ICG had been washed out from the cell suspensions of the Gram-positive bacteria (*Staph. aureus* and *Strep. pyogenes*), significant kills were attained. Substantial kills of 99.87% and 99.78% for *Staph. aureus* and *Strep. pyogenes* respectively were achievable after washing ICG from the cell suspensions. These kills were still significantly lower than those attained when the ICG remained in the suspensions. Kills of 99.999% were achieved for those organisms when ICG was not washed from the cell suspensions. In contrast,

washing out ICG from *P. aeruginosa* cell suspensions considerably reduced the effectiveness of lethal photosensitization. Only 4% kills of *P. aeruginosa* were achieved after washing ICG compared to 99.999% kills without washing the PS. These results were supported by the spectrophotometric analysis of the lysed *Staph. aureus* and *Strep. pyogenes* pellets in 2% SDS which displayed a high peak absorbance at 795 nm which matches the absorbance of ICG. Yet, no such peak absorbance was detected in the case of lysed *P. aeruginosa* cells. These results suggest that ICG uptake by *Staph. aureus* and *Strep. pyogenes* was much greater than that by *P. aeruginosa*. Demidova & Hamblin, (2005) established that the photo-cidal effect exerted after washing the excess PS from the cell suspension of three microbial species (*Staph. aureus*, *E. coli* and *C. albicans*) was mainly dependent on the chemical nature of the PS. For example, with pL-ce6 comparable kills were achieved whether pL-ce6 was washed from the suspension or not for all three microbial species. For TBO, kills were dramatically reduced when TBO was washed out of the cell suspensions, especially for *Staph. aureus* and *E. coli*. RB-mediated photo-killing decreased significantly after washing the PS from suspensions of *E. coli* and *C. albicans* but not for *Staph. aureus* (Demidova & Hamblin, 2005).

In clinical practice, providing an effective cooling system for the skin surface during PDT (Altshuler *et al.*, 1999) or the use of a pulsed laser may avoid tissue hyperthermia. During the lethal photosensitization experiments at high fluence rates and the highest concentration of ICG (200 µg/mL), the temperature of the bacterial suspensions increased to 47°C, whereas at lower ICG concentrations the temperature did not exceed 37°C. In order to ascertain whether this temperature rise was responsible for, or contributed to, the kills achieved, the test organisms were exposed to a slightly higher temperature than that which occurred during lethal photosensitization and for longer periods of time. As exposure to these temperatures resulted in no, or only slight, reductions in bacterial viability, it can be concluded that the observed kills were attributable almost exclusively to the light-induced generation of singlet oxygen (and possibly free radicals) rather than to thermal effects. Even though the temperature of *Staph. aureus* suspensions containing 200 µg/mL ICG increased up to 47°C, there was no significant reduction in the viable count of *Staph. aureus* in 50% HS at a higher temperature of 50°C. It may be that

protein molecules in the serum provide protection against the heating effect. Furthermore, no kills were observed in the case of *P. aeruginosa* and *E. coli* when the bacteria were exposed to similar temperatures to those recorded during lethal photosensitization using 100-200 µg/mL ICG. It should be noted that heating has some synergic effect when applied together with photosensitization (Henderson *et al.*, 1985). It was noted that maximum photosensitization appears to occur with temperatures in the range of 42.5-47°C (Waldow *et al.*, 1985; Gomer *et al.*, 1988). This heat increases the membrane permeability of bacteria which allows further diffusion of the PS into the cell, thus more effective bacterial killing can be achieved. The results of these experiments suggest that killing of wound-infecting bacteria was more likely attributable to the products of lethal photosensitization. In addition, irradiating the bacteria with low fluence rates also resulted in significant reductions in the viability of the Gram-positive bacteria and *P. aeruginosa* (see Chapter 3). These results support the hypothesis that the cytotoxic species produced from the photosensitization process are responsible for bacterial killing.

In summary, ICG is an effective light-activated antimicrobial agent capable of producing $^1\text{O}_2$. The greater the light energy absorbed by the ICG molecules, the greater the extent of $^1\text{O}_2$ generation. Increasing the life-time of $^1\text{O}_2$ enhanced bacterial killing while quenching this ROS reduced killing. These results suggest that photo-inactivation of micro-organisms using ICG is mediated by $^1\text{O}_2$, with the possible involvement of other ROS. ICG was taken up by the Gram-positive bacteria *Staph. aureus* and *Strep. pyogenes* but not by the Gram-negative bacterium *P. aeruginosa*. Significant kills of both Gram-positive bacteria were achieved when ICG was washed out from the cell suspensions. However, the number of cells killed was still lower than when the PS was not washed from the suspensions. No photosensitization of *P. aeruginosa* was achievable after removal of unbound ICG. Exposing the target organisms to similar temperatures to those recorded during lethal photosensitization, resulted in no reduction in bacterial viability. Therefore, it can be concluded that the observed kills were mediated by the light-induced generation of singlet oxygen (and, possibly, free radicals) rather than to thermal effects.

Chapter 9
**The effect of lethal
photosensitization on bacterial
virulence factors**

9.1 Introduction

Pathogenic bacteria display a variety of virulence factors that enable them to overcome host defences as well as damage host tissue during the course of an infection. The term virulence factor can be defined as a trait (i.e., a gene product) that enables a micro-organism to establish itself within a host and augment its ability to cause disease and tissue destruction (Chen *et al.*, 2005). Virulence factors include bacterial toxins (i.e. endotoxins and exotoxins), hydrolytic enzymes, cell surface proteins that mediate bacterial attachment (e.g. flagella and pili/fimbria), and cell surface carbohydrates and proteins such as capsular polysaccharides that protect a bacterium from host defences (Brogden *et al.*, 2000). These factors play a key role in all stages of an infection from invasion-adherence-colonization to dissemination and their levels determine the pathogenicity of the organism.

During critical colonization of bacteria in a wound, excreted toxins and exoenzymes are believed to play a major role in delaying the healing process (Ovington, 2003). Exotoxins are soluble extracellular proteins secreted by both Gram-positive and Gram-negative bacteria during active growth. These subsequently diffuse into the host environment, binding to and degrading specific target substrates causing damage to the host by destroying cells or disrupting normal cellular metabolism (Schaechter & Lederberg, 2004). Exotoxins produced by common wound-infecting genera (e.g. *Staphylococcus*, *Streptococcus*, *Pseudomonas*) attack several types of cells and tissues, resulting in generalized tissue necrosis at the wound surface (Ovington, 2003). Endotoxins are the lipopolysaccharide components of the outer membrane of Gram-negative bacteria. However, endotoxins may also be released in small amounts from proliferating bacterial cells; they discharge in significant quantities when the bacterial cells are lysed in response to effective phagocytosis or antimicrobial agents (Todar, 2008). Hence, bacterial toxins, both soluble and cell-associated, may be transferred via blood and lymph and cause cytotoxic effects at remote tissues from the original wound site (Ovington, 2003). The presence of endotoxins in the wound environment stimulates the production of inflammatory mediators such as TNF- α and the interleukins, which in turn induce the production of endogenous MMPs (Konturek *et al.*, 2001; Power *et al.*, 2001). Different types of non-healing

wounds show increased levels of MMPs (Tarnuzzer & Schultz, 1996; Trengove *et al.*, 1999) which contribute to the local damage of tissue components, growth factors and protease inhibitors (Mast & Schultz, 1996). As a consequence, wound remodelling will be unbalanced due to increased degradation simultaneously with decreased construction of the ECM. It has been reported that the presence of copious amounts of endotoxins in surgical wounds decreases collagen deposition and cross-linking consequently reducing its tensile strength which in turn results in failure of wound healing (Metzger *et al.*, 2002). Chronic wounds are halted in a prolonged phase of inflammation. This extended inflammatory phase causes increased levels of proteases such as MMPs, elastase, plasmin and thrombin that destroy the ECM components and damage the growth factors and their receptors which are crucial for healing (Mast & Schultz, 1996).

The ubiquitous organism, *P. aeruginosa*, is a major cause of fatal burn infections, while *Staph. aureus* is the most common organism isolated from infected wounds. Numerous virulence factors of these organisms (Figure 9-1) contribute to the pathogenesis of wound infection. Well-known virulence factors of *P. aeruginosa* including pili, flagella, LPS, proteases, QS, exotoxin A and S exoenzymes (Lyczak *et al.*, 2000) help to establish infection in wounds. Surface protein adhesins (e.g. clumping factor, fibronectin-, fibrinogen- and collagen-binding proteins)(Foster & Höök, 1998); antiphagocytic factors such as serotype 5 and 8 capsules (Cunnion *et al.*, 2001; Luong & Lee, 2002); exoenzyme as hyaluronate lyase, proteases, lipases (Lowy, 1998); and exotoxins (e.g. haemolysins and toxic shock syndrome toxin-1)(Dinges *et al.*, 2000) are common examples of *Staph. aureus* virulence factors. An antimicrobial therapy which can neutralise virulence factors in addition to exerting a cidal effect against the infecting organism would be highly advantageous. In this study, the effect of ICG/NIR laser light on the activity of the V8 protease from *Staph. aureus* and *P. aeruginosa* LPS and proteases was studied.

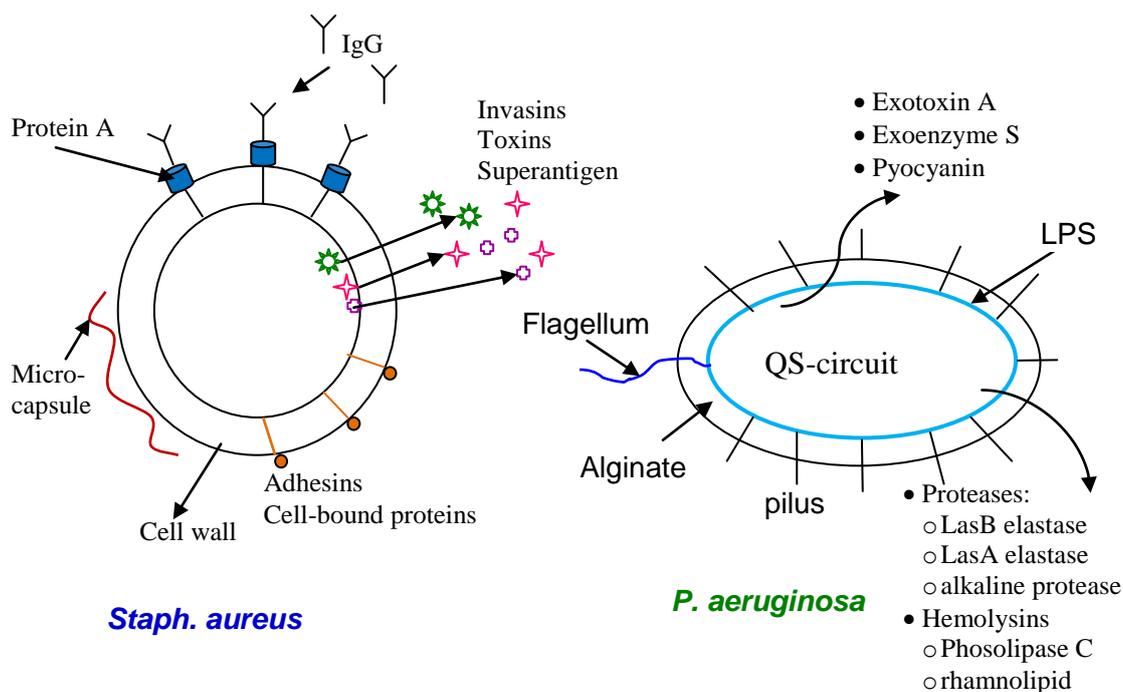


Figure 9-1: The main virulence factors produced by *Staph. aureus* and *P. aeruginosa*

9.2 Materials and Methods

9.2.1 Photosensitizer preparation and irradiation system

In the case of studies of protease photosensitization, the preparation of ICG was described in section 2.1.4. For LPS photosensitization, fresh stock solutions of 1 mg/mL ICG were prepared in LAL reagent water (LRW), immediately prior to each experiment. Afterwards, this was diluted in LRW to the desired concentration. Irradiation was carried out using the 0.4 W diode laser (Ondine laser), which emits continuous wave light with a wavelength of 808 ± 5 nm.

9.2.2 Azocasein assay for the assessment of the activity of *Staph. aureus* V8 protease

The procedures for the azocasein assay to measure the activity of *Staph. aureus* V8 protease are described in section 2.5.1.

Final ICG concentrations of 25 or 100 $\mu\text{g/mL}$ were used in these experiments. Proteases samples were exposed to light energies of 3, 15, 30, 45 or 90 J/cm^2 delivered at a low fluence rate of 0.05 W/cm^2 from the NIR 808 nm Ondine laser. Control V8 protease samples incubated with PBS or ICG were kept in the dark.

9.2.2.1 Azocasein assay for determination of total proteolytic activity of *P. aeruginosa* culture supernatant

The procedures for the azocasein assay to detect the total proteolytic activity of *P. aeruginosa* strain PA01 are described in section 2.5.2.

Final ICG concentrations of 200 µg/mL were used in these experiments. The light energy of 90 J/cm² was delivered to the supernatant either at a high (0.3 W/cm²) or at a low (0.05 W/cm²) fluence rate from the NIR 808 nm laser.

9.2.3 Assessment of LPS by the Limulus amoebocyte lyase (LAL) assay

The procedures for the LAL assay to evaluate the concentration of LPS from *P. aeruginosa* serotype 10 are described in section 2.5.3.

At first, the effect of different ICG concentrations combined with NIR laser light on the potency of *P. aeruginosa* LPS was investigated. Various ICG concentrations ranging from 25 µg/mL to 200 µg/mL were exposed to a light dose of 90 J/cm² delivered at a fluence rate of 0.3 W/cm² from the NIR 808 nm Ondine laser. Secondly, the effect of increasing the light energy coupled with 200 µg/mL ICG on LPS potency was studied. Here, LPS samples were exposed to light energies of 90 or 180 J/cm² delivered at a high fluence rate of 0.3 W/cm² from the NIR 808 nm Ondine laser. Control LPS specimens incubated with PBS or ICG were kept in the dark.

9.2.3.1 Calculation and interpretation of LPS concentration

LPS is quantified in the LAL assay by finding the endpoint in a series of specimen dilutions. The endpoint is the lowest concentration of LPS able to give a positive test. The concentration of LPS in the specimen was calculated by multiplying the Pyrotell[®] sensitivity ($\lambda=0.25$ EU/mL) by the reciprocal of the dilution at the endpoint. The assay was performed in six-replicates; therefore the sensitivity was expressed as the geometric mean (GM) of the individual sensitivities:

GM = antilog ($(\sum e)/f$), where $\sum e$ is the sum of the log endpoint concentrations of the dilution series used, and f is the number of replicates ($n=6$). The GM endpoint concentration is the measured sensitivity of the LAL reagent (in EU/mL).

9.3 Statistical analysis

The data were analysed using the Univariate General Linear Model to determine if there was a difference between groups and between similar experiments performed on different occasions. A Post-Hoc Test in the form of a Bonferroni correction was applied to detect where the difference occurred.

9.4 Results

9.4.1 The effect of ICG and NIR laser light on the activity of V8 protease

The effect of ICG and low intensity NIR laser light on the proteolytic activity of the V8 protease as established by the azocasein-hydrolysis assay is shown in Figures 9-2, 9-3 and 9-4. The proteolytic activity was expressed as units of activity (i.e. one unit of activity was defined as that which caused a change in absorbance of 0.001 in one hour at 450 nm). When a range of light energies of 1-30 J/cm² was coupled with 25 µg/mL ICG, no inhibition of the proteolytic activity was observed (Figure 9-2). Increasing the light energy to 45 J/cm² to photo-activate the same ICG concentration of 25 µg/mL resulted in a significant decrease (P = 0.001) in the proteolytic activity of 23% (Figure 9-3). However, when the concentration of ICG was increased to 100 µg/mL and the light dose to 90 J/cm², the activity of the V8 protease was only decreased by 8.1% as shown in Figure 9-4. This reduction in the proteolytic activity was significant (P = 0.00002). Throughout the course of the experiments, neither the light nor the PS alone reduced the proteolytic activity of the V8 protease.

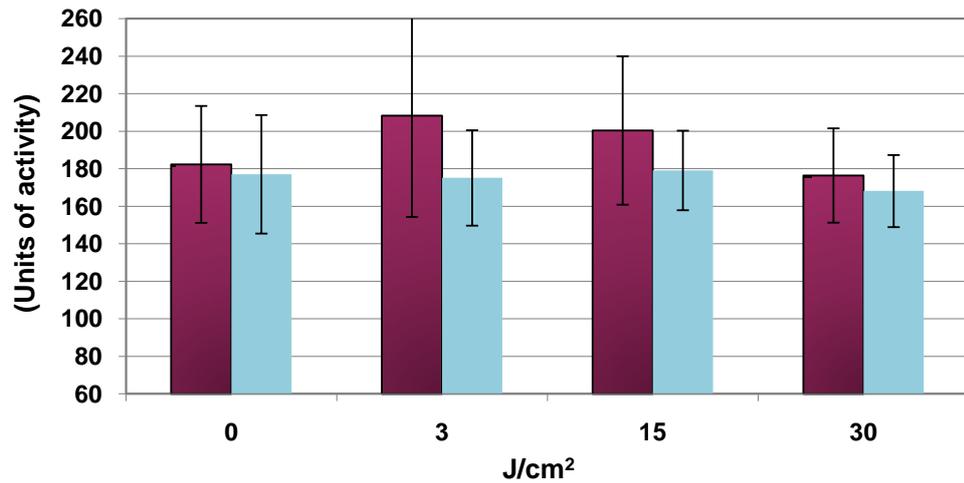


Figure 9-2: The effect of 25 µg/mL ICG and NIR laser light on the proteolytic activity of V8 protease. The protease in PBS (■) or in 25 µg/mL ICG (□) was irradiated at a fluence rate of 0.05 W/cm² with light energies of 3, 15, or 30 J/cm² from the NIR 808 nm Ondine laser. Control samples in PBS or ICG were kept in the dark (0 J/cm²). Bars represent mean values and error bars represent standard deviations (n = 6)

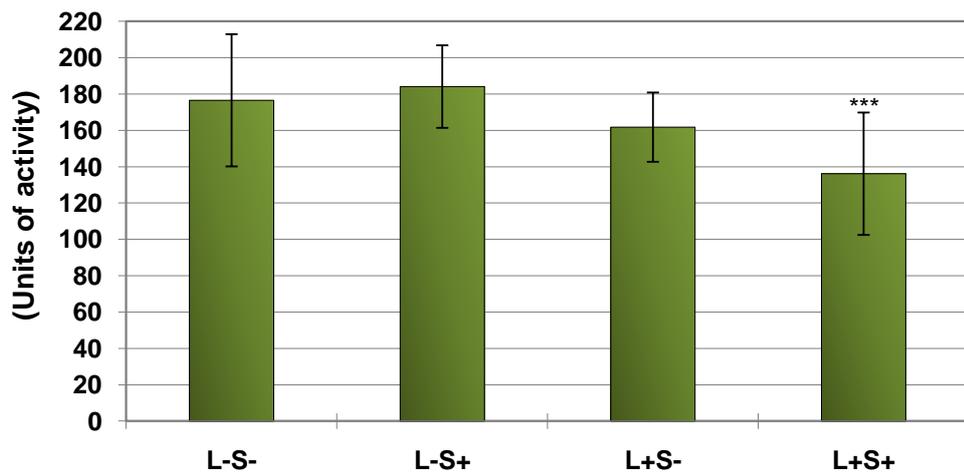


Figure 9-3: The effect of 25 µg/mL ICG and NIR laser light on the proteolytic activity of the V8 protease. Samples were irradiated at a fluence rate of 0.05 W/cm² and a light dose of 45 J/cm² from the NIR 808 nm Ondine laser in the absence of ICG (L+S-) or in the presence of ICG (L+S+). Control samples in PBS (L-S-) or in ICG (L-S+) were kept in the dark. Bars represent mean values and error bars represent standard deviations (n = 6).

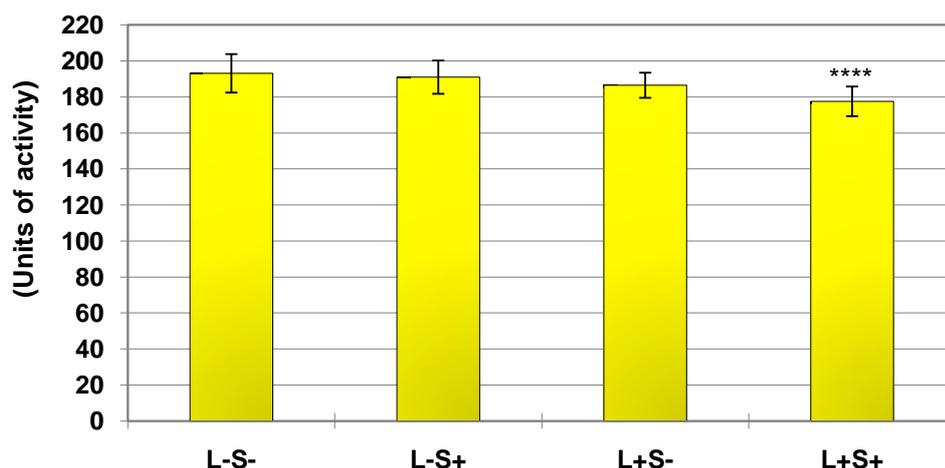


Figure 9-4: The effect of 100 µg/mL ICG and NIR laser light on the proteolytic activity of the V8 protease. Samples were irradiated at a fluence rate of 0.05 W/cm² and a light dose of 90 J/cm² from the NIR 808 nm Ondine laser in the absence of ICG (L+S-) or in the presence of ICG (L+S+). Control samples in PBS (L-S-) or in ICG (L-S+) were kept in the dark. Bars represent mean values and error bars represent standard deviations (n = 6).

9.4.2 The effect of ICG and NIR laser light on the total proteolytic activity of *P. aeruginosa*

Figures 9-5 and 9-6 show the proteolytic activity of *P. aeruginosa* treated with 200 µg/mL ICG and a light dose of 90 J/cm². When the light energy was delivered at a low fluence rate of 0.05 W/cm², no significant reduction in the total proteolytic activity of *P. aeruginosa* was detected (Figure 9-5). In contrast, when the same light dose was delivered at a high fluence rate of 0.3 W/cm², the same ICG concentration of 200 µg/mL ICG resulted in a small but significant reduction of 8.22% (P = 0.000004) in the proteolytic activity of *P. aeruginosa* as seen in Figure 9-6. Neither low nor high light irradiation alone, without ICG, decreased the proteolytic activity of *P. aeruginosa*. ICG had no effect on the proteolytic activity of *P. aeruginosa* when incubated in the dark.

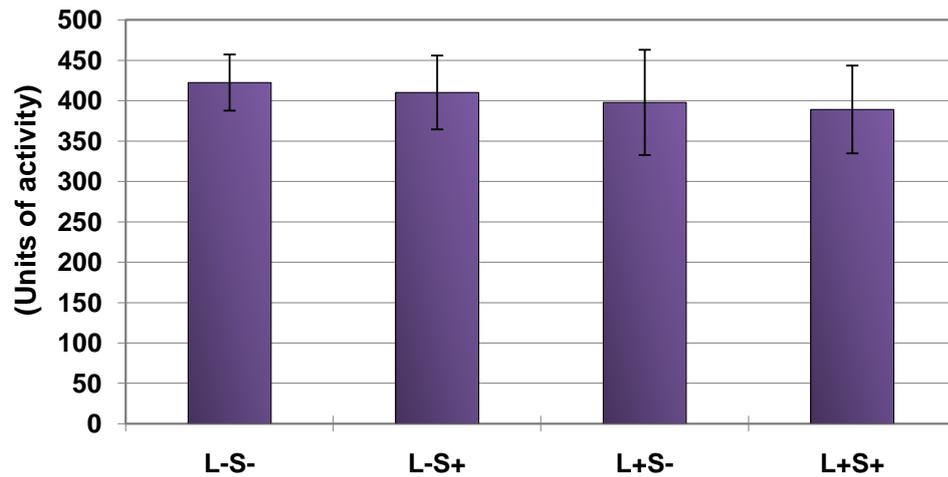


Figure 9-5: The effect of 200 µg/mL ICG and low intensity NIR laser light on the total proteolytic activity of *P. aeruginosa*. Samples were irradiated at a fluence rate of 0.05 W/cm² and a light dose of 90 J/cm² from the NIR 808 nm Ondine laser in the absence of ICG (L+S-) or in the presence of ICG (L+S+). Control samples in PBS (L-S-) or in ICG (L-S+) were kept in the dark. Bars represent mean values and error bars represent standard deviations (n = 6).

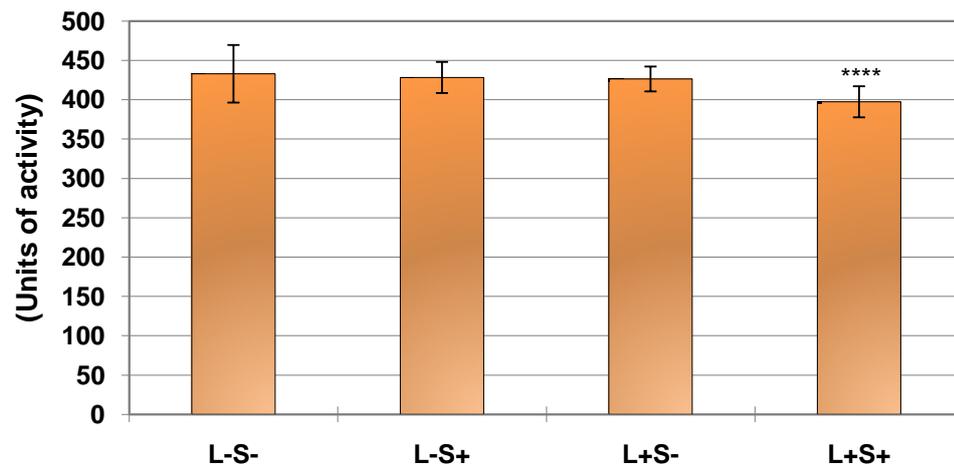


Figure 9-6: The effect of 200 µg/mL ICG and high intensity NIR laser light on the total proteolytic activity of *P. aeruginosa*. Samples were irradiated at a fluence rate of 0.3 W/cm² and a light dose of 90 J/cm² from the NIR 808 nm Ondine laser in the absence of ICG (L+S-) or in the presence of ICG (L+S+). Control samples in PBS (L-S-) or in ICG (L-S+) were kept in the dark. Bars represent mean values and error bars represent standard deviations (n = 6).

9.4.3 The effect of ICG and NIR laser light on the LPS of *P. aeruginosa*

Figure 9-7 shows the effect of various concentrations of ICG combined with NIR laser light on the activity of LPS. Irradiation of LPS in the presence of ICG reduced the potency of the LPS at all ICG concentrations. The ICG concentrations ranged from 25 to 200 $\mu\text{g}/\text{mL}$ when combined with a high intensity light of $90 \text{ J}/\text{cm}^2$, they reduced the potency of the LPS from 15 EU/mL to 2.5-3.5 EU/mL (76-83% reduction) ($P < 0.00001$). The highest ICG concentration of 200 $\mu\text{g}/\text{mL}$ did not reduce the LPS activity when incubated in the dark. The NIR laser light, in the absence of ICG, significantly inhibited ($P=0.001$) the activity of the LPS by 52% as seen in Figure 9-7.

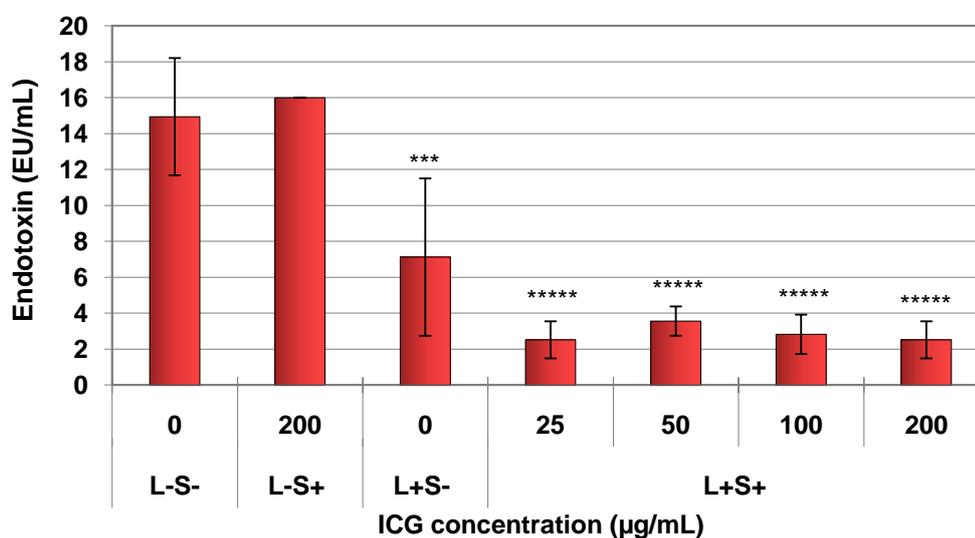


Figure 9-7: The effect of various ICG concentrations on the bio-activity of *P. aeruginosa* LPS. LPS samples were irradiated at a fluence rate of $0.3 \text{ W}/\text{cm}^2$ and at a light dose of $90 \text{ J}/\text{cm}^2$ from the NIR 808 nm Ondine laser in PBS (L+S-) or in ICG (L+S+). Control samples in PBS (L-S-) or in ICG (L-S+) were kept in the dark. Bars represent geometric mean values and error bars represent standard deviations ($n = 6$).

Figure 9-8 shows a light dose-dependent reduction in the biological activity of the LPS. A light dose of $90 \text{ J}/\text{cm}^2$ coupled with 200 $\mu\text{g}/\text{mL}$ ICG resulted in a significant decrease ($P < 0.00001$) in biological activity of 82.32%, whereas irradiation with a light dose of $180 \text{ J}/\text{cm}^2$ attained a 86% decrease in LPS potency ($P < 0.00001$). In the absence of ICG, the biological activity reduced significantly ($P < 0.001$) by 55.46 and 64.64% upon exposure to light energies of 90 and $180 \text{ J}/\text{cm}^2$, respectively (Figure 9-8).

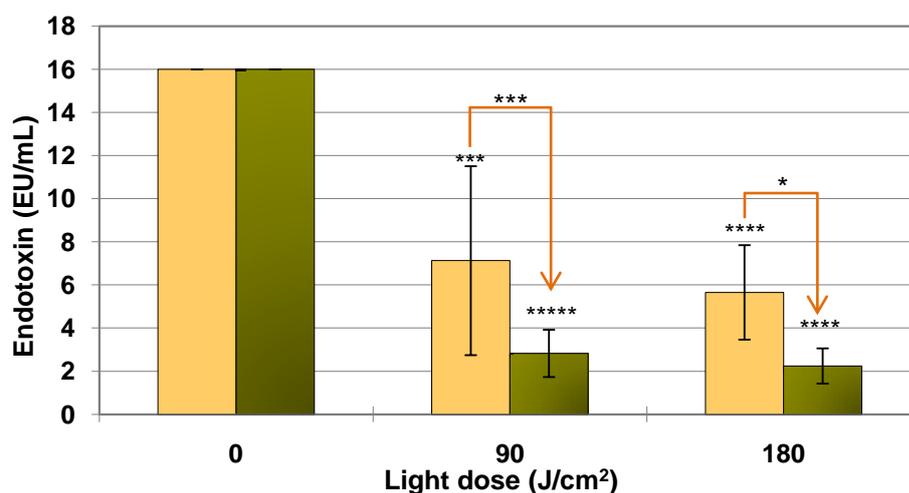


Figure 9-8: The effect of 200 $\mu\text{g/mL}$ ICG activated with various light doses on the bio-activity of *P. aeruginosa* LPS. LPS samples in PBS (□) or in 200 $\mu\text{g/mL}$ ICG (■) were irradiated with light doses of 90 and 180 J/cm^2 at a fluence rate of 0.3 W/cm^2 from the NIR 808 nm Ondine laser. Control samples in PBS or ICG were kept in the dark (0 J/cm^2). Bars represent geometric mean values and error bars represent standard deviations ($n = 6$).

9.5 Discussion

Throughout the infection process, bacterial virulence factors may inflict significant damage on host tissue. Inactivation of these factors using a light-activated antimicrobial agent may reduce the extent of host tissue destruction, in addition to killing the infecting organism. The results of this study have shown that it is feasible to reduce the activity of *Staph. aureus* V8 protease, *P. aeruginosa* proteases and LPS using ICG in combination with NIR light.

The pathogenicity of *Staph. aureus* relies mainly on the synchronized action of more than 40 different virulence factors (Arvidson & Tegmark, 2001). These include several proteases such as serine-, cysteine- and metalloenzymes. Proteases are able to interfere with the host defence mechanisms and destroy tissue components as well as amend other pathogen-derived virulence factors (Dubin, 2002). The main function of these enzymes is thought to be the conversion of local host tissues into low-molecular weight nutrients necessary for bacterial growth (Dinges *et al.*, 2000). The V8 protease (glutamylendopeptidase) is a specific serine protease and plays a very limited role as a supplier of nutrients for bacterial growth because of its narrow substrate specificity (Popowicz *et al.*, 2006). This protease can promote bacterial survival and spread through the inactivation of important host proteins

in particular immunoglobulins and α_1 -proteinase inhibitor (elastase inhibitor) in a way that could attenuate host defence against the bacterium and assist in degradation of the host tissue via the uncontrolled activity of host elastase (Arvidson, 2000). Also, the V8 protease is capable of generating kinin directly from high-molecular weight kininogen, ultimately leading to pain and oedema (Molla *et al.*, 1989). Inactivation of such an enzyme would be an attractive feature for any antimicrobial treatment. Packer *et al.*, (2000) established that proteolytic enzymes of the periodontal pathogen *P. gingivalis* could be inactivated using TBO and visible light of 633 nm. A recent study has shown that the proteolytic activity of *Vibrio vulnificus* culture supernatants was reduced after treatment with TBO and non-coherent light of 560 to 780 nm (Wong *et al.*, 2005). The findings of this study are in agreement with the results of the previous studies. Herein, the treatment of *Staph. aureus* V8 protease with 25 $\mu\text{g/mL}$ ICG combined with 45 J/cm^2 of NIR light delivered at 0.05 W/cm^2 , achieved a significant 23% reduction in the activity of this enzyme. When V8 protease was exposed to 100 $\mu\text{g/mL}$ ICG and a light dose of 90 J/cm^2 , a significant 8.1% inhibition of the enzyme activity was observed. This smaller inactivation at the higher light and ICG concentration may be owing to the presence of excess free non-reacted ICG molecules which acted as a shield (Bhatti *et al.*, 1997; Komerik, 2000). ICG in the absence of light did not inhibit the activity of V8 protease. On the contrary, TBO in the absence of light resulted in a 19% decrease in the proteolytic activity of *P. gingivalis* (Packer *et al.*, 2000). This difference may be attributed to the charge of the PS molecule, as TBO is a cationic dye while ICG is an anionic dye. The unique tandem tertiary structure of V8 protease along with the presence of a positively charged N-terminus that determines the substrate-specificity may play a role in its interaction with the PS (Dubin, 2002; Prasad *et al.*, 2004). It is possible that the loss of V8 protease activity after irradiation with the NIR light in the presence of ICG is due to protein cross-linking and oxidation reactions (Al-Mutairi *et al.*, 2007). The average level of proteases activity in acute wound fluids has been estimated to be 0.75 $\mu\text{g/mL}$ -seven times lower than the V8 protease level used in this study (5 $\mu\text{g/mL}$) (Tarnuzzer & Schultz, 1996).

Proteases are thought to play a major role during the acute phase of *P. aeruginosa* infection (Snell *et al.*, 1978). *P. aeruginosa* secretes quite a few

proteases including, LasB elastase, LasA elastase, alkaline and IV protease (Rumbaugh *et al.*, 1999; Caballero *et al.*, 2001). These enzymes are secreted by *P. aeruginosa* to overcome the host's initial defence mechanisms (Caballero *et al.*, 2001). Until now, the role of the alkaline protease in tissue invasion and systemic infections remains unclear; albeit, it contributes significantly to the development of corneal infections (Van Delden, 2004). Proteases, mainly elastase, are thought to be essential for virulence in burn wound infections (Pavlovskis & Wretling, 1979) as they degrade collagen and non-collagen host proteins including elastin (Bejarano *et al.*, 1989). Elastin is an important component of blood vessels as it is responsible for maintaining their resilience. The collaborative activity of LasB elastase (zinc metalloprotease) and LasA elastase (serine protease) is responsible for elastolytic activity (Van Delden & Iglewski, 1998). The level of proteolytic enzymes such as elastase and matrix metalloproteinases (MMPs) is higher in chronic wounds than in acute wounds (Edwards *et al.*, 2004; Schönfelder *et al.*, 2005). Although all wounds require a certain level of elastase and proteases for proper healing, too high a concentration is damaging (Edwards *et al.*, 2004). Herein, ICG coupled with NIR light was found to reduce the total proteolytic activity of *P. aeruginosa*. This activity was reduced significantly upon treatment with 200 µg/mL ICG and a light dose of 90 J/cm² delivered at a high fluence rate of 0.3 W/cm², achieving a small, but significant, reduction in activity of 8.22%. However, when the same light energy was delivered at a low fluence rate of 0.05 W/cm², no significant reduction in the total proteolytic activity of *P. aeruginosa* was observed. The findings presented in this study are consistent with the results of previous work which showed that TBO combined with visible light of 632.8 nm, inactivated 55-60% of the extracellular proteolytic activity in *P. aeruginosa* (Komerik *et al.*, 2000). The lower inactivation in the total proteolytic activity of *P. aeruginosa* using ICG compared to that achieved using TBO may be due to the charge of the PS molecule, as TBO is a cationic dye while ICG is an anionic dye. Proteases produce several adverse effects on the innate and acquired host immune response. For example, elastase inhibits monocyte chemotaxis, (Schultz & Miller, 1974; Wilkinson, 1980) which could adversely affect early clearance of *P. aeruginosa* from wound sites by phagocytosis. Therefore reducing their level in an infected wound would help accelerate the healing process. Treatment of wounds using photo-activated ICG may help to control

infection dissemination by reducing the level of bacterial proteases. An *in vivo* study has shown that a mutant defective in the *lasR* regulatory gene (a strain defective in the synthesis of multiple virulence factors, including two different elastases, LasA and the LasB elastase, along with exotoxin A, and alkaline protease) is incapable of disseminating to distal host sites from a colonized burn wound (Rumbaugh *et al.*, 1999). Reducing the total proteolytic activity of *P. aeruginosa* using ICG-photosensitization may be mediated by protein cross-linking and oxidation reactions (Al-Mutairi *et al.*, 2007).

Another virulence factor of *P. aeruginosa* that has been reported to be involved in the pathogenesis of burn wound infection is LPS (Cryz *et al.*, 1983; Goldberg *et al.*, 1995). LPS is a typical characteristic component of the cell wall of Gram-negative bacteria, and basically consists of a hydrophobic lipid A, a hydrophilic core polysaccharide chain, and a hydrophilic O-antigenic polysaccharide side chain (Pier, 2007). However, lipid A is the active toxic part of the molecule, the nature and number of attached saccharide residues and substituents has a considerable effect on modifying this activity (Cryz *et al.*, 1984; Erridge *et al.*, 2002). LPS is a potent mediator of bacterial septic shock through the stimulation of host pyrogenic responses (Rietschel *et al.*, 1994). Reducing the level of LPS may prevent the spreading of infection to the circulation and thus prevent bacteraemia or toxemia (Cryz *et al.*, 1983). In this study, ICG activated with NIR laser light was capable of reducing the potency of *P. aeruginosa* LPS, the effect may be induced via lipid peroxidation which is mainly caused by the accompanying generation of singlet oxygen and other ROS (Wolnicka-Glubisz *et al.*, 2009).

In scrape wounds of human airway epithelial cells, high concentrations of LPS were toxic and decreased wound repair. Conversely, lower concentrations of LPS accelerated wound repair via a surface signalling cascade that causes epidermal growth factor receptor (EGFR) activation (Koff *et al.*, 2006). The endotoxin concentrations in the lymph fluid of rats suffering from *P. aeruginosa* burn wound infection increased significantly compared to the contralateral uninfected burn wound. The level of the endotoxin was 2.813 ± 0.116 EU/mL for infected burns versus 0.316 ± 0.287 EU/mL for non-infected burn wounds 6 hours post-infection (Wang *et al.*, 2008). Another study demonstrated that

there are increased levels of endotoxin in fluid from non-healing chronic leg ulcers compared with wound fluid from the same ulcers in the healing phase. The endotoxin level in fluid from non-healing chronic leg ulcers ranged from 1.5-5 EU/mL while it ranged from 0-2.5 EU/mL in the healing ulcers (Stacey & Trengove, 1999). In this study, irradiation of LPS in the presence of ICG reduced the endotoxin potency at a range of ICG concentration of 25 to 200 µg/mL. At an initial LPS concentration of 15 EU/mL (3 times higher than those present in a chronic wound), a light energy of 90 J/cm² delivered at 0.3 W/cm² reduced the LPS activity by 76-83% to 2.5-3.5 EU/mL. This reduction in the LPS potency was found to be light dose-dependent. Light energies of 90 and 180 J/cm² combined with 200 µg/mL ICG caused reductions in the LPS potency of 82.32% and 86%, respectively. In the absence of ICG, reductions of 55.46 and 64.64% in the LPS level were achieved after exposure to NIR light energies of 90 and 180 J/cm², respectively, yet these reductions were lower than those achieved in the presence of ICG. In support of the findings presented here, Komerik *et al.*, (2000) demonstrated that TBO combined with visible light of 632.8 nm, reduced *E. coli* LPS potency. Also the authors observed that the addition of TBO to LPS reduced its potency even in the dark.

In conclusion, ICG reduces the proteolytic activity of V8 protease when irradiated with NIR light of 808 nm, achieving a maximum reduction of 23% under the conditions used. A light dose of 45 J/cm² or more was necessary to inactivate the V8 protease when the light was delivered at a low fluence rate of 0.05 W/cm². In the case of the total proteolytic activity of *P. aeruginosa*, only delivering the light at a high fluence rate of 0.3 W/cm² to activate ICG was capable of inactivating the enzymes, achieving a reduction of only 8.22%. A substantial reduction in the potency of *P. aeruginosa* LPS was achieved upon exposure to NIR laser light in the presence and the absence of ICG. The light alone resulted in a 55.46- 64.64% reduction in LPS potency. Combining the NIR light with ICG resulted in greater reductions of 76-86% in LPS potency. These results suggest that ICG in combination with NIR light is an effective method of reducing the activities of two virulence factors of bacteria (proteases and LPS) implicated in delayed wound healing in addition to its cidal effect. Thus the application of PDT as a treatment for wound infection would have a major advantage over conventional antibiotic therapy.

Chapter 10

***In vivo* studies of the
effectiveness of photodynamic
therapy for the treatment of
wound infections**

10.1 Introduction

The results described in Chapter 7 have shown that under physiological conditions mimicking the wound environment, the common wound-infecting organisms can be photo-inactivated using ICG combined with NIR laser light. In this part of the study the focus was to ascertain if ICG-PDT can reduce the bacterial load in infected wounds *in vivo*.

Lately, several research groups have studied the potential use of PDT as an antimicrobial strategy to combat infections in a number of wound models *in vivo*. The most investigated PSs to treat wound infections in animal models include chlorin e6 (Hamblin *et al.*, 2002b & 2003), porphyrin (Lambrechts *et al.*, 2005c), TBO (Wong *et al.*, 2005; de Carvalho *et al.*, 2008; Lin *et al.*, 2010) and MB (Zolfaghari *et al.*, 2009). These previously-investigated PSs absorb light in the visible range of the spectrum which penetrates human tissue as deep as 2 cm (Stolik *et al.*, 2000). In this study, the use of NIR light to activate ICG offers the advantage of deeper tissue penetration of 2.5-4 cm (Stolik *et al.*, 2000). This makes ICG-PDT ideal for the treatment of deep, infected wounds that may be complicated with soft tissue or bone infections.

A plethora of studies have established the effectiveness of antimicrobial PDT as a treatment for infected wounds and related soft tissue and bone infections. The Gram-positive bacterium *Staph. aureus* was the most studied organism in infected wounds in animal models. Burn wounds infected with *Staph. aureus* have been successfully treated using PTMPP-PDT in mice (Lambrechts *et al.*, 2005c). MB-PDT has been shown to be capable of significantly reducing the number of EMRSA-16 in infected excision and superficial scarified wounds in mice (Zolfaghari *et al.*, 2009). Other *Staph. aureus* wound infections such as soft tissue infection (Gad *et al.*, 2004b) and osteomyelitis (Bisland *et al.*, 2006) have been effectively treated using PDT. Another study has shown that TBO-mediated PDT was able to kill 97% of *Streptococcus* spp. and *Actinomyces viscosus* in infected oral wounds in rats (Lin *et al.*, 2010).

Further investigations revealed the potential of PDT in the control and treatment of infected wounds with different highly invasive Gram-negative species. Wounds infected with *P. aeruginosa*, *E. coli* and *V. vulnificus* have

been effectively treated by PDT using chlorin e6 or TBO (Berthiaume *et al.*, 1994; Hamblin *et al.*, 2002b & 2003; Wong *et al.*, 2005). PDT not only reduced the bacterial load in wounds but also stimulated the healing process (Hamblin *et al.*, 2002b & 2003). The promising results of these studies imply the potential of antimicrobial PDT as a novel alternative to conventional antibiotics.

In animal wound models, Wilson and co-workers determined the extent of infection in wounds by viable counting (Zolfaghari *et al.*, 2009), while Hamblin and colleagues monitored the bioluminescence of genetically-engineered bacteria (Hamblin *et al.*, 2002b & 2003; Gad *et al.*, 2004b; Lambrechts *et al.*, 2005c).

Up until now, no study has explored the effect of a PS that can be activated by NIR light on wound infections. Therefore, the main aim of this study was to investigate the bactericidal effect induced by ICG-PDT in partial-thickness wounds infected with either *Staph. aureus*, *Strep. pyogenes* or *P. aeruginosa*. The use of a long-pulsed dye laser and intense pulsed light, as alternatives to continuous laser light sources enhance PDT efficacy and provide rapid treatment and recovery, while diminishing unwanted side effects (Babilas *et al.*, 2007). Thus, another aim was to study the effectiveness of ICG coupled with either pulsed or continuous irradiation modes against *Staph. aureus* infected wounds in mice.

10.2 Materials and Methods

10.2.1 Target organisms and growth conditions

The organisms used were: *Staph. aureus* NCTC 8325-4, *Strep. pyogenes* ATCC 12202 and *P. aeruginosa* strain PA01. All organisms were grown as described previously in section 2.1.2. The bacterial culture was washed twice with PBS and readjusted to contain 4×10^9 CFU/mL (1×10^8 CFU/25 μ L) or 4×10^8 CFU/mL (1×10^7 CFU/25 μ L).

10.2.2 Photosensitizer preparation and irradiation system

Fresh ICG solutions of 1 mg/mL were prepared in sterile H₂O as described in section 2.1.4.

Irradiation was carried out using the GaAIA's Velopex diode laser system which was described in sections 2.6.2 and 2.1.3. The infected wounds were irradiated at a fluence rate of 0.3 W/cm² using an optical fiber with a diameter of 400 µm, either in continuous mode or a repeated pulse duration mode which was selected to switch on and off between 100 – 100 msec or 150 – 50 msec. Several PDT protocols were investigated in this study and are shown in Table 10-1.

Table 10-1: PDT protocols used in this study

Protocol	Mode of irradiation	Light energy (J/cm ²)	Irradiation time (minutes)	ICG concentration	Pairs
1	Continuous	108	6	1 mg/mL	L-S- & L+S+
2	Continuous	216	On:6 - Off:1 - On: 6	1 mg/mL	L-S- & L+S+
3	Continuous	162	On:3-Off:1- On:3- Off:1- On: 3	1 mg/mL	L-S- & L+S+
4	Continuous	162	9	1 mg/mL	L-S- & L+S- L-S+ & L+S+
5	Pulsed	108	12 (on & off : 100 msec - 100 msec)	1 mg/mL	L-S- & L+S+
6	Pulsed	162	12 (on & off : 150 msec - 50 msec)	1 mg/mL	L-S- & L+S+

10.2.3 Experimental procedure *in vivo*

10.2.3.1 Anaesthesia

Mice were anaesthetised as described in section 2.6.3.1.

10.2.3.2 Superficial wound model

The anatomically matched paired wounds (25 mm²) were created as described in section 2.6.3.2. The wounds were inoculated with 25 µL of bacterial suspension contain approximately 1 x 10⁸ CFU for *Staph. aureus* or 1 x 10⁷ for *Staph. aureus*, *Strep. pyogenes* and *P. aeruginosa*. The number of viable bacteria in each inoculum was determined by serial dilution and colony counting immediately before each experiment.

10.2.3.3 Photodynamic therapy

PDT was performed 1 hour after inoculating the wounds with the bacterial suspension. The wounds were treated with 25 μ L of ICG at a concentration of 1 mg/mL then the experimental procedures were carried out as described in section 2.6.3.3. Figure 10-1 shows the irradiation of the ICG-treated wound with the 810 nm NIR laser light.

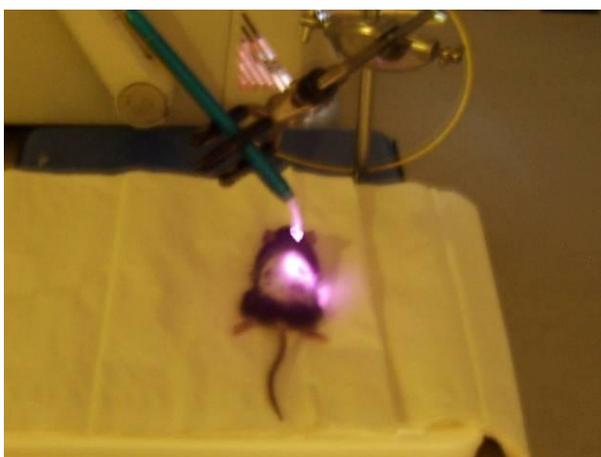


Figure 10-1: The application of ICG-PDT to treat the infected wound in a mouse

10.2.3.3.1 *In vivo* killing of *Staph. aureus* in a wound model in mice

The effect of continuous and pulsed ICG-PDT protocols (Table 10-1) was investigated on wounds inoculated with 1×10^8 CFU of *Staph. aureus*. Protocol 4 was further studied on wounds infected with a *Staph. aureus* inoculum containing 1×10^7 CFU. The effect of a light energy dose of 162 J/cm^2 was also investigated in the absence of ICG on wounds inoculated with either 1×10^8 or 1×10^7 CFU *Staph. aureus*.

It was important to study the effect of multiple ICG-PDT treatments on the infected wounds. Therefore, the effect of treating the wounds, inoculated with 1×10^7 CFU *Staph. aureus*, twice a day for one day or for 3 consecutive days using PDT protocol 4 was investigated. A one hour interval was allowed between each PDT treatment applied on the same day.

In these experiments a group of 3-6 mice was used for each pair of wounds i.e. total number of wounds was 6-12 i.e. pairs are arranged as following (L-S- & L+S+), (L-S- & L+S-), and (L-S+ & L+S+).

10.2.3.3.2 *In vivo* killing of *Strep. pyogenes* in a wound model in mice

Protocol 4 was studied on wounds inoculated with 1×10^7 CFU of *Strep. pyogenes*. The effect of a light energy dose of 162 J/cm^2 was also investigated

in the absence of ICG on wounds inoculated with the same number of this organism. In these experiments a group of 8 mice was used for each pair i.e. total number of wounds was 16 for each pair (pair 1: L-S- & L+S- and pair 2: L-S+ & L+S+).

10.2.3.3.3 *In vivo* killing of *P. aeruginosa* in a wound model in mice

Protocol 4 was studied on wounds inoculated with 1×10^7 CFU of *P. aeruginosa*. The effect of a light energy dose of 162 J/cm^2 was also investigated in the absence of ICG on wounds inoculated with the same number of this organism. In these experiments a group of 8 mice was used for each pair i.e. total number of wounds was 16 for each pair (pair 1: L-S- & L+S- and pair 2: L-S+ & L+S+).

10.2.3.4 Processing of tissue samples

The wound tissue specimens were processed as described in section 2.6.3.4. The number of viable bacteria isolated from the control and treated wounds was determined by viable counting using the appropriate non-selective and selective agar for the three targeted organisms (see Chapter 3, Table 3-2).

10.2.3.5 Measurement of wound temperature

This was carried out as described in section 2.6.3.5. Figure 10-2 shows the measurement of the core temperature for an animal during PDT treatment.



Figure 10-2: The measurement of the core temperature of the animal during ICG-PDT

10.3 Statistical analysis

For all experiments, the survival colony counts isolated from each wound (CFU/wound) were transformed into \log_{10} to normalize the data. In some experiments, the data were expressed as percentage of bacteria retrieved from

each group of wounds. The percentage of bacteria retrieved from the wound was calculated using the following formula:

$$\left[\frac{\text{The number of isolated CFU per wound}}{\text{The initial number of bacteria inoculated into the wound}} \times 100 \right]$$

A paired Student's *t*-test was employed to compare the number of viable microorganisms recovered per wound with those recovered from the matched paired control wound in the same mouse. The independent samples *t*-test was used to compare the number of viable bacteria recovered per wound with those recovered from control wounds in another group of mice. After consulting Levene's test for equality of variances, the Student's *t*-test parameters for the *Staph. aureus* data was a two-tailed distribution assuming equal variance and for the *Strep. pyogenes* and *P. aeruginosa* data sets unequal variance. The mean difference (P) was significant at the level of 0.05 (*P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 and *****P < 0.00001). All statistical analyses were performed using SPSS software.

10.4 Results

10.4.1 The effect of ICG-PDT on the viability of *Staph. aureus* infected partial-thickness wounds in mice

10.4.1.1 The effect of continuous mode of irradiation during ICG-PDT

Figure 10-3 shows the number of viable *Staph. aureus* isolated from the treated partial-thickness wounds compared to their anatomically matched-paired control wounds that neither received ICG nor light irradiation. When the wounds were treated with ICG and 108 J/cm² delivered in continuous mode for 6 minutes, an average of 1.1 log₁₀ reduction in the viable counts was achieved. The number of bacteria killed was in the range 0.7-1.6 log₁₀ based on the kill achieved in each mouse, approximately 79-97% kills. The number of *Staph. aureus* isolated from the PDT-treated wounds was significantly less (P=0.0002) than those isolated from the matched control wounds.

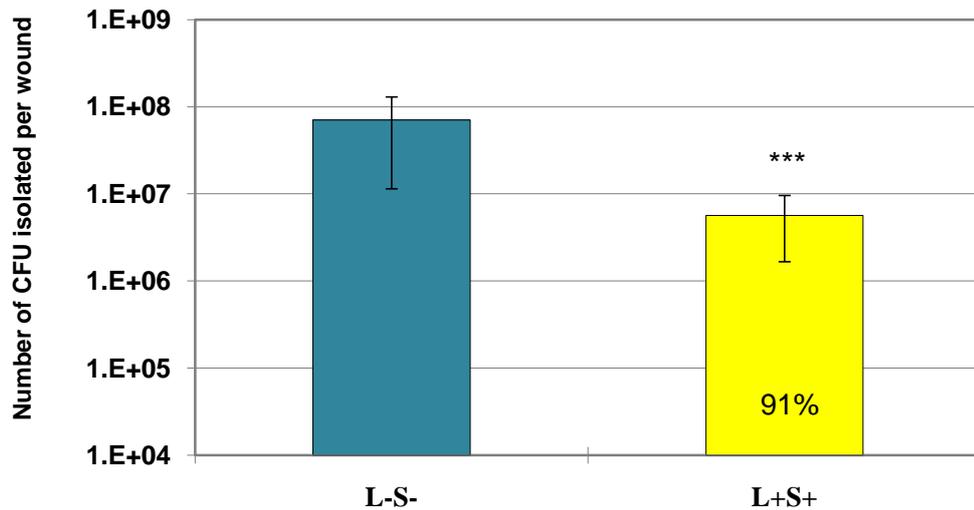


Figure 10-3: The number of viable *Staph. aureus* isolated from partial-thickness wounds treated with ICG continuous-PDT protocol 1. Each wound was inoculated with 1×10^8 CFU for one hour then treated with 1 mg/mL ICG combined with continuous light energy of 108 J/cm^2 from the 810 nm laser (L+S+). Control wounds received PBS instead of ICG (L-S-) and did not receive light. Error bars represent standard deviation from the mean of 6 wounds.

In an attempt to optimize the PDT treatment, a light dose of 216 J/cm^2 delivered intermittently in 6 minutes irradiation, 1 minute relaxation, followed by another 6 minutes of irradiation (Figure 10-4). However, when the light energy used was increased, no enhancement of *Staph. aureus* kill was observed. The kills of *Staph. aureus* achieved ranged from 78.2% to 94% with a mean of 90.2%. These reductions in the viable counts were in the range 0.7-1.2 \log_{10} and a mean of 1.01 \log_{10} . The number of viable *Staph. aureus* isolated from the PDT-treated wounds was significantly less ($P=0.001$) than those isolated from the matched control wounds which did not receive any treatment.

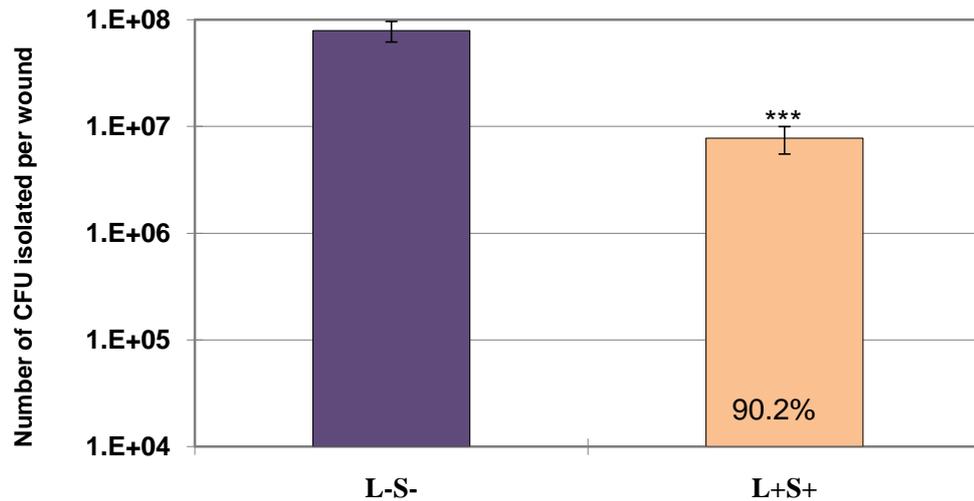


Figure 10-4: The number of viable *Staph. aureus* isolated from partial-thickness wounds treated with ICG continuous-PDT protocol 2. Each wound was inoculated with 1×10^8 CFU for one hour then treated with 1 mg/mL ICG combined with continuous light energy of 216 J/cm^2 delivered intermittently in 6 minutes on, 1 minute relaxation, followed by another 6 minutes of light exposure (L+S+). Control wounds received PBS instead of ICG and did not receive light (L-S-). Error bars represent standard deviation from the mean of 5 wounds.

A third PDT protocol was tested the data for which is presented in Figure 10-5. In this protocol, ICG-treated wounds were exposed to a light energy of 162 J/cm^2 . This light dose was delivered intermittently as follows: 3 minutes irradiation, 1 minute relaxation, 3 minutes irradiation, 1 minute relaxation followed by another 3 minutes of light irradiation. A mean reduction in the viable count of $1.06 \log_{10}$ and a range of $0.84\text{-}1.33 \log_{10}$ was obtained when *Staph. aureus* infected wounds were treated with this PDT protocol. The kills of *Staph. aureus* in PDT treated wounds averaged 90.94% with a range of 85.68-95.32%. The number of viable *Staph. aureus* isolated from the PDT-treated wounds was significantly ($P=0.0002$) less compared to that isolated from the matched control wound in each mouse.

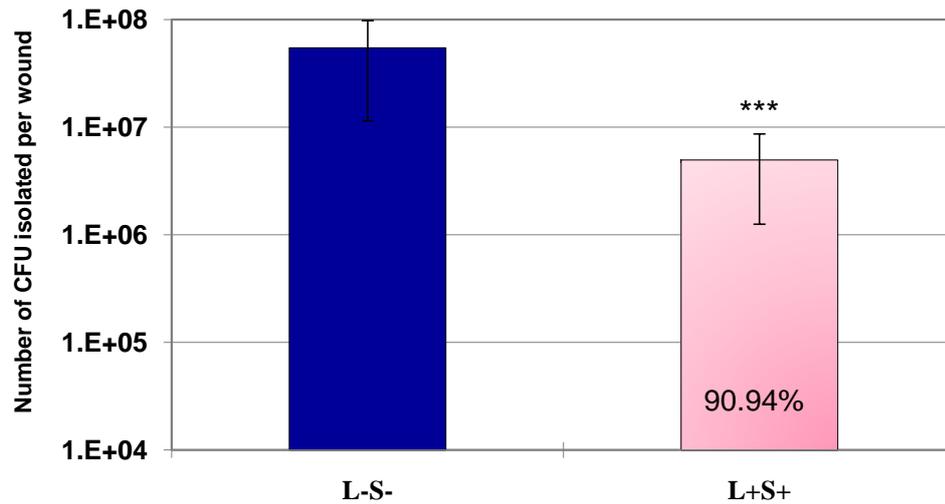


Figure 10-5: The number of viable *Staph. aureus* isolated from partial-thickness wounds treated with ICG continuous-PDT protocol 3. Each wound was inoculated with 1×10^8 CFU for one hour then treated with 1 mg/mL ICG combined with continuous light energy of 162 J/cm^2 delivered intermittently in 3 minutes on, 1 minute relaxation, 3 minutes on, 1 minute relaxation followed by another 3 minutes of light exposure (L+S+). Control wounds received PBS instead of ICG and did not receive light (L-S-). Error bars represent standard deviation from the mean of 5 wounds.

Figure 10-6 shows the bactericidal effect exerted by ICG-PDT on wounds infected with an initial load of 1×10^8 of *Staph. aureus* treated either by ICG activated with the NIR light of 162 J/cm^2 for 9 minutes or by the NIR light alone. The mice receiving light alone had a matched contra-lateral control wound which did not receive any treatment. The mice receiving ICG-PDT had a matched contra-lateral control wound which received ICG without the NIR light. Wounds treated with the NIR light showed no significant difference in the number of *Staph. aureus* isolated compared to their own control wound. The number of *Staph. aureus* isolated from the PDT-treated wounds were significantly ($P=0.01$) less compared to those isolated from the matched control wound in each mouse. A mean reduction of $1.1 \log_{10}$ was achieved after treating the wounds with ICG-PDT. The number of *Staph. aureus* isolated from the PDT-treated wounds were significantly ($P=0.03$) less compared to those isolated from the control wounds which did not receive any treatment in the other group of mice.

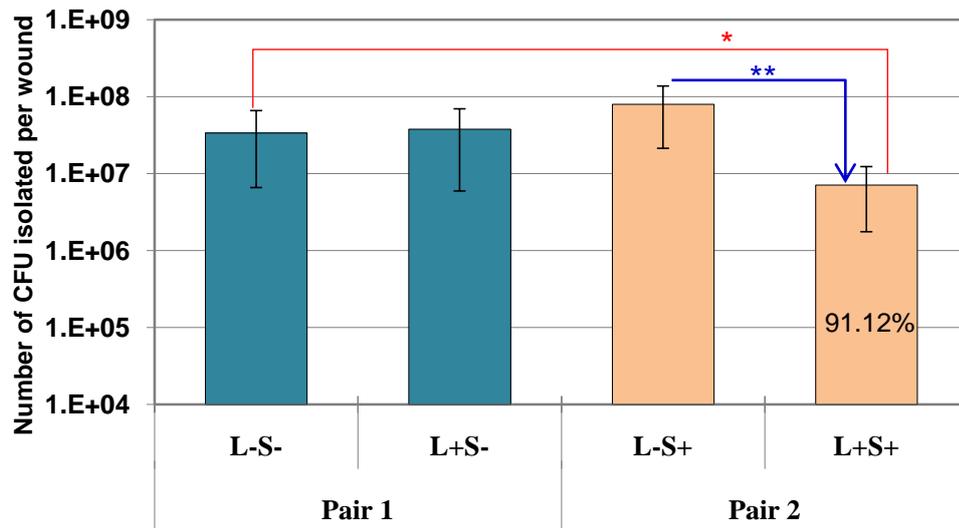


Figure 10-6: The number of viable *Staph. aureus* isolated from partial-thickness wounds treated with ICG continuous-PDT protocol 4. Wounds infected with 1×10^8 CFU exposed to a continuous light energy dose of 162 J/cm^2 delivered in 9 minutes after treatment with PBS (L+S-) or 1 mg/mL ICG (L+S+). Control wounds received PBS (L-S-) or ICG (L-S+) but were not exposed to light. Error bars represent standard deviation from the mean of 5 wounds.

The bactericidal effect mediated by ICG-PDT protocol 4, when the initial *Staph. aureus* inoculum used to infect the wounds was reduced to 1.4×10^7 is displayed in Figures 10-7a and 10-7b. The numbers of *Staph. aureus* isolated from the PDT-treated wounds were significantly ($P=0.046$) less compared to those isolated from the matched control wound in each mouse. A mean reduction of $0.85 \log_{10}$ and a range of $0.4\text{-}1.4 \log_{10}$ were achieved after treating the wounds with ICG-PDT (Figure 10-7a). The numbers of *Staph. aureus* isolated from the PDT-treated wounds were significantly ($P=0.047$) less compared to those isolated from the control wounds which did not receive any treatment in the other group of mice. A mean reduction of $0.89 \log_{10}$ and a range of $0.3\text{-}1.8 \log_{10}$ were achieved comparing the PDT treated wounds with the control wounds which did not receive any treatment in the other group of mice (Figure 10-7a). As shown in Figure 10-7a, however, wounds treated with the NIR light alone showed a 51.2% reduction in the viable count of *Staph. aureus*, this reduction was not significant when compared to their own control wound (neither received light nor ICG) (Figure 10-7a).

Figure 10-7b shows the percentage of viable bacteria retrieved from wounds in relation to the original inoculated number of *Staph. aureus*. The percentage of viable *Staph. aureus* retrieved from PDT-treated wounds amounted to $13.40 \pm 13.80\%$ compared to $60.26 \pm 33.79\%$ and $72.40 \pm 42.88\%$ retrieved, respectively, from control wounds receiving ICG but no light (L-S+) and wounds not receiving any treatment (L-S-). This percentage was significantly less than those retrieved from the control wounds ($P= 0.04$ for L-S- and $P= 0.05$ for L-S+).

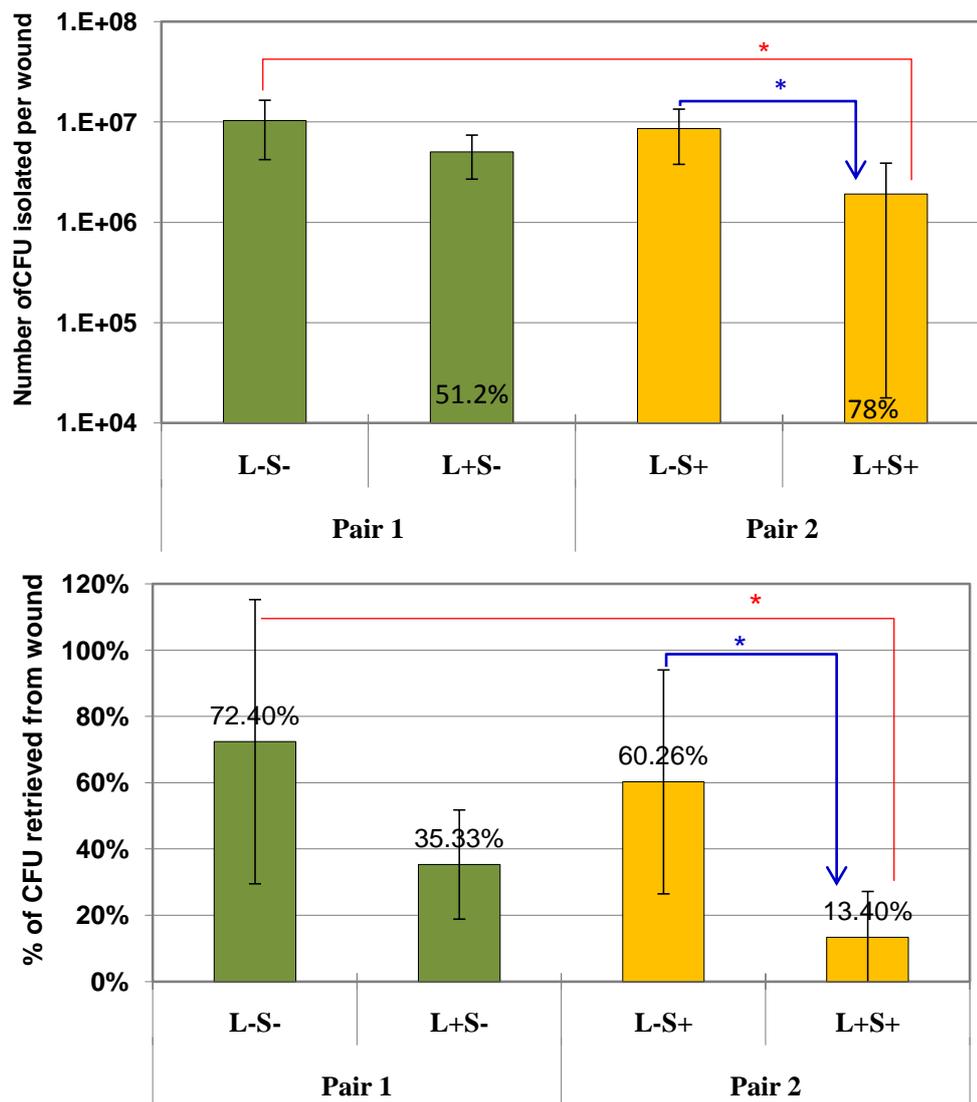


Figure 10-7: Treatment of *Staph. aureus* infected-wounds with ICG continuous-PDT protocol 4, (a) the number and (b) the percentage of *Staph. aureus* retrieved from the wounds. Wounds infected with 1.43×10^7 CFU exposed to a continuous light energy dose of 162 J/cm^2 delivered in 9 minutes after treatment with PBS (L+S-) or 1 mg/mL ICG (L+S+). Control wounds received PBS (L-S-) or ICG (L-S+) but were not exposed to light. Error bars represent the standard deviation from the mean of 4 wounds.

10.4.1.2 The effect of repeated continuous mode ICG-PDT

Figure 10-8 shows the bactericidal effect of repeated ICG-PDT treatments (protocol 4) on *Staph. aureus* infected wounds. When wounds infected with 1.59×10^7 CFU of *Staph. aureus* then treated with ICG-PDT twice a day for one day, a significant 77.73% reduction in the number of viable bacteria ($P=0.04$) was observed. When the animals received ICG-PDT twice a day for 3 consecutive days, a significant 85.13% reduction in the number of viable bacteria ($P=0.01$) was achieved. A 0.6 \log_{10} significant growth of *Staph. aureus* ($P=0.04$) was observed in the control wounds after 3 days of infection. However, a growth of 0.2 \log_{10} was observed in the wounds treated with ICG-PDT 3 days post-infection, this growth was not significant compared to the wounds treated for one day.

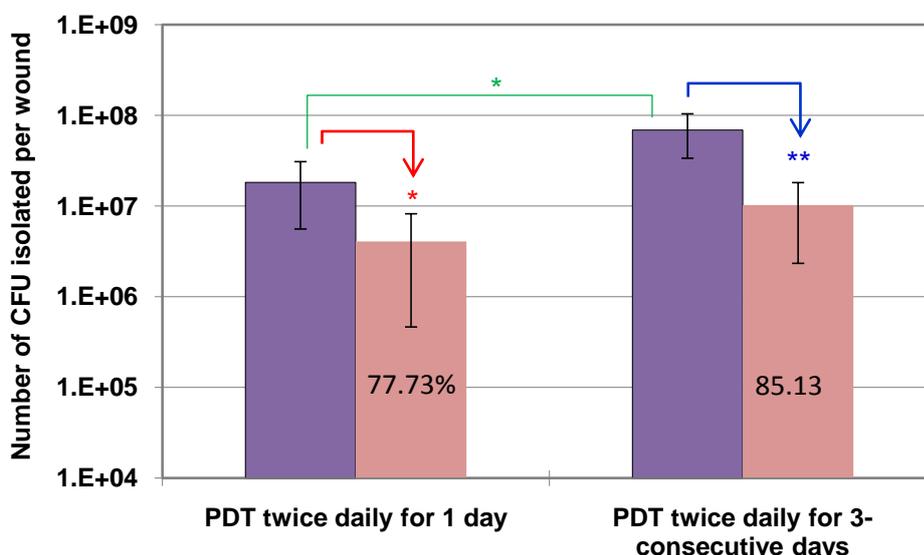


Figure 10-8: The effect of repeated treatment using ICG continuous-PDT on controlling *Staph. aureus* infected-wounds. The number *Staph. aureus* isolated after two PDT treatment/day for 1 day or for 3 consecutive days. Wounds infected with 1.59×10^7 CFU exposed to a continuous light energy dose of 162 J/cm^2 delivered in 9 minutes after treatment with 1 mg/mL ICG (■). Control wounds received ICG but were not exposed to light (■). Error bars represent the standard deviation from the mean of 4 wounds.

10.4.1.3 The effect of pulsed mode of irradiation during ICG-PDT

Figure 10-9 shows the effect of pulsed PDT using ICG to treat wounds infected with *Staph. aureus* with an initial load of 1×10^8 . When a light dose of 108

J/cm² was pulsed (pulse duration was 100 msec: 100 msec), the kills of *Staph. aureus* achieved ranged from 60.62% to 87.1% with a mean of 79.4%. These reductions in the viable counts were in the range 0.4-0.9 log₁₀ and a mean of 0.66 log₁₀. The number of *Staph. aureus* isolated from the pulsed PDT-treated wounds was significantly less (P=0.042) than that isolated from the matched control wounds which did not receive any treatment.

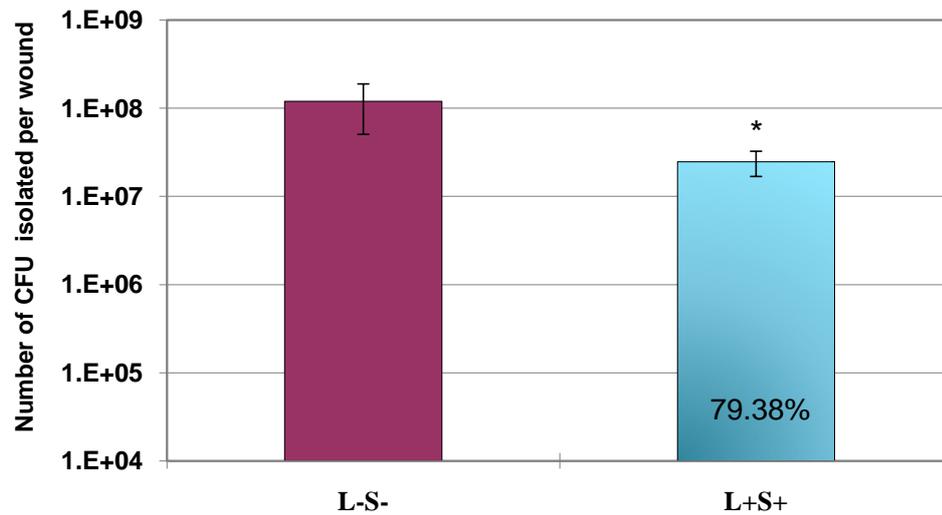


Figure 10-9: The number of viable *Staph. aureus* isolated from partial-thickness wounds treated with ICG pulsed-PDT protocol 5. Each wound was inoculated with 1 X 10⁸ CFU for one hour then treated with 1 mg/mL ICG combined with a pulsed light energy dose of 108 J/cm² from the 810 nm laser (L+S+). Control wounds received PBS instead of ICG (L-S-) and did not receive light. Error bars represent standard deviation from the mean of 3 wounds.

When the light dose was increased to 162 J/cm² and was pulsed (pulse duration was 150 msec: 50 msec), the kills of *Staph. aureus* achieved ranged from 51.34% to 75.4% with a mean of 60.86% as seen in Figure 10-10. These reductions in the viable counts were in the range 0.31-0.61 log₁₀ and a mean of 0.43 log₁₀. The number of viable *Staph. aureus* isolated from the pulsed PDT-treated wounds was significantly less (P=0.044) than those isolated from the matched control wounds which did not receive any treatment.

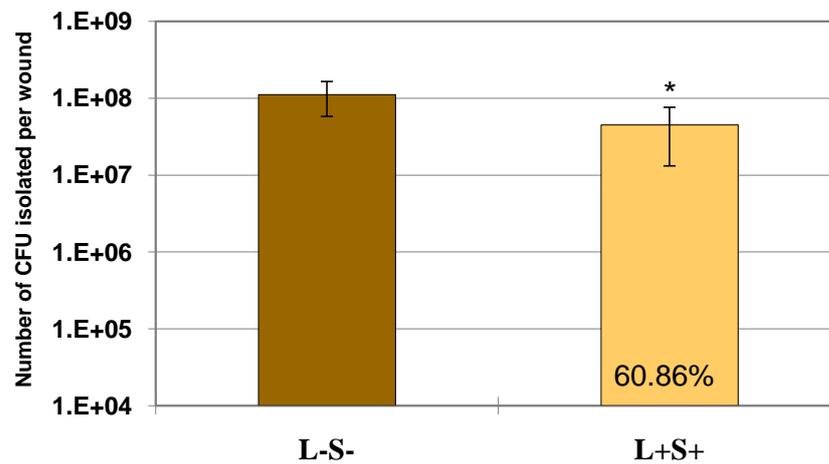


Figure 10-10: The number of viable *Staph. aureus* isolated from partial-thickness wounds treated with ICG pulsed-PDT protocol 6. Each wound was inoculated with 1×10^8 CFU for one hour then treated with 1 mg/mL ICG combined with pulsed light energy of 162 J/cm^2 from the 810 nm laser (L+S+). Control wounds received PBS instead of ICG (L-S-) and did not receive light. Error bars represent the standard deviation of the mean of 3 wounds.

10.4.2 The effect of continuous mode ICG-PDT on the viability of *Strep. pyogenes* infected partial-thickness wounds in mice

Figures 10-11a and 10-11b shows the bactericidal effect mediated by continuous mode ICG-PDT (protocol 4), on wounds inoculated with 1.6×10^7 CFU of *Strep. pyogenes*. The kills of *Strep. pyogenes* ranged from 82.92% to 99.95% with a mean of 93% when compared to the number of bacteria isolated from the matched control wound in each mouse (Figure 10-11a). These significant reductions ($P=0.002$) in the viable counts were in the range $0.8\text{-}3.3 \log_{10}$ and had a mean of $1.42 \log_{10}$. A significant mean reduction of $1.4 \log_{10}$ and a range of $0.6\text{-}3.2 \log_{10}$ ($P=0.002$) were achieved comparing the PDT treated wounds with the control wounds which did not receive any treatment in the other group of mice (Figure 10-11a). The numbers of viable *Strep. pyogenes* isolated from the PDT-treated wounds were significantly ($P=0.007$) less compared to those isolated from the control wounds which received light but no ICG in the other group of mice.

As shown in Figure 10-11b, all control wounds showed significant growth of the bacteria in the wound tissue, yet ICG-PDT treated wounds showed a significant reduction in the number of viable *Strep. pyogenes* retrieved as seen in Figure 10-11b. ICG-PDT induced a greater than 75.4% reduction in the number of

viable *Strep. pyogenes* retrieved from the wounds compared to the initial number of *Strep. pyogenes* inoculated into the wounds. The percentage of viable *Strep. pyogenes* retrieved from PDT-treated wounds amounted to $24.60 \pm 25.03\%$ compared to $329.82 \pm 129.06\%$, $294.44 \pm 107.61\%$ and $206.43 \pm 146.64\%$ retrieved, respectively, from control wounds receiving ICG but no light (L-S+), wounds not receiving any treatment (L-S-) and wounds receiving light only (L+S-). This percentage was significantly less than those retrieved from all control wounds ($P= 0.0001$ for L-S-, $P= 0.0002$ for L-S+ and $P= 0.01$ for L+S-).

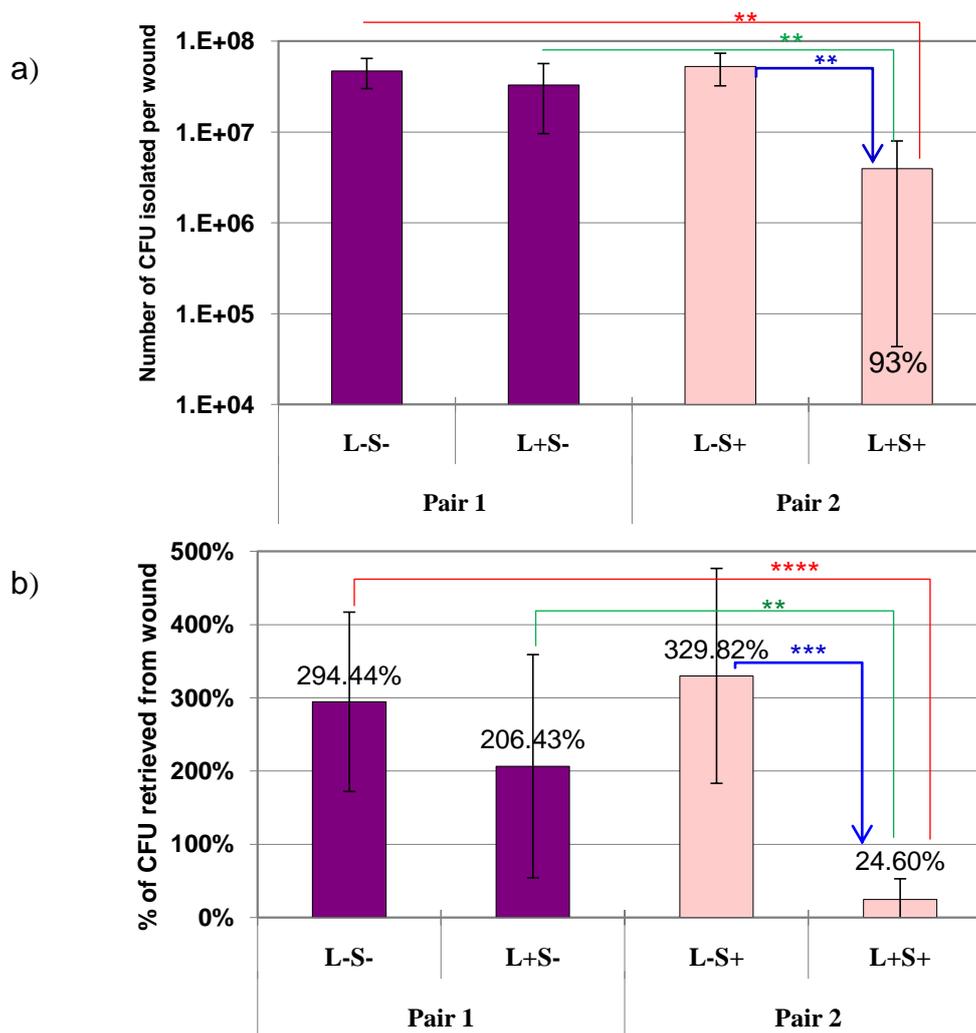


Figure 10-11: Treatment of *Strep. pyogenes* infected-wounds with ICG continuous-PDT protocol 4, (a) the number and (b) the percentage of *Strep. pyogenes* retrieved from the wounds. Wounds infected with 1.6×10^7 CFU exposed to continuous light energy of 162 J/cm^2 delivered in 9 minutes after treatment with PBS (L+S-) or 1 mg/mL ICG (L+S+). Control wounds received PBS (L-S-) or ICG (L-S+) but were not exposed to light. Error bars represent the standard deviation of the mean of 8 wounds.

10.4.3 The effect of continuous mode ICG-PDT on the viability of *P. aeruginosa* infected partial-thickness wounds in mice

Figures 10-12a and 10-12b shows the bactericidal effect mediated by continuous mode ICG-PDT (protocol 4), on wounds inoculated with 1.6×10^7 CFU of *P. aeruginosa*. The kills of *P. aeruginosa* ranged from 87.77% to 99.81% with a mean of 96.45% when compared to the number of bacteria isolated from the matched control wound which received ICG but no light in the same mouse (Figure 10-12a). These significant reductions ($P=0.0001$) in the viable counts had a range of 1.0-2.72 \log_{10} and a mean of 1.65 \log_{10} . A significant mean reduction of 1.69 \log_{10} and a range of 1.2-2.64 \log_{10} ($P=0.00005$) were achieved comparing the PDT treated wounds with the control wounds which did not receive any treatment in the other group of mice (Figure 10-12a). The numbers of viable *P. aeruginosa* isolated from the PDT-treated wounds were significantly ($P=0.0001$) less compared to those isolated from the control wounds which received light but no ICG in the other group of mice. Interestingly, a 26% significant reduction in the number of *P. aeruginosa* isolated from the light-treated wounds ($P=0.02$) compared to those isolated from the matched control wound (L-S-) in each mouse (Figure 10-12a).

As shown in Figure 10-12b, all control wounds showed rapid growth of the bacteria in wound tissue, yet ICG-PDT treated wounds showed a significant reduction in the number of *P. aeruginosa* retrieved as seen in Figure 10-12b. ICG-PDT induced a greater than 94.4% reduction in the number of viable *P. aeruginosa* retrieved from the wounds compared to the initial number of bacteria inoculated into the wounds. The percentage of viable *P. aeruginosa* retrieved from PDT-treated wounds amounted to $5.75 \pm 4.55\%$ compared to $161.71 \pm 53.35\%$, $170.86 \pm 15.84\%$ and $126.96 \pm 42.40\%$ retrieved, respectively, from control wounds receiving ICG but no light (L-S+), wounds not receiving any treatment (L-S-) and wounds receiving light only (L+S-). This percentage was significantly less than those retrieved from all control wounds ($P < 0.00001$ for L-S-, $P = 0.0001$ for both L-S+ and L+S-). The percentage of viable *P. aeruginosa* retrieved from light-treated wounds was significantly less ($P = 0.01$) than those retrieved from the matched control wound (L-S-) in each mouse (Figure 10-12b).

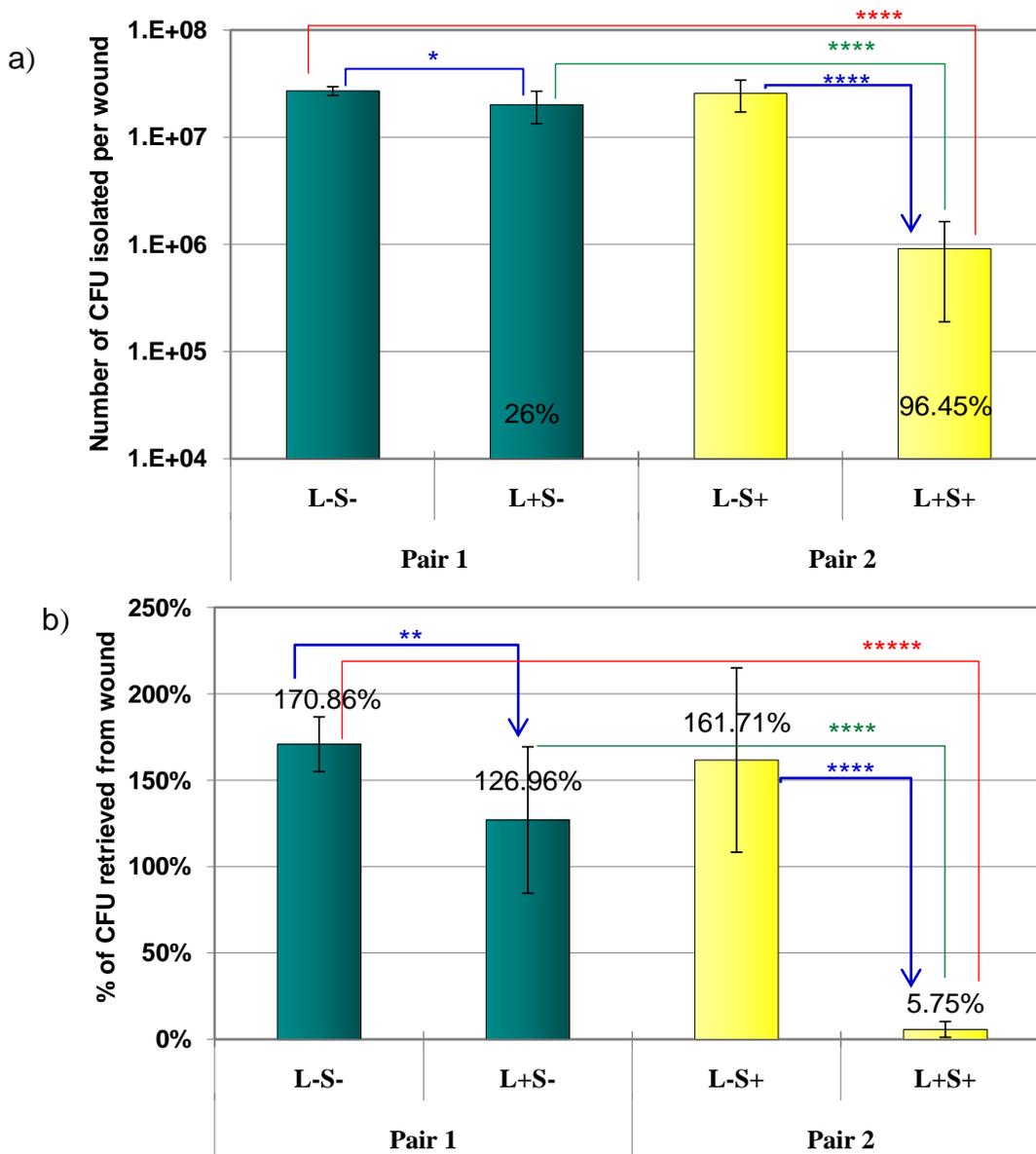


Figure 10-12: Treatment of *P. aeruginosa* infected-wounds with ICG continuous-PDT protocol 4, (a) the number and (b) the percentage of *P. aeruginosa* retrieved from the wounds. Wounds infected with 1.6×10^7 CFU exposed to a continuous light energy dose of 162 J/cm^2 delivered in 9 minutes after treatment with PBS (L+S-) or 1 mg/mL ICG (L+S+). Control wounds received PBS (L-S-) or ICG (L-S+) but were not exposed to light. Error bars represent standard deviation of the mean of 8 wounds.

10.4.4 Effect of PDT on the temperature of the wounds

Tables 10-2, 10-3, 10-4 show the subcutaneous temperature in the wounds infected with *Staph. aureus* then treated with different PDT protocols. As shown in Table 10-2 the wound temperatures rose by a maximum of $13.04 \pm$

2.63°C during continuous irradiation ICG-PDT and the maximum temperature achieved in this group of wounds was 42.5°C.

When continuous irradiation ICG-PDT was repeated twice a day with a one hour interval between the treatments, a maximum subcutaneous wound temperature of 38.6°C was recorded and the temperature rose by a maximum of $11.28 \pm 1.5^\circ\text{C}$ (Table 10-3).

Table 10-4 shows that the wound temperature recorded during the two pulsed PDT protocols used in this study were lower than those achieved during continuous-PDT. When a light dose of 108 J/cm^2 was delivered at a pulse duration of (100msec on: 100msec off) (protocol 5), the wound temperature rose by a maximum of $2.4 \pm 1.28^\circ\text{C}$ and the maximum recorded temperature of the wound was 34.5°C . Increasing the light dose to 162 J/cm^2 delivered at a pulse duration of (150msec on: 50msec off) (protocol 5) resulted in a maximum wound temperature rise of $5.2 \pm 0.06^\circ\text{C}$ and the maximum recorded temperature of the wound was 35.7°C . The core temperature of the animals remained consistently stable throughout the treatment.

Table 10-2: The subcutaneous wound temperature recorded during several continuous ICG-PDT protocols to treat *Staph. aureus* infection

Protocol	1 PDT	2 PDT	3 PDT	4	
				Laser only	PDT
Initial temperature	31.63 ± 0.47	26.36 ± 0.6	29.54 ± 2.2	29.82 ± 1.9	27.38 ± 1.8
Final temperature	41.43 ± 0.71	39.4 ± 2.25	38.6 ± 1.6	32.22 ± 2.4	36.18 ± 2.2
Temperature rise	9.8 ± 1.18	13.04 ± 2.63	9.06 ± 2.76	2.4 ± 1.5	8.8 ± 2.8

Table 10-3: The subcutaneous wound and core temperature recorded during repeated continuous ICG-PDT to treat *Staph. aureus* infection

Protocol		Initial temperature	Final temperature	Temperature rise
Repeated PDT treatments				
1st-PDT dose	Subcutaneous	30.47 ± 0.89	37.1 ± 1.16	6.63 ± 0.84
	Core	29.75 ± 1.02	28.0 ± 1.42	-1.75 ± 0.87
2nd-PDT dose	Subcutaneous	23.87 ± 1.23	35.15 ± 1.9	11.28 ± 1.5
	Core	24.38 ± 1.52	24.63 ± 1.35	0.25 ± 1.4

Table 10-4: The subcutaneous wound temperature recorded during pulsed ICG-PDT protocols to treat *Staph. aureus* infections

Protocol	5 Pulsed-PDT	6 Pulsed-PDT
Initial temperature	31.0 ± 0.3	30.3 ± 0.25
Final temperature	33.4 ± 1.5	35.5 ± 0.26
Temperature rise	2.4 ± 1.28	5.2 ± 0.06

Table 10-5 shows the subcutaneous temperature in the wounds infected with *Strep. pyogenes* or *P. aeruginosa* then treated with the continuous PDT protocol 4. The subcutaneous temperature of wounds infected with *Strep. pyogenes* rose by a maximum of 14.86 ± 1.1°C during continuous irradiation ICG-PDT and the maximum temperature achieved in this group of wounds was 39.5°C. A similar pattern was observed for wounds infected with *P. aeruginosa*, a maximum wound temperature of 41.5°C was recorded and the temperature rose by a maximum of 11.98 ± 4.0°C (Table 10-5).

The core temperature of the animals remained stable throughout the treatment. The maximum subcutaneous temperature of the wounds treated with light in the absence of ICG was 35.3°C as shown in Tables 10-2 and 10-5.

Table 10-5: The subcutaneous wound and core temperature recorded during continuous ICG-PDT protocol 4 to treat *Strep. pyogenes* and *P. aeruginosa* infections

Infected organism	Type of temperature	Laser only			PDT protocol 4		
		Initial	Final	Rise	Initial	Final	Rise
<i>Strep. pyogenes</i>	Subcutaneous	23 ± 2.0	31 ± 3.1	8	23 ± 1.1	37.86 ± 1.2	14.86
	Core	23.64 ± 2.2	23.8 ± 1.45	0.16	23.2 ± 1.1	23.65±0.77	0.45
<i>P. aeruginosa</i>	Subcutaneous	23.3 ± 2.2	30.1 ± 5.2	6.8	25.9 ± 1.7	37.88 ± 3.3	11.98
	Core	24 ± 2.6	24 ± 1.9	0	25.7 ± 1.6	25.3 ± 0.74	-0.4

10.5 Discussion

In this part of the study, the investigation has been taken further to determine the ability of ICG-PDT to kill the most common wound-infecting organisms during the early phase of colonization in a wound model in mice. These *in vivo* studies have demonstrated that ICG and the NIR light could reduce the microbial burden in wounds infected with *Staph. aureus*, *Strep. pyogenes* or *P. aeruginosa*.

In this study, different ICG-PDT protocols were employed to treat *Staph. aureus* infected wounds. When infected wounds were treated with ICG and irradiated with continuous NIR light (108 J/cm²), significant reductions in the viable counts of *Staph. aureus* ranging from 79 to 97% with a mean of 91%. Under the same conditions, pulsed light (100 msec on: 100 msec off) of 108 J/cm² resulted in a kill range of 60.6-87.1% with a mean of 79.4%. A higher light dose of 162 J/cm² applied with a pulse duration of 150 msec on: 50 msec off, achieved a slightly lower range of kills 51.34-75.4%. These results revealed that the pulse duration may play a role in the effectiveness of PDT. Nussbaum *et al.*, (2002) inferred that the NIR laser light mediated growth stimulation of *Staph. aureus* was dependent on the pulse frequency. Thus, the continuous mode of irradiation was more effective than the pulsed mode in killing *Staph. aureus* infected wounds. A previous *in vitro* study has shown that the cytotoxic effect mediated from using the pulsed light for the treatment of mammalian cells was significantly less than that of using continuous light and this was possibly related to the reduced oxygen consumption and ROS production when pulsed irradiation is employed (Kawauchi *et al.*, 2004).

A growing number of *in vivo* studies have shown that PDT is an effective antimicrobial strategy to treat and control infections due to *Staph. aureus*. In this study, when wounds were inoculated with 1 x 10⁸ CFU of *Staph. aureus*, the average kills achieved after treating the wounds with a single dose of continuous-PDT was always more than 90% (1.1 log₁₀), regardless of the PDT protocol used. In support of these results, Zolfaghari *et al.*, (2009), demonstrated that the treatment of *Staph. aureus* infected wounds using MB activated with 360 J/cm² of 665 nm light resulted in significant reductions of 1.40 log₁₀ and 1.15 log₁₀ in the number of viable bacteria recovered, respectively, from excision and superficial wounds in mice. Another study has reported that TBO-PDT significantly reduced the bacterial load of *Staph. aureus* by 96.4%, while red light of 660 nm alone achieved a reduction of 84.5% in a wound model in rats (de Carvalho *et al.*, 2008). In contrast, in this study the NIR laser light did not reduce the number of *Staph. aureus* significantly at a light dose of 162 J/cm². This difference may be attributed to the different wavelength of light and the high fluence rate of 1.4 W/cm² at which the micro-organism was irradiated in the study of de Carvalho *et al.*, compared to 0.3

W/cm² in this study. Both of the above-mentioned studies determined the number of viable bacteria per wound using the viable counting method, the same method used in this study.

In this study, wounds infected with 1×10^7 CFU of *Staph. aureus* showed lower kills compared to those infected with 1×10^8 CFU. Significant reductions of approximately $0.85 \log_{10}$ or 78% in the number of viable bacteria recovered from wounds treated with a single dose of ICG coupled with a continuous light energy of 162 J/cm². The lower rate of kill may be due to the inability to retrieve all the bacteria inoculated into the control wounds. For example in this experiment, only $60.26 \pm 33.79\%$ and $72.40 \pm 42.88\%$ were retrieved, respectively, from control wounds which received ICG but no light and wounds which did not receive any treatment. Thereafter, when *Staph. aureus* infected wounds were treated with the same ICG-PDT protocol twice a day for one day, similar reductions of 78% in the number of viable bacteria were observed. However, when the animals received ICG-PDT twice a day for 3 consecutive days, a significant 85.13% reduction in the number of viable bacteria was achieved. In this group of mice, 3 days post-infection, untreated wounds showed a significant growth of $0.6 \log_{10}$ while a small insignificant growth of $0.2 \log_{10}$ was noted in the PDT-treated wounds. A similar bacterial re-growth has been reported after treating infected burn wounds (Lambrechts *et al.*, 2005c) and soft tissue infections (Gad *et al.*, 2004b) with PTMPP and poly-L-lysine chlorin e6 conjugate PDT, respectively. Both studies have shown that even though > 98% of the bacteria were killed using a light dose of 160-210 J/cm² in the presence of PS, bacterial re-growth was detected (Gad *et al.*, 2004b; Lambrechts *et al.*, 2005c).

Strep. pyogenes invades the host mainly through an open wound resulting in streptococcal toxic shock syndrome, necrotizing fasciitis and/or bacteraemia (Morales *et al.*, 2006). *Strep. pyogenes* is one of the most invasive organisms infecting wounds. The effect of antimicrobial PDT has not previously been investigated on killing this organism *in vivo*. In this study, irradiation of the wounds infected with *Strep. pyogenes* in the presence of ICG resulted in a significant reduction of 93% in the number of viable bacteria recovered from the wounds. In contrast to *Staph. aureus*, *Strep. pyogenes* showed a very

rapid growth in the wounds. The percentage of viable *Strep. pyogenes* retrieved from control wounds amounted to $329.82 \pm 129.06\%$, $294.44 \pm 107.61\%$ and $206.43 \pm 146.64\%$ recovered, respectively, from control wounds receiving ICG but no light, wounds which did not receive any treatment and wounds receiving light alone. While a very small percentage of $24.60 \pm 25.03\%$ bacteria were retrieved from wounds treated with a single dose of ICG photo-activated with continuous light energy of 162 J/cm^2 . Hope & Wilson, (2006) have shown that cell death was induced in biofilms of *Strep. pyogenes* when treated with a Sn (IV) chlorin e6 (SnCe6) at a concentration of $50 \mu\text{g/mL}$ and exposed to laser light with wavelengths of 488 nm and 543 nm. The cumulative cell damage was most likely caused by the generation of $^1\text{O}_2$ by SnCe6 on exposure to laser light.

Wong *et al.*, (2005) treated lethal *V. vulnificus*-infected wounds with $100 \mu\text{g/mL}$ TBO and exposed them to a broad-spectrum red light (150 J/cm^2 at 80 mW/cm^2), 53% of PDT-treated mice survived, even though the infection had been established with a bacterial inoculum 100-times the 50% lethal dose. Hamblin *et al.*, (2003) have demonstrated that 90% of poly-L-lysine-ce6 conjugate PDT-treated mice survived an invasive *P. aeruginosa* infection in excision-wounds in mice. In contrast, all of the mice that had not received any treatment died within 5 days post-infection. The authors optically monitored the infection through bioluminescence imaging (Hamblin *et al.*, 2003). Both studies have implied that PDT can control and reduce the bacterial load in wounds and prevent fatal complications. Another study has reported 75% kills of *P. aeruginosa* after the treatment of infected skin with tin (IV) chlorin e6 - monoclonal antibody conjugate and a light dose of 160 J/cm^2 at a fluence rate of 100 mW/cm^2 (Berthiaume *et al.*, 1994). In accordance with the previous studies, this study has shown significant reductions of 96.45% in the number of viable *P. aeruginosa* recovered from wounds treated with a single dose of ICG activated with a continuous light energy dose of 162 J/cm^2 . *P. aeruginosa* multiplied rapidly in wound tissues that did not receive ICG-PDT. The percentages of viable *P. aeruginosa* retrieved from control wounds were $161.71 \pm 53.35\%$, $170.86 \pm 15.84\%$ and $126.96 \pm 42.40\%$, respectively, from control wounds receiving ICG but no light, wounds not receiving any treatment and wounds receiving light alone. In comparison, only $5.75 \pm 4.55\%$ of viable

P. aeruginosa were retrieved from PDT-treated wounds. Interestingly, continuous NIR laser light of 162 J/cm² in the absence of ICG resulted in a very small but significant reduction of 26% in the number of viable *P. aeruginosa* isolated from light-treated wounds. These data are consistent with the *in vitro* results which have shown that the high fluence 808 nm light in the absence of ICG exerted a cidal effect of 0.7-1.2 log₁₀ against *P. aeruginosa*, achieving 79.3% and 93.2% kills in saline and serum, respectively (see Chapter 7).

Although substantial kills of the three wound-infecting organisms were achieved *in vivo*, these kills were considerably lower than those achieved *in vitro*. Several factors may account for the reduced bactericidal effect observed *in vivo* including: (1) binding of the PS to wound tissues thus reducing its effectiveness and resulting in the generation of ROS in sites far from the target bacteria; (2) absorption of laser light by PS bound to host tissues – this would cause shielding of any PS bound to bacteria preventing light activation and (3) scavenging of ROS by host molecules thereby protecting bacteria from its damaging effects. Using a PS covalently linked to a bacterial targeting moiety may overcome some of these problems, and hence enable greater kills to be achieved *in vivo* (Hamblin *et al.*, 2003; Gad *et al.*, 2004b).

A change in temperature of the wound tissues during the PDT procedure has been reported in only one study. Zolfaghari *et al.*, (2009) reported a rise in the wound temperature - the average maximum temperature at the centre of the wounds being 42.7 ± 1.8°C when wounds were irradiated with 360 J/cm² of red light in the presence of MB. The slightly lower temperatures reported in this study may be due to the lower light energy used. In this study, the wound temperature rose significantly upon continuous irradiation of the wounds with 162 J/cm² in the presence of ICG - the average temperature at the centre of the wounds being 36.2 ± 2.2; 37.9 ± 1.2 and 37.9 ± 3.3°C, respectively, for *Staph. aureus*, *Strep. pyogenes* and *P. aeruginosa*-infected wounds. Using a higher light dose of 216 J/cm² the wound temperature increased to 39.4 ± 2.3 °C. These temperatures are unlikely to account for the bacterial kills observed. Furthermore, such a temperature would not induce collateral tissue damage because normal tissues can tolerate temperatures up to 45°C via heat dissipation and augmentation of blood flow (Storm *et al.*, 1979). In the current

study, the wound temperature was at 40°C for no longer than 9 minutes and did not reach 45°C. Microscopic examination of wound biopsies immediately following MB-PDT treatment using 360 J/cm² of red light and after 24 hours did not reveal any tissue necrosis regardless of the experimental treatment applied (Zolfaghari *et al.*, 2009).

In summary, *in vivo* studies employing a partial-thickness wound model in mice showed that ICG and continuous-NIR light could achieve kills of 96.43%, 93% and 78-91% for *P. aeruginosa*, *Strep. pyogenes* and *Staph. aureus* respectively. Lower kills of 61-79.4% were observed using ICG and pulsed-NIR light. Repeated ICG-PDT treatments can control *Staph. aureus* infections- the average kills being 85.13%. The results of these *in vivo* studies imply that ICG-PDT could be an effective means of decreasing the microbial burden in wounds.

Chapter 11

Final discussion and conclusions

The current worldwide rise in antibiotic-resistant bacteria and, simultaneously, the downward trend in the development of new antibiotics mean that the treatment of infected wounds is becoming increasingly difficult. Infections are responsible for the failure of wounds to heal and may cause other complications including septicaemia that may lead to death (Vazquez, 2006). It is clear that there is a need to develop novel antimicrobial strategies to which pathogens will not easily develop resistance. One promising alternative to conventional antibiotics is PDT. The *in vitro* and *in vivo* results of the research project described in this thesis imply that the light-activated antimicrobial agent ICG may be able to contribute to reducing the microbial load in infected wounds and consequently also reduce the rate of cross-infection and transmission of bacteria in hospital environments.

The ideal light-activated antimicrobial agent would have the following characteristics:

- (i) very low toxicity,
- (ii) would not induce hypersensitivity reactions,
- (iii) rapid tissue clearance
- (iv) activated by near-infrared light to enable maximum tissue penetration,
- (v) easy to apply,
- (vi) effective at killing a range of micro-organisms.

ICG is a NIR-absorbing water-soluble dye, which has been approved by the FDA of the USA for medical diagnostic studies. ICG is a safe dye of very low toxicity, with no reported hypersensitivity, rapidly cleared from the body via the biliary pathway and has a high absorption at wavelengths around 800 nm. It has been safely used for diagnostic angiography and PDT treatment of macular degeneration at concentrations as high as 20 mg/mL (Yoon *et al.*, 2007). Recently, PDT with topically applied ICG has been used to treat acne vulgaris (Tuchin *et al.*, 2003; Genina *et al.*, 2004). However it has, as yet, not been investigated for the PDT of any other infectious disease. This project was concerned with evaluating its effectiveness against wound-infecting organisms *in vitro* and *in vivo*.

The initial aim of the study was to determine whether the common wound-infected organisms *Staph. aureus*, *Strep. pyogenes*, *P. aeruginosa* and *E. coli* are susceptible to lethal photosensitization using the dye ICG coupled with NIR laser light. Based on an initial absorption scanning of ICG in saline and serum, light with a wavelength of 808 ± 5 nm was chosen to activate the dye. The investigations revealed that all species were susceptible to killing, the bactericidal effect being dependent on both the concentration of ICG and the light dose. Although ICG at a concentration of 25 $\mu\text{g/mL}$ enabled the killing of the Gram-positive species (*Staph. aureus* and *Strep. pyogenes*), higher concentrations of 100-200 $\mu\text{g/mL}$ were necessary to achieve substantial kills of the Gram-negative species (*P. aeruginosa* and *E. coli*). The fluence rate at which the light was delivered was another variable that proved to be an important aspect of the antimicrobial efficacy. Nevertheless, both high and low fluences were able to kill large proportions (99.999% of suspensions containing 1×10^7 CFU/mL) of the Gram-positive bacteria, high fluence irradiation was necessary to kill 99.99% of suspensions containing 1×10^8 - 1×10^9 CFU/mL of the Gram-negative bacteria. Low light energies were capable of killing the Gram-positive species, while high light energies were necessary to kill the Gram-negative species. These experiments demonstrated that Gram-negative bacteria are less susceptible to ICG-photosensitization than the Gram-positive species. In addition, the results of this study have shown that exposure of mixed bacterial cultures to NIR laser light in the presence of ICG results in a dose-dependent decrease in bacterial viability, indicating that PDT could be useful in the treatment of burn and wound infections caused by mono or multi-species. Furthermore, the bacterial kills achieved were independent of the pre-irradiation time which is an important consideration for the clinical application of PDT. Interestingly, 808 nm light alone at both high and low fluences was able to achieve a small but significant kill of *P. aeruginosa* which may be attributable to the endogenous pigments (pyoverdinin and pyocyanin) that may absorb the light and result in the production of bactericidal species. A related hypothesis is that the NIR laser light may interfere with bacterial metabolic processes such as cellular respiration resulting in a reduction in ATP production which in turn causes bacterial death (Nandakumar *et al.*, 2003). Certainly, this killing effect was not heat mediated as the temperature of the bacterial suspensions did not

exceed 33°C during irradiation. The current data suggest that NIR laser light irradiation by itself would also inhibit growth of *P. aeruginosa* in infected wounds.

It has been claimed that fractionation of the light delivered helps to maintain a high level of tissue oxygenation during PDT and allows deeper tissue penetration (Dougherty *et al.*, 1998). Furthermore, the use of a pulsed laser with longer pulse duration allows adequate thermal diffusion and so may avoid tissue necrosis (Shokrollahi *et al.*, 2004). Therefore, once the bactericidal activity of ICG combined with the NIR light had been established, the next step was to evaluate the efficacy of pulsed mode in comparison with continuous mode of irradiation in the presence of ICG to photosensitize common organisms responsible for wound infections. The pulsed-mode of irradiation was as effective as the continuous-mode for killing the Gram-positive species. At a low concentration of 15.5 µg/mL ICG, pulsed NIR laser light resulted in slightly greater kills of the Gram-positive organisms *Staph. aureus* and *Strep. pyogenes*, however, increasing the concentration of ICG to 25 µg/mL or more removed any difference in bacterial photo-toxicity attributed to the modulation of the light wave. These kills were not significantly different from kills induced by the continuous light. Nevertheless, only the continuous-mode of irradiation was able to kill *P. aeruginosa* in the presence of 100 µg/mL and a light energy dose of 63 J/cm². At this combination of ICG and the NIR light energy, the temperature of the bacterial cultures increased up to 43.7 °C. Such temperatures would not affect the viability of the Gram-negative bacterium but may assist in the diffusion of ICG through the cell membrane to initiate the phototoxic effect (Leyko & Bartosz, 1986; Dougherty *et al.*, 1998). These data suggest that both modes of irradiation combined with ICG are capable of killing the Gram-positive species, but only the continuous-mode is able to kill the Gram-negative species.

In an attempt to enhance the killing mediated by ICG-photosensitization, three different strategies were investigated to enhance the effectiveness of *Staph. aureus* lethal photosensitization. First, Au nanoparticles were found to

enhance the photo-bactericidal capability of a low concentration of 10 µg/mL ICG by 47-fold compared to the free ICG. Secondly, antioxidants such as vitamins C and E were also found to improve the efficiency of antimicrobial-PDT. Despite this fact, caution should be exercised when choosing the appropriate antioxidant because it can act either way as an anti-oxidant or a pro-oxidant. Vit C combined with Fe (II) resulted in a 14-fold enhancement, while TroloxTM resulted in a 1000-fold increase in the number of *Staph. aureus* killed. Finally, the use of NaSCN resulted in only a 4-fold enhancement of *Staph. aureus* photo-inactivation. These data imply that the non-covalent interaction of either AuNPs or antioxidants with ICG were significantly effective in increasing photosensitization-mediated bacterial killing and show potential as antimicrobial-PDT enhancers.

It was established that ICG, in conjunction with NIR laser light, is an effective light activated-antimicrobial agent which acts against a wide range of planktonic bacterial cells responsible for wound infections. However, many bacteria responsible for diseases in humans exist as biofilms. Hence, aggregated microcolonies of *Staph. aureus* and *P. aeruginosa* have been found attached to damaged host tissue surrounded by an extracellular matrix (Bjarnsholt *et al.*, 2008; Davis *et al.*, 2008; James *et al.*, 2008). Consequently, the ability of NIR laser light alone and in combination with ICG to disrupt the EPS of *Staph. aureus* and *P. aeruginosa* biofilms, as well as to kill the bacterial cells was investigated. Remarkably, the light alone disrupted approximately 41 % of the EPS of *P. aeruginosa* biofilms. When a light dose of 90 J/cm² was used to activate ICG approximately 55 % and 38% disruption was detected for *P. aeruginosa* and *Staph. aureus* biofilms, respectively. The production of ROS and other free radicals accelerate the decomposition of proteins, lipids and carbohydrates which may cause photo-oxidation of the main constituents of the biofilms resulting in significant disruption of the EPS (Lyons & Jenkins, 1997). The NIR laser light killed approximately 99.9 % of 2.7 x 10¹⁷ CFU *P. aeruginosa* cells encased in biofilms, while a combination of both light and dye resulted in a 99.3% kill. A shielding effect may reduce the kill in the presence of 200 µg/mL ICG (Bhatti *et al.*, 1997). Exposure of *Staph. aureus* biofilms to

200 µg/mL ICG in combination with NIR laser light or to the NIR light alone resulted in significant 99.9% and 99.7% reductions in the number of viable bacteria when compared to the control biofilms containing 7.3×10^{11} CFU. NIR laser light is absorbed mainly by water (Shokrollahi *et al.*, 2004) which constitutes 50-90% of the matrix material of the biofilm. *Staph. aureus* and *P. aeruginosa* produce staphyloxanthin and pyocyanin pigments, respectively. The biosynthesis of these pigments is always increased when bacterial cells are grown as a biofilm (Resch *et al.*, 2005; Mavrodi *et al.*, 2006). The NIR laser light absorbed within the biofilm may cause excitation of these pigments that may trigger a burst of ROS resulting in killing the bacteria. In clinical practice, the disruption of the EPS by the NIR laser light alone or in combination with ICG may help to counteract much of the risk posed by infections caused by bacterial biofilms in wounds. This method has been widely used for the removal of dental plaque (Cortes, 2003).

It was essential to determine whether ICG in combination with the NIR laser light would be able to kill the investigated organisms under conditions similar to those found in infected wounds. It was found possible to achieve substantial kill of the targeted bacteria under physiological conditions mimicking the wound environment. In the presence of serum, high and low light intensities were able to achieve killing of *Staph. aureus* at high concentrations of ICG. ICG-mediated photo-cytotoxicity was slightly inhibited in the case of *E. coli*, but was unaffected for *P. aeruginosa*. Furthermore, pulsed and continuous modes of irradiation resulted in substantial kills of *Staph. aureus* and *Strep. pyogenes*. Only continuous irradiation was capable of photosensitizing *P. aeruginosa*, both in the absence and presence of serum, achieving substantial kills of this organism. If these kills are achievable *in vivo*, ICG in combination with NIR light may be an effective means of eradicating bacteria from wounds and burns. The decrease in the effectiveness of lethal photosensitization of bacteria under anaerobic conditions confirms that the greater the oxygen concentration present in the environment, the greater the photolethal effect of ICG. These findings imply that the level of tissue oxygenation is an important factor to consider during the attempted eradication of bacteria from wounds. Although PDT is known to consume oxygen (Ochsner, 1997), the relative superficiality of

the infection in the wound allows free diffusion of atmospheric oxygen during PDT treatment. Otherwise in ischemic wounds, the application of supplementary hyperoxygenation i.e. 100% normobaric or hyperbaric oxygen, during PDT treatment may improve its antimicrobial effect (Huang *et al.*, 2003).

The next logical step was to explore the underlying mechanisms involved in bacterial killing mediated by the photo-activated ICG. The results of mechanistic studies have shown that ICG is an effective light-activated antimicrobial agent capable of producing $^1\text{O}_2$. The SOSG assay revealed that the greater the light energy absorbed by the ICG molecules, the greater the extent of $^1\text{O}_2$ generation. Increasing the life-time of $^1\text{O}_2$ enhanced bacterial killing while quenching this ROS reduced killing. These results suggest that photo-inactivation of micro-organisms using ICG is mediated by $^1\text{O}_2$, with the possible involvement of other ROS. ICG was taken up by the Gram-positive bacteria *Staph. aureus* and *Strep. pyogenes* but not by the Gram-negative bacterium *P. aeruginosa*. Significant kills of both Gram-positive bacteria were achieved when unbound ICG was washed out from the cell suspensions. However, the number of cells killed was still lower than when the PS was not washed from the suspensions. No photosensitization of *P. aeruginosa* was achievable after removal of unbound ICG. Exposing the target organisms to similar temperatures to those recorded during lethal photosensitization, resulted in no reduction in bacterial viability. Therefore, it can be concluded that the observed kills were mediated by the light-induced generation of singlet oxygen (and, possibly, free radicals) rather than to thermal effects.

A treatment modality that can reduce the activities of virulence factors released by the infecting organisms as well as killing them is potentially a great advantage of any antimicrobial agent. ICG was found to reduce the proteolytic activity of the V8 protease of *Staph. aureus* when irradiated with NIR light of 808 nm, achieving a 23 % reduction under the conditions used. A light dose of 45 J/cm^2 or more was necessary to inactivate the V8 protease when the light was delivered at a low fluence rate of 0.05 W/cm^2 . In the case of the total proteolytic activity of *P. aeruginosa*, only delivering the light at a high fluence rate of 0.3 W/cm^2 in the presence of ICG was capable of inactivating the

enzymes, achieving a reduction of only 8.22%. A substantial reduction in the potency of *P. aeruginosa* LPS was achieved upon exposure to NIR laser light in the presence and the absence of ICG. The light alone resulted in a 55.46-64.64% reduction in LPS potency. Combining the NIR light with ICG resulted in greater reductions of 76-86% in LPS potency. These results suggest that ICG in combination with NIR light is an effective method of reducing the activities of two virulence factors of bacteria (proteases and LPS) implicated in delayed wound healing. Thus the application of PDT as a treatment for wound infection would have a major advantage over conventional antibiotic therapy.

PDT has a number of advantages over conventional antibiotics. Firstly, as the mechanism of killing is non-specific, with ROS causing damage to many bacterial components, resistance is unlikely to develop from repeated use (Jori *et al.*, 2006). Secondly, both the PS and the light are applied locally to the target tissue; therefore reducing the risk of adverse systemic effects (Hamblin & Hasan, 2004). Superficial wound infections are particularly appropriate for treatment by PDT due to their easy accessibility to both a topical PS and light. Therefore, once the activity of the ICG activated with the NIR laser light had been extensively tested *in vitro*, the final stage of the project was to assess their efficacy *in vivo*. A partial thickness wound model in mice was chosen on the basis of its good reliability and reproducibility of the infection (Zolfaghari *et al.*, 2009). This *in vivo* model imitates the early stages of an infectious process i.e. the initial colonisation of a wound by a potential pathogen. The results of these *in vivo* studies demonstrated that ICG and continuous-NIR light could achieve kills of 96.43%, 93% and 78-91% for *P. aeruginosa*, *Strep. pyogenes* and *Staph. aureus* infected wounds, respectively. These wounds were initially inoculated with bacterial suspension contain approximately 1×10^7 - 1×10^8 CFU for *Staph. aureus* or 1×10^7 CFU for *Strep. pyogenes* and *P. aeruginosa*. Pulsed-PDT treatment did not enhance the bactericidal effect of ICG-PDT. Lower kills of 61-79.4% were observed using ICG and pulsed-NIR light. Repeated ICG-PDT treatments were able to control *Staph. aureus* infections- the average kills being 85.13% from an initial inoculum of 1×10^7 CFU. The

results of these *in vivo* studies imply that ICG-PDT could be an effective means of decreasing the microbial burden in wounds.

A potential problem with PDT however, is its lack of specificity. The generated ROS may damage host tissue as well as bacterial cells (Lambrechts *et al.*, 2005c). Antimicrobial PDT will need further optimization to the point at which bacterial cells can be killed rapidly and efficiently, while minimizing normal cell and tissue damage. Conjugation of the PS to an antibody targeting the bacteria may overcome this problem and improve the specificity of the PS (Berthiaume *et al.*, 1994). Delayed wound healing has been reported after infected burn wounds treated with the photosensitizer PTMPP and high energies (211–423 J/cm²) of non-coherent light (Lambrechts *et al.*, 2005c). In contrast, Komerik *et al.*, (2002 & 2003) showed no necrotic or inflammatory changes were found on the adjacent tissues in the buccal mucosa of the rats after the treatments with 200 µg/ml TBO and 340 J/cm² from 632.8 nm laser light. Recently, Luan *et al.*, (2009) reported no adverse effects after treating periodontal tissues using 1 mg/mL TBO and a light energy dose of 60 J/cm² from 665 nm laser light. In a wound model in mice, laser light energy doses up to 360 J/cm² and 100 µg/mL MB did not cause any collateral tissue damage (Zolfaghari *et al.*, 2009). The results of these studies suggest that PDT could be a safe antimicrobial approach for topical infections without damaging the adjacent normal tissue. Due to time constraints, the effects of ICG-PDT on normal and infected tissues were not investigated. This point needs to be addressed in future work and its effect on wound healing will be another variable to study.

In this study, the effect of ICG-PDT has been evaluated in animal models, but more in depth investigation is necessary on its effect on human tissues (normal and infected tissues). More specifically, random controlled clinical trials are needed to determine the optimal light energies and the PS concentration and their effects in humans.

In conclusion, the extensive *in vitro* and *in vivo* studies carried out in this research project have demonstrated that the light-activated antimicrobial agent ICG could consistently reduce the microbial burden present in infected wounds. The bactericidal effect was shown to be mediated by the light-induced generation of singlet oxygen and free radicals rather than to thermal effects. Additional investigations might include carrying out histological analysis after treating normal and infected tissues with ICG-PDT, studying the course of wound healing during ICG-PDT, application of ICG-PDT under hyperoxygenation conditions or enhancing the killing *in vivo* using antioxidants and AuNPs.

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Lethal photosensitization of wound-associated microbes using indocyanine green and near-infrared light

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Abstract

Background: The increase in resistance to antibiotics among disease-causing bacteria necessitates the development of alternative antimicrobial approaches such as the use of light-activated antimicrobial agents (LAAAs). Light of an appropriate wavelength activates the LAAA to produce cytotoxic species which can then cause bacterial cell death via loss of membrane integrity, lipid peroxidation, the inactivation of essential enzymes, and/or exertion of mutagenic effects due to DNA modification. In this study, the effect of the LAAA indocyanine green excited with high or low intensity light (808 nm) from a near-infrared laser (NIR) on the viability of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* was investigated.

Results: All species were susceptible to killing by the LAAA, the bactericidal effect being dependent on both the concentration of indocyanine green and the light dose. Indocyanine green photosensitization using both high (1.37 W cm⁻²) and low (0.048 W cm⁻²) intensity NIR laser light was able to achieve reductions of 5.6 log₁₀ (>99.99%) and 6.8 log₁₀ (>99.99%) in the viable counts of *Staph. aureus* and *Strep. pyogenes* (using starting concentrations of 10⁶–10⁷ CFU ml⁻¹). Kills of 99.99% were obtained for *P. aeruginosa* (initial concentration 10⁸–10⁹ CFU ml⁻¹) photosensitized by the high intensity light (1.37 W cm⁻²); while a kill of 80% was achieved using low intensity irradiation (0.07 W cm⁻²). The effects of L-tryptophan (a singlet oxygen scavenger) and deuterium oxide (as an enhancer of the life span of singlet oxygen) on the survival of *Staph. aureus* was also studied. L-tryptophan reduced the proportion of *Staph. aureus* killed; whereas deuterium oxide increased the proportion killed suggesting that singlet oxygen was involved in the killing of the bacteria.

Conclusion: These findings imply that indocyanine green in combination with light from a near-infrared laser may be an effective means of eradicating bacteria from wounds and burns.

Background

Wound infections are an ongoing problem not only for patients but also for healthcare providers worldwide. Infected wounds are responsible for significant morbidity and mortality, and an increase in the duration and the cost of hospital stay [1-3]. The growing resistance to con-

ventional antibiotics among organisms that infect wounds and burns makes such infections difficult to treat [4-6]. Therefore there is a drive to develop novel antimicrobial strategies to which pathogens will not easily develop resistance.

One promising alternative is photodynamic therapy (PDT). Photodynamic therapy is the application of a non-toxic compound, termed a photosensitizer (PS) or light-activated antimicrobial agent (LAAA), which can be activated by light of an appropriate wavelength to produce reactive oxygen species (ROS) (i.e. singlet oxygen and free radicals) which can then exert a microbicidal effect [7,8]. Light of the appropriate wavelength excites the PS molecule into a triplet state which reacts with either a substrate to produce radical ions which in turn react with oxygen to produce cytotoxic species such as superoxide and hydroxyl radicals (type I reaction), or reacts directly with molecular oxygen to produce singlet oxygen (1O_2) (type II reaction). PDT has a number of advantages over conventional antibiotics. Firstly, as the mechanism of killing is non-specific, with reactive oxygen species causing damage to many bacterial components, resistance is unlikely to develop from repeated use [9,10]. Secondly, both the PS and the light are applied locally to the target tissue; therefore reducing the risk of adverse systemic effects [11]. Cutaneous wound infections are particularly appropriate for treatment by PDT due to their easy accessibility to both a topical PS and light. The organisms most frequently responsible for infections of wounds and burns are *Streptococcus pyogenes*, staphylococci, such as methicillin-resistant *Staphylococcus aureus* (MRSA), and the Gram-negative bacterium *Pseudomonas aeruginosa* [1]. *Staphylococcus aureus* is a common wound-infecting organism which results in delayed epithelial closure of the wound, possibly due to its interaction with fibronectin and inhibition of keratinocyte migration [12]. The eradication of wound-infecting bacteria using lethal photosensitization has been reported in the literature, e.g. *Staph. aureus* [13-15], *P. aeruginosa* [16-19], *Strep. pyogenes* [20].

In this study we have investigated whether common wound-associated organisms are sensitive to lethal photosensitization using the dye indocyanine green (ICG) coupled with light from a near-infrared (NIR) laser emitting at 808 nm. This combination of PS and light source has a number of desirable characteristics over those described in the literature [13-20]. NIR laser light has a greater capacity to penetrate tissues than light of lower wavelengths whilst ICG is a NIR-absorbing water-soluble tricarbocyanine dye, which has been approved by the United States Food and Drug Administration (US FDA) for medical diagnostic studies. ICG has a very low toxicity and a high absorption at wavelengths around 800 nm. Recently, PDT with ICG has been used to treat tumors [21,22]. However it has, as yet, not been used for the PDT of infections with the exception of its topical use for the treatment of acne vulgaris [23-25].

Methods

Bacteria

Two Gram-positive organisms were used in this study; *Staph. aureus* strain 8325-4 and *Strep. pyogenes* strain 12202. In addition, the Gram-negative bacterium *Pseudomonas aeruginosa* strain PA01 was used. Gram-positive bacteria were maintained by weekly subculture on blood agar base (Oxoid Ltd, UK) supplemented with 5% horse blood whereas *P. aeruginosa* was subcultured on nutrient agar (Oxoid). *Staph. aureus* and *P. aeruginosa* were grown aerobically in nutrient broth (Oxoid) with shaking at 37°C for 16 h. *Strep. pyogenes* was grown in brain heart infusion broth (Oxoid) for 16 h at 37°C in an atmosphere of 5% CO₂/95% air. Cells were then harvested by centrifugation and resuspended in phosphate buffered saline (PBS) to an optical density of 0.05 at 600 nm which corresponded to approximately 10⁶-10⁷ colony forming units per ml (CFU ml⁻¹) for the Gram-positive bacteria and 10⁸-10⁹ CFU ml⁻¹ for *P. aeruginosa*.

Photosensitizer

4,5-benzoindotricarbocyanine (Indocyanine green) C₄₃H₄₇N₂NaO₆S₂ is a negatively-charged polymethine dye and was obtained from Sigma-Aldrich-UK. Fresh stock solutions were prepared immediately prior to each experiment in either PBS or sterile distilled water (H₂O) or deuterium oxide (D₂O) and kept in the dark.

Laser light

A 500 mW Gallium-aluminum-arsenide (Ga-Al-As) NIR-Laser (Thor International Ltd - UK) emitting light with a wavelength of 808 ± 5 nm was used for irradiation. For experimental purposes, the distance of the laser probe to the plate surface was adjusted to give fluence rates of 1.37, 0.07 or 0.048 W cm⁻² with an actual power output of 470, 225 and 150 mW respectively.

Effect of photosensitizer concentration on lethal photosensitization

Aliquots (50 µl) of a suspension of *Staph. aureus*, *Strep. pyogenes* (containing approximately 10⁶-10⁷ CFU ml⁻¹) or *P. aeruginosa* (10⁸-10⁹ CFU ml⁻¹) in sterile PBS were transferred into a 96-well plate and an equal volume of ICG in PBS was added to each well to give final concentrations ranging from 25 µg ml⁻¹ to 250 µg ml⁻¹. After addition of the ICG, the wells were left in the dark for 10 or 30 minutes (pre-irradiation time) and then exposed to a measured dose of high intensity laser light at a fluence rate of 1.37 W cm⁻². In this system, an exposure of 5 minutes corresponded to a light dose of 411 J cm⁻². Each experimental condition was tested in at least triplicate and each experiment was carried out on at least two occasions. The conditions tested were; 1) controls which contained neither ICG nor received irradiation (L- S-), 2) incubation with ICG in the dark (L- S+), 3) irradiation in the absence of

ICG (L+ S-) and 4) the test which was irradiated in the presence of ICG (L+S+).

To enumerate the surviving bacteria, serial 10-fold dilutions were plated in duplicate either on blood agar (*Staph. aureus* and *Strep. pyogenes*) or nutrient agar (*P. aeruginosa*).

Effect of low intensity laser light

To avoid any heating effect that may occur during high intensity irradiation, the experiments were repeated using a laser power output of 150 mW to irradiate the samples at a low fluence rate of 0.048 W cm⁻². Aliquots (50 µl) of a suspension of *Staph. aureus* or *Strep. pyogenes* (containing approximately 10⁶-10⁷ CFU ml⁻¹) in sterile PBS were transferred into a 96-well plate and an equal volume of ICG in PBS was added to each well to give final concentration of 25 µg ml⁻¹. After addition of the ICG, the wells were exposed to a measured dose of low intensity laser light for 15 or 30 minutes corresponding to light doses of 43 and 86 J cm⁻² respectively. Similar experiments were performed with *P. aeruginosa* except that the concentration of ICG was 200 µg ml⁻¹ and using a laser power output of 225 mW with a fluence rate of 0.07 W cm⁻² for 35 minutes which corresponded to a light dose of 147 J cm⁻².

Effect of laser light dose

The effect of laser light dose on bacterial killing was investigated by varying the exposure time whilst the distance from the light source remained constant. The bacterial suspensions were prepared as described above. A photosensitizer concentration of 25 µg ml⁻¹ was used for photosensitizing the Gram-positive organisms while 200 µg ml⁻¹ was used for *P. aeruginosa*. Survival was determined after 1, 3, and 5 minutes irradiation at a high fluence rate of 1.37 W cm⁻² which corresponded to energy doses of 28, 85 and 141 J and energy densities of 82, 247 and 411 J cm⁻² respectively.

Effect of serum on the lethal photosensitization of *Staph. aureus*

In order to investigate the lethal photosensitization of *Staph. aureus* in an environment similar to that which would exist in a wound, lethal photosensitization experiments were performed in the presence of 50% horse serum (HS). *Staph. aureus* was suspended in HS (~10⁷ CFU ml⁻¹) and ICG (in PBS) added at concentrations of 25 or 200 µg ml⁻¹ (bacteria were not washed after incubation with serum). After addition of ICG, samples of 100 µl were irradiated at a fluence rate of 1.37 W cm⁻² and light dose of 411 J cm⁻².

Effect of an enhancer of singlet oxygen life span on lethal photosensitization

To determine if singlet oxygen was involved in the lethal photosensitization process, D₂O (Sigma-Aldrich, UK) was

used to extend the life span of any singlet oxygen generated by exposure of ICG to laser light. *Staph. aureus* was suspended in D₂O or H₂O (~2 × 10⁷ CFU ml⁻¹) and ICG added to give a final concentration of 25 µg ml⁻¹. Samples of 100 µl in triplicate wells were exposed to laser light for 1 minute at a fluence rate of 1.37 W cm⁻². Control wells were also prepared and either were not exposed to light but received ICG or did not receive ICG and were not exposed to light or did not receive ICG and were exposed to light.

Effect of a free-radical and singlet oxygen scavenger on lethal photosensitization

Staph. aureus was suspended in PBS (~2 × 10⁷ CFU ml⁻¹) or different concentrations of L-tryptophan (10 µM-10 mM) dissolved in PBS and incubated at 37°C for 1 h to detect any bactericidal activity of the scavenger itself. No bactericidal effect of the scavenger alone was detected. Samples of 100 µl in triplicate wells containing *Staph. aureus* and 25 µg ml⁻¹ ICG (L+ S+) in H₂O (control) or the scavenger at a concentration of 10 mM were exposed to laser light for 1 minute at a fluence rate of 1.37 W cm⁻². Control wells containing *Staph. aureus* and 10 mM L-tryptophan were also prepared and either did not receive ICG and were not exposed to light (L-S-) or were not exposed to light but received ICG (L-S+) or did not receive ICG and were exposed to light (L+S-).

Measurement of bacterial suspension temperature during lethal photosensitization and the effect of these temperatures on the viability of *Staph. aureus* and *P. aeruginosa*

100 µL of the bacterial suspension, with final concentrations of 25, 100 or 200 µg ml⁻¹ ICG, were exposed to a measured dose of high intensity laser light for 1, 3 or 5 minutes (L+S+) to determine the temperature elevation during lethal photosensitization. Three additional wells contained the microbial suspension plus PBS instead of the photosensitizer were exposed to the same light doses to determine the rise of temperature in the absence of ICG (L+S-). The temperatures of the L+S- and L+S+ aliquots were recorded immediately before and after irradiation using an immersion thermocouple probe connected to a Fluke 179 digital multimeter (Fluke, USA). The experiments were carried out in triplicate.

Aliquots of *Staph. aureus* either in sterile PBS or in HS (containing approximately 10⁷ CFU ml⁻¹) were added to an equal volume of ICG to give a final concentration of 25 or 200 µg ml⁻¹, whereas *P. aeruginosa* suspension in PBS was added to ICG to give a final concentration of 200 µg ml⁻¹. Controls were prepared by adding an equal volume of PBS instead of ICG. The *Staph. aureus* suspensions were incubated either at 40°C (suspension in PBS) or 50°C (suspension in 50% HS) whereas *P. aeruginosa* was incu-

bated at 50°C. All aliquots were incubated for 10 minutes in the dark. The survivors were enumerated by viable counting.

Statistics

All experiments were performed twice and at least in triplicate except for lethal photosensitization experiments in horse serum with 25 µg ml⁻¹ which were performed twice but in duplicate. Values are expressed as means ± standard deviation. Comparisons between means of groups were analyzed using the Mann-Whitney U test. P = 0.05 was considered statistically significant.

Results

Indocyanine green is an effective photosensitizer of wound-infecting organisms

When *Staph. aureus* was treated with different concentrations of ICG and exposed to 411 J cm⁻² of high intensity NIR light a significant reduction in the viable count was achieved even with the minimum concentration of ICG used. For instance, when 25 µg ml⁻¹ of ICG was used there was a significant (P < 0.01) 5.56 log₁₀ reduction in the viable count of the suspension which contained 2.55 × 10⁷ CFU ml⁻¹ corresponds to 99.99% efficacy. Suspensions of *Staph. aureus* treated with ICG but not irradiated (L- S+) showed a small (0.5 log₁₀) but significant (P < 0.05) reduction in the viable count (Figure 1).

When *Strep. pyogenes* was treated with different concentrations of ICG and exposed to 411 J cm⁻² of NIR light, a sig-

nificant (P < 0.001) reduction in the viable count was achieved even with the lowest concentration of ICG. Treatment with 25 µg ml⁻¹ of ICG gave approximately a 4.7 log₁₀ (99.99%) reduction in the viable count. This amounted to a kill of 5.66 × 10⁶ CFU ml⁻¹. Bacteria treated with ICG but not irradiated (L- S+), or those not treated with ICG but irradiated with NIR light did not show a significant reduction in viability (Figure 1).

When *P. aeruginosa* was treated with ICG, the concentration used to obtain a significant kill with the Gram-positive bacteria above was not sufficient to achieve killing upon irradiation. However, increasing the concentration of ICG to 200 µg ml⁻¹ gave a significant (P < 0.001) 2 log₁₀ (99.1%) reduction in the viable count on irradiation. This equated to killing of approximately 5.99 × 10⁸ CFU ml⁻¹. Interestingly, irradiation of *P. aeruginosa* with NIR light in the absence of ICG resulted in a significant (P < 0.05) reduction in the viability of this organism (approximately 0.4 log₁₀ reduction) albeit not as great as that achieved in the presence of ICG (Figure 1).

The effect of various dye concentrations

The bactericidal effect in all species was dependent on the ICG concentration. Figure 2 shows the log₁₀ unit reduction in the viable count in the three micro-organisms when treated with different concentration of the PS and using a light dose of 411 J cm⁻² at a fluence rate of 1.37 W cm⁻². The minimum effective concentration for killing the Gram-positive organisms was 25 µg ml⁻¹ whilst 200 µg ml⁻¹ was needed to kill *P. aeruginosa*.

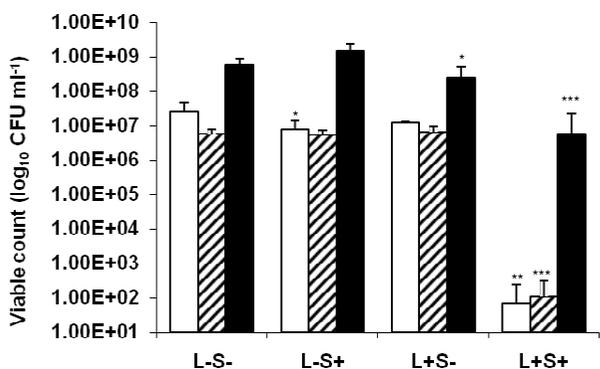


Figure 1 High intensity lethal photosensitization of *Staph. aureus* □ and *Strep. pyogenes* ■ with 25 µg ml⁻¹ ICG or *P. aeruginosa* ■ with 200 µg ml⁻¹ ICG. An equal volume of either 50 µg ml⁻¹ (in the case of *Staph. aureus* and *Strep. pyogenes*) or 400 µg ml⁻¹ (in the case of *P. aeruginosa*) ICG (L-S+ and L+S+) or PBS (L-S- and L+S-) was added to each bacterial suspension, samples were left for 10 minutes in the dark and then irradiated at a fluence rate of 1.37 W cm⁻² with a light dose of 411 J cm⁻² from a NIR 808 nm laser (L+S- and L+S+) or kept in the dark (L-S+ and L-S-).

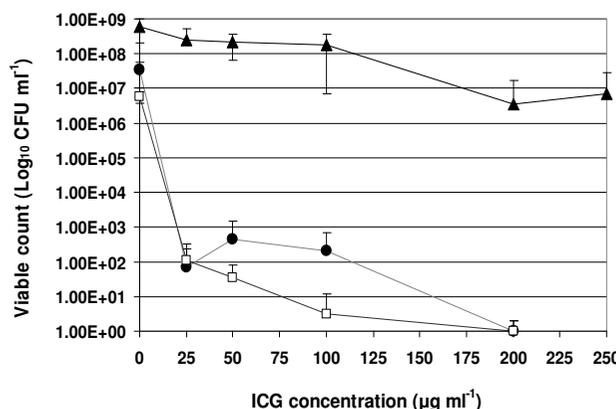


Figure 2 Lethal photosensitization of *Staph. aureus* ●, *Strep. pyogenes* □ with 25, 50, 100 and 200 µg ml⁻¹ ICG, and *P. aeruginosa* ▲ with 25, 50, 100, 200 and 250 µg ml⁻¹ ICG. An equal volume of the appropriate ICG concentration (L+S+) was added to each bacterial suspension, samples were left for 10 minutes in the dark and then irradiated at a fluence rate of 1.37 W cm⁻² with light dose of 411 J cm⁻² from a NIR 808 nm laser.

The effect of low intensity light

Irradiation of *Staph. aureus* in the presence of ICG for 15 or 30 minutes with a low fluence rate of 0.048 W cm⁻² achieved a significant ($P < 0.001$) reduction of 3.8 and 4.3 log₁₀ in the viable count respectively (Figure 3A). Using the same conditions, irradiation of *Strep. pyogenes* resulted in significant ($P < 0.001$) reductions of 3.7 and 5.4 log₁₀ in the viable count respectively (Figure 3A). For both gram positive-bacteria, kills amounting to 99.98 and 99.99% reductions in the viable count were achieved depending on the irradiation time. In the case of *P. aeruginosa*, a significant ($P = 0.001$) reduction of 0.7 log₁₀ (80%) in the viable count was achieved in the presence of 200 µg ml⁻¹ ICG which was irradiated for 35 minutes at a fluence rate of 0.07 W cm⁻², corresponding to a light dose of 147 J cm⁻².

Amongst the three bacteria, only irradiation of *P. aeruginosa* with NIR light in the absence of ICG resulted in a sig-

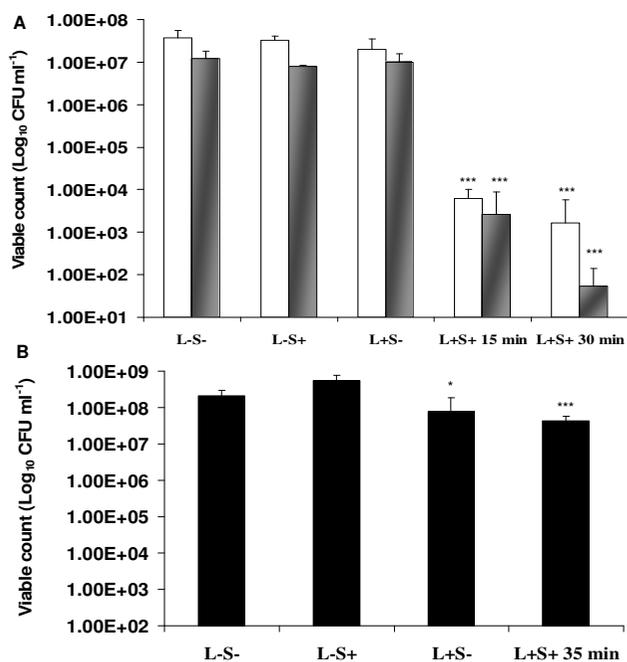


Figure 3

(A) Lethal photosensitization of *Staph. aureus* □ and *Strep. pyogenes* ■ using a final concentration of 25 µg ml⁻¹ ICG. Samples were left for 10 minutes in the dark and then irradiated using a fluence rate of 0.048 W cm⁻² for 15 and 30 minutes, corresponding to light doses of 43 & 86 J cm⁻² respectively. (B) Lethal photosensitization of *P. aeruginosa* ■ using a final concentration of 200 µg ml⁻¹ ICG. Samples were left for 10 minutes in the dark and then irradiated using a fluence rate of 0.07 W cm⁻² for 35 minutes, corresponding to light dose of 147 J cm⁻². L-S- and L+S- were kept in the dark.

nificant ($P < 0.05$) 0.44 log₁₀ (64%) reduction in the viability of this organism (Figure 3B).

The effect of light dose

During high intensity irradiation, the bactericidal effect was dependent on the light dose delivered. Significant ($P < 0.01$) reductions of 2.54 (99.7%), 3.36 (99.94%), and 5.15 log₁₀ (99.99%) in the viable count of *Staph. aureus* were achieved using exposure times of 1, 3 and 5 minutes respectively. Significant ($P < 0.001$) log₁₀ reductions in the viable count of *Strep. pyogenes* were 3.94 (99.99%), 6.82 (99.99%) and 6.13 (99.99%) using light doses of 1, 3 and 5 minutes respectively. In the case of *P. aeruginosa*, lethal photosensitization using exposure times of 1, 3 and 5 minutes achieved significant ($P < 0.001$) kills of 1.35, 1.75 and 4.74 log₁₀ corresponding to 95.5, 98.2 and 99.99% reductions in the viable count respectively. However, in the absence of ICG, irradiation of *P. aeruginosa* also resulted in significant kills, with exposure times of 1, 3 and 5 minutes achieving log₁₀ reduction in the viable count of 0.66 (78.2%), 0.85 (85.93%) and 1.36 (95.65%) respectively (Figure 4).

Lethal photosensitization of *Staph. aureus* in the presence of serum

In the presence of HS, lethal photosensitization of *Staph. aureus* using a high fluence rate was significantly inhibited (Figure 5). Lethal photosensitization using 25 µg ml⁻¹ ICG and an irradiation time of 5 minutes in 50% HS gave a significant ($P < 0.05$) 0.3 log₁₀ reduction in the viable count

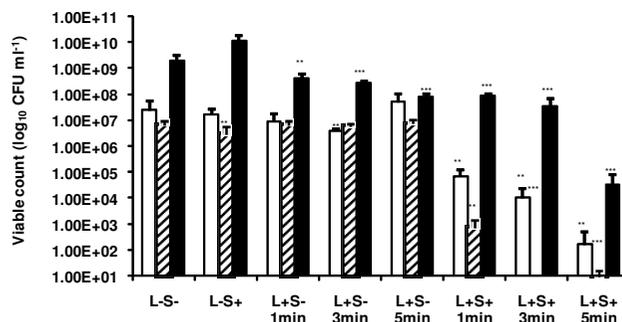


Figure 4

High intensity lethal photosensitization of *Staph. aureus* □, *Strep. pyogenes* ■ with 25 µg ml⁻¹ ICG and *P. aeruginosa* ■ with 200 µg ml⁻¹ ICG. An equal volume of either 50 µg ml⁻¹ (in the case of *Staph. aureus* and *Strep. pyogenes*) or 400 µg ml⁻¹ (in the case of *P. aeruginosa*) ICG (L-S+ and L+S+) or PBS (L-S- and L+S-) was added to each bacterial suspension. Samples were left in the dark for 30 minutes and then (L+S- and L+S+) irradiated at a fluence rate of 1.37 W cm⁻² for 1, 3 or 5 minutes with light from a NIR 808 nm laser, corresponding to light doses of 82, 247, or 411 J cm⁻² respectively. Samples L-S+ and L-S- were kept in the dark.

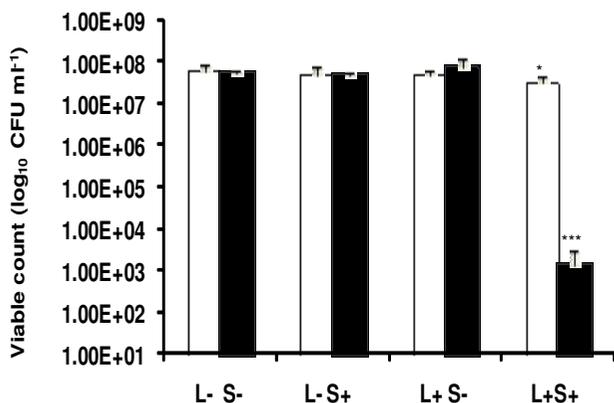


Figure 5
Lethal photosensitization of *Staph. aureus* in 50% horse serum (HS) using 25 □ or 200 ■ μg ml⁻¹ ICG. An equal volume of either 50 or 400 μg ml⁻¹ ICG (L-S+ and L+S+) or PBS (L-S- and L+S-) was added to *Staph. aureus* suspensions in HS and samples were left for 10 minutes in the dark and then irradiated at a fluence rate of 1.37 W cm⁻² and a light dose of 411 J cm⁻² using a NIR 808 nm laser (L+S- and L+S+) or kept in the dark (L-S+ and L-S-).

compared to a 5 log₁₀ reduction in the absence of serum. However, increasing the concentration of ICG to 200 μg ml⁻¹ and exposure to the same light dose achieved a 4.61 log₁₀ reduction in the viable count in the presence of serum ($P < 0.001$).

Lethal photosensitization of *Staph. aureus* in the presence of a singlet oxygen scavenger or enhancer

The singlet oxygen scavenger, L-tryptophan, significantly reduced the lethal photosensitization of *Staph. aureus* ($P < 0.01$). One log₁₀ reduction in the number of *Staph. aureus* killed was found in the presence of L-tryptophan over that in the absence of this scavenger (Figure 6A). Conversely, D₂O considerably enhanced the killing of *Staph. aureus* with a 1.62 log₁₀ greater reduction in viable counts compared to those achieved in the absence of D₂O ($P < 0.01$) (Figure 6B).

Temperature elevation during lethal photosensitization and its effect on the viability of bacteria

The temperature of the bacterial suspensions was elevated during high intensity lethal photosensitization with ICG. The rises in temperature depended on the light dose delivered and the concentration of the photosensitizer. The temperature of the bacterial suspensions increased from 22°C to 37°C during irradiation with the highest light dose and a dye concentration of 25 μg ml⁻¹, whereas using 200 μg ml⁻¹ of ICG in either PBS or 50% HS, the temperature increased to a maximum of 47°C. No change in the viable count was observed after incubation of *Staph.*

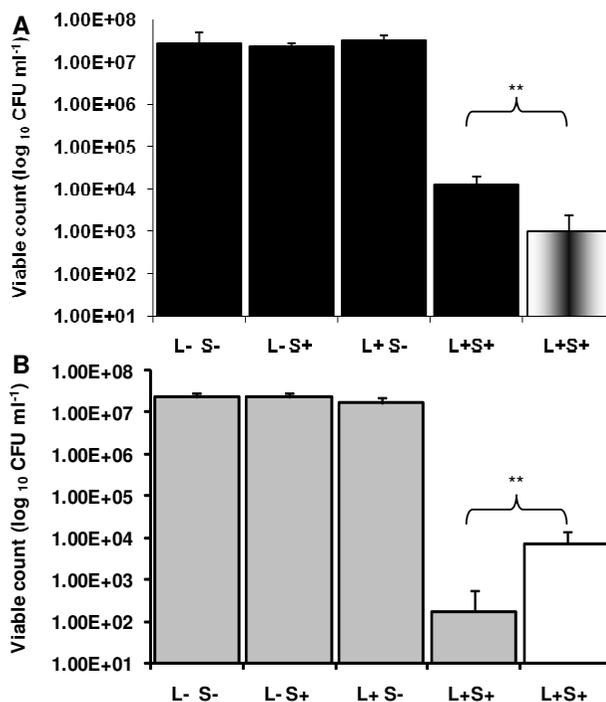


Figure 6
High intensity lethal photosensitization of *Staph. aureus* (A) suspended in 10 mM L-tryptophan ■ or suspended in H₂O ▒ and (B), suspended in D₂O ▒ or suspended in H₂O □. In this experiment an ICG concentration of 25 μg ml⁻¹ was used and samples were pre-incubated in the dark for 10 minutes before being irradiated at a fluence rate of 1.37 W cm⁻² and light dose of 82 J cm⁻².

aureus suspended in PBS, in the absence or presence of 25 μg ml⁻¹ ICG, and incubated for 10 minutes at 40°C in the dark. Horse serum provided a protective effect for *Staph. aureus*, a slight increase or no change in the viable count was noted after *Staph. aureus* was suspended in PBS containing 50% HS in the absence or presence of 200 μg ml⁻¹ ICG and incubation for 10 minutes at 50°C. A statistically non-significant reduction was observed in the viable count of *P. aeruginosa* suspended in PBS, in the absence or presence of 200 μg ml⁻¹ ICG, and incubated for 10 minutes at 50°C. The viable count reduction was always less than 0.4 log₁₀ (data not shown).

Discussion

Indocyanine green is a water-soluble anionic photosensitizer which is widely used in medical diagnosis. It is approved by the US FDA for use in humans, in ophthalmology for retinal angiography as well as being used for diagnostic assessment of liver function and cardiac output and determining burn depth [26,27]. A number of *in vitro*

and *in vivo* studies of the potential use of ICG-mediated PDT have been carried out. *In vitro* researches reported an inhibitory effect of photoactivated ICG on pancreatic cancer cells [28], colonic cancer cells [29], human (SKMEL 188) and mouse (S91) melanoma cells [30]. These studies suggested that ICG was a promising photosensitizer for clinical PDT but that further *in vivo* investigations were needed. Several authors have investigated ICG cyto-toxicity and photo-toxicity using *in vitro*, [31-34], *in vivo*, [35,36] and *ex vivo* models [37]. Some of these have reported phototoxicity in eukaryotic cells which was dependent on pre-irradiation time, ICG concentration, and light dose. Abels et al., 2000 reported that ICG phototoxicity depends on the pre-irradiation time [38]. After 1 h pre-irradiation time, no significant phototoxicity on HaCaT keratinocytes was detected using an ICG concentration as high as 50 μM and light doses of 48 J/cm^2 (80 mW/cm^2). Phototoxicity of ICG at concentrations of 25 μM was detected after 4 h pre-irradiation while a concentration as low as 10 μM was phototoxic after 24 h [38]. However, in the current study the pre-irradiation time was no more than 30 minutes which implies that keratinocyte phototoxicity would not be a problem under these conditions.

In humans, ICG-mediated PDT has been used in the treatment of acne vulgaris [23-25]. A recent pilot study was carried out by Tuchin *et al*, (2003) on the effects of ICG photodynamic and photothermolysis treatment on acne vulgaris. In this study, the authors used a very high concentration of ICG (1.0 mg/ml), a pre-irradiation time of 5 or 15 minutes and very high light dose and fluence rates of NIR laser-diode light (803 or 809 nm). For soft acne treatment, the low intensity (803 nm, 10–50 mW/cm^2 , 5–10 minutes) or the medium-intensity (809 nm, 150–190 mW/cm^2 , 15 minutes) protocols were used. Single and multiple (up to 8–9) treatments were studied. The individual acne lesions were photothermally treated at 18 W/cm^2 (803 nm, 0.5 seconds) without skin surface cooling or at 200 W/cm^2 (809 nm, 0.5 seconds) with cooling. As no adverse effects were reported, the investigators concluded that such high light intensities and ICG concentrations were safe for use in humans [23].

The purpose of this study was to investigate whether ICG in combination with light from a NIR laser is effective at killing common wound-infecting organisms and to determine the fluence rates needed to photosensitize wound-infecting organisms. The results of the study have shown that all three organisms tested are susceptible to lethal photosensitization using ICG as a photosensitizer in combination with 808 nm light using both high and low fluence rates. At high fluence rates the Gram-negative bacterium *P. aeruginosa* showed no resistance to lethal photosensitization with ICG in the presence of high con-

centrations of the photosensitizer. However at the low fluence rate *P. aeruginosa* did show resistance to the lethal photosensitization process while the Gram-positive organisms (*Staph. aureus* and *Strep. pyogenes*) were not resistant. The lethal photosensitization of these wound-infecting organisms with ICG was dependent on the light dose and ICG concentration.

The differing susceptibilities of the Gram-negative and Gram-positive organisms to lethal photosensitization in this study are probably attributable to differences in cell wall structures. Gram-negative bacteria have an outer membrane that may reduce the uptake of reactive oxygen species by the bacterium [9]. In addition the presence of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria acts as a very effective permeability barrier to many of the molecules in the external environment and contributes to the development of drug-resistance in these organisms [39]. In contrast, Gram-positive bacteria have a porous outer layer of peptidoglycan which is a less effective permeability barrier [40]. Gram-negative bacteria are known to be relatively impermeable to neutral or anionic drugs and ICG is an anionic dye [41].

Interestingly, both high and low intensity 808 nm light alone was able to exert a cidal effect against *P. aeruginosa*. This killing effect was not heat mediated as the temperature of the bacterial suspensions did not exceed 33°C during irradiation. This finding is supported by the results of a previous study in which irradiation of the organism with 1–80 J/cm^2 laser light at a wavelength of 810 nm and using an irradiance rate of 0.03 W/cm^2 resulted in a significant inhibition of bacterial growth [42]. A possible explanation for this observation is that *P. aeruginosa* has endogenous pigments (pyoverdinin and pyocyanin) that may absorb the light and result in the production of bactericidal species [16]. The current data suggest that NIR laser light irradiation by itself would also inhibit growth of *P. aeruginosa* in infected wounds.

A prerequisite for successful antimicrobial PDT to treat wound infection is its efficiency in the presence of wound fluid. To more closely mimic the conditions likely to be experienced *in vivo*, we investigated the effect of serum on the lethal photosensitization of *Staph. aureus*. Horse serum reduced the effectiveness of lethal photosensitization with ICG possibly because serum proteins in the environment bind to the ICG thereby preventing its uptake by the organism [43] or else they may act as quenchers of the singlet oxygen produced thereby protecting the bacterial cells from their lethal effects [44]. However, increasing the concentration of ICG overcame the inhibitory effect of serum suggesting that killing of the organism may be achievable *in vivo*. Our findings were confirmed by the results of Lambrechts *et al.* which

showed that albumin inhibited the photodynamic inactivation (PDI) of *Staph. aureus* and exerted a dose dependent protective effect against the PDI of the microorganism which was dependent on the albumin content of the suspending fluid [45].

To reveal something of the underlying mechanisms involved in killing of the wound-infecting organisms by ICG, lethal photosensitization experiments were carried out in the presence of an enhancer of $^1\text{O}_2$ life span (D_2O) or a quencher of $^1\text{O}_2$ (L-tryptophan) [46]. The augmentation of *Staph. aureus* killing in the presence of D_2O demonstrated that enhancement of the life span of $^1\text{O}_2$ increases the bactericidal effect thereby suggesting the involvement of this cytotoxic species in the lethal photosensitization process. Further evidence of the involvement of singlet oxygen is provided by the protective effect of the singlet oxygen scavenger, tryptophan.

However during the lethal photosensitization experiments at high fluence rate and the highest concentration of ICG ($200 \mu\text{g ml}^{-1}$), the temperature of the bacterial suspensions increased to 47°C , whereas at lower concentrations the temperature did not exceed 42°C . According to the literature, temperatures of a few degrees above the physiological norm, i.e., $42\text{--}45^\circ\text{C}$ can induce cell apoptosis. However, as normal tissues approach this temperature, intrinsic heat dissipation occurs (possibly due to augmented blood flow) so that temperatures up to 45°C could be tolerated by the tissues [47]. More prolonged heating can be associated with secondary necrosis of apoptotic cells, where the cells retained some of the features of apoptosis but had superimposed features of necrosis including membrane disintegration and swelling of cytoplasmic organelles. After a few days, apoptotic cells are phagocytosed by tissue mononuclear phagocytes. Temperatures greater than 45°C ($45\text{--}48^\circ\text{C}$) cause prolonged necrosis, uniformly affecting all cells in tissue structure [48]. It also should be noted that heating has some synergic effect when applied together with PDT [49]; therefore more effective bacterial killing can be achieved. In clinical practice, providing an effective cooling system for the skin surface [50] or else the use of a pulsed laser may avoid tissue necrosis.

In order to ascertain whether this temperature rise was responsible for the kills achieved, the test organisms were exposed to a slightly higher temperature than that which occurred during lethal photosensitization and for longer periods of time. As exposure to these temperatures resulted in no, or only slight, reductions in bacterial viability, it can be concluded that the observed kills were attributable to the light-induced generation of singlet oxygen (and, possibly, free radicals) rather than to thermal effects. Even though the temperature of *Staph. aureus* sus-

pensions containing $200 \mu\text{g ml}^{-1}$ ICG increased up to 47°C , there was no significant reduction in the viable count of *Staph. aureus* in 50% HS at a similar temperature (50°C). It may be that protein molecules in the serum provide protection against the heating effect. This observation suggests that killing of *Staph. aureus* was more likely attributable to the products of lethal photosensitization. In addition, irradiating the bacteria with low fluence rates of 0.048 or 0.07 W cm^{-2} resulted in significant reduction in the viability of the three bacteria. These results support the hypothesis that the cytotoxic species produced from the photosensitization process are responsible for bacterial kill.

The notion of disinfecting burns and wounds using a non-invasive and localized strategy such as PDT with limited damage to the host tissue is well documented in the literature [10,51,52]. Lambrechts et al achieved 3.6 or 4.8 \log_{10} units reduction in the viability of *Staph. aureus* using 635 nm light with a light dose of 0.6 or 1.5 J cm^{-2} and $1.56 \mu\text{M}$ 5-phenyl-10,15,20-tris(N-methyl-4-pyridyl) porphyrin chloride (PTMPP) [10]. Taking into account the variation in experimental design, we achieved a 5.5 \log_{10} unit reduction in the viability of *Staph. aureus* using $25 \mu\text{g ml}^{-1}$ ICG ($32 \mu\text{M}$) and a light dose of 411 J cm^{-2} from a coherent light source (808 nm) which allows deeper tissue penetration than the light source ($635 \pm 15 \text{ nm}$) used in the above mentioned study. In another study, Orenstein et al. used a mixture of deuteroporphyrin and hemin which successfully disinfected burns infected with *Staph. aureus* even in the dark without illumination [15]. In contrast, the ICG used in the current study had minimal dark toxicity against the organisms tested. ICG is a comparatively weak photosensitizer because the yields of the triplet state appear low compared with other photosensitizers used in PDT [29]. Nevertheless, the data obtained in this study have shown that significant kills of *Staph. aureus* and *Strep. pyogenes* can be achieved using a low concentration of the photosensitizer of $25 \mu\text{g ml}^{-1}$ and a low light dose of 43 J cm^{-2} . The gram-negative organism, *P. aeruginosa*, appeared to be less susceptible as higher ICG concentrations and higher light doses were needed to achieve substantial kills. Interestingly, however, appreciable kills of the organism were attained by irradiation in the absence of the ICG. It is well established that humans can tolerate systemic ICG concentrations in the mg/ml range i.e. 5-fold greater than those used in this study.

The phototherapeutic window of many PSs effective against bacteria is $600\text{--}780 \text{ nm}$ [53]. The most frequently studied PSs are activated by light wavelengths between $632.8\text{--}665 \text{ nm}$ [54]. However, light of such wavelengths has limited tissue penetration compared to light with a wavelength of 808 nm [55] which we used to activate ICG in the present study. In fact, the depth of penetration dou-

bles from 4 mm at 500–600 nm to 8 mm at 800 nm [56]. This may enable killing of bacteria within deeper wounds and burns. In addition, ICG is of very low toxicity and is rapidly excreted from the body so that any possible damage to host tissue would be limited.

Conclusion

In summary, the results of the present study suggest that indocyanine green in combination with near-infrared light is a promising candidate for the photodynamic therapy of burn and wound infections. Furthermore, the NIR laser light itself may be able to disinfect wounds contaminated with *P. aeruginosa*. Use of this approach would reduce the requirements for systemic antibiotics in the management of skin infections and thereby help to reduce the emergence of antibiotic resistance. Although the results of these *in vitro* studies are promising, *in vivo* studies are needed to ascertain whether appreciable bacterial kills can be obtained in a wound model.

Abbreviations

D2O: Deuterium Oxide; DNA: Deoxyribonucleic acid; HS: Horse serum; ICG: Indocyanine green; LAAA: Light activated antimicrobial agent; NIR: Near Infra-red; PBS: Phosphate buffered saline; PDT: Photodynamic therapy; PS: Photosensitizer; ROS: Reactive oxygen species; US FDA: United State Food and Drug Administration.

Authors' contributions

GSO: conceived of the study, participated in the study design, carried out the microbiological studies, performed the statistical analysis and drafted the manuscript. MW: conceived of the study, participated in its design and coordination and helped to draft the manuscript. SPN: conceived of the study, participated in its design and coordination, provided technical support and helped to draft the manuscript. All authors read and approved the final manuscript.

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