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Light-activated antimicrobial coatings for reducing microbial contamination of surfaces

Submitted by

Valérie Decraene BSc

For the degree of Doctor of Philosophy in the Faculty of Medicine,

University of London

2007

Division of Microbial Diseases

UCL Eastman Dental Institute

University College London

256 Gray's Inn Road

London WC1X 8LD

United Kingdom

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated.

Valérie Decraene

Acknowledgements

First and foremost I would like to thank the Charles Wolfson Charitable Trust for funding this research. I am also grateful to my supervisors Dr. Jon Pratten and Professor Mike Wilson for their continued support and guidance throughout my PhD; Jon for his practical and editorial assistance and Mike for his technical advice and unrelenting enthusiasm and encouragement which were an inspiration during even the most stressful times.

I am also indebted to Dr. Derren Ready for his constant patience and invaluable expertise in microbiology, to Dr. Adam Roberts for his advice regarding molecular techniques and to Miss Aviva Petrie for her help and guidance with statistical analyses.

I would like to offer my sincere thanks to all of my colleagues in the Division of Microbial Diseases who supported me throughout the highs and lows of my PhD and whose friendship and cake therapy were indispensable. In particular, I would like to acknowledge Dr. Stefania Baldursdottir for her encouragement and help in the early days of my research and Dr. Florent Chang-Pi-Hin for his help (and humour) during the final stages of writing-up.

Of course, as with all of the major accomplishments in my life, undertaking and completing this PhD would not have been possible without the unwavering support of my parents and family, who have always believed in me (even when I have not).

Finally I would like to thank my best friend and partner, Robin, who provided a refuge of calm throughout the stress of writing-up. His moral support and continuous encouragement together with his knowledge of computers were invaluable.

Abstract

Environmental surface contamination can contribute to cross-infection within hospitals by acting as a reservoir from which personnel and patients can soil their hands. It is important, therefore, to develop effective means of reducing the microbial load on such surfaces. One possible approach involves the use of photosensitisers, compounds which generate antimicrobial moieties (e.g. singlet oxygen), when irradiated with an appropriate light source. The overall aim of this study was to develop a coating containing photosensitisers that is able to kill microbes when irradiated with light generated by the type of lamp recommended for use in hospitals in the United Kingdom i.e. a 28 W General Electric® fluorescent lamp. After comparing the absorption spectra of candidate compounds with the emission spectrum of the light source, the following photosensitisers were selected for study: Toluidine Blue O (TBO), Methylene Blue (MB), Methylene Violet (MV), Rose Bengal (RB), and Erythrosine B (ErB). The singlet oxygen ($^1O_2$) producing ability of the selected compounds was then tested using the uric acid assay. All photosensitisers had comparable rates of $^1O_2$ production but both ErB and RB became photobleached over time. Based on these results, TBO, MB and MV were selected for further study and lethal photosensitisation experiments were carried out using aqueous suspensions of *Staphylococcus aureus, Escherichia coli, Candida albicans* and Bacteriophage ΦX174. All three photosensitisers produced significant kills against these four organisms but, while 2 hours of irradiation was sufficient for bacteria, longer (6 hour) irradiation was necessary to achieve substantial kills of the bacteriophage and yeast. These photosensitisers were then incorporated into cellulose acetate coatings, and the antimicrobial activity of these coatings was investigated. MV was found to be inactive when immobilized in the polymer and

was therefore replaced by RB. The optimal coating of those tested contained a combination of 25 μM TBO and 25 μM RB. This coating was able to generate appreciable kills of a range of different microbes both when aliquoted on to the coating as a suspension and when sprayed on using a nebuliser. Significant kills were also achieved in the presence of horse serum and saliva and following prolonged irradiation of the coatings.

A study of the environmental contamination present in a dental clinic was carried out using settle plates and revealed that both the number and variety of bacteria were significantly greater on days when clinics were held compared to days when there was no clinical activity. The most frequently encountered species were *P. acnes*, *M. luteus*, and *S. epidermidis* and the mean total microbial load was 21.9 x 10^2 cfu/m^2/h. The final stage of the project involved testing the activity of the TBO/RB coatings in this clinical setting. When compared to control (photosensitiser-free) coatings, the TBO/RB coatings achieved reduced counts on 12 out of the 15 days, with median reductions of 63.8 % and 81.8 % for aerobic and anaerobic bacteria respectively.

Overall, these coatings show potential as a renewable, self-disinfecting surface and may have a range of applications in the hospital environment.

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Abbreviations

ABA	Anaerobe basal agar
AOB	Active oxygen-based
BC	Benzalkonium chloride
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
°C	Degree Celsius
CBA	Columbia base agar
cfu	Colony-forming units
cm	Centimetre
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
E-MRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
Er B	Erythrosine B
h	Hour
HAIs	Hospital-acquired infections
HIV	Human immunodeficiency virus
LPS	Lipopolysaccharide
µg	Microgram
µL	Microlitre
µm	Micrometer
µM	Micro-molar
mg	Milligram
mL	Millilitre
mm	Millimetre
min	Minute
MB	Methylene blue
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MV	Methylene violet
nm	Nanometer
$^1O_2$	Singlet oxygen
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction

PDT	Photodynamic therapy
QACs	Quaternary ammonium compounds
RB	Rose Bengal
REA	Restriction enzyme analysis
RNA	Ribonucleic acid
s	Second
SARS	Severe acute respiratory syndrome
TBO	Toluidine blue O
UA	Uric acid
UV	Ultraviolet
VRE	Vancomycin resistant enterococci
VSE	Vancomycin-susceptible enterococci
VSV	Vesicular stomatitis virus

CHAPTER 1

Introduction

1.1 Hospital-acquired infections

Hospital-acquired infections (HAIs) afflict healthcare systems worldwide, and dealing with the control, prevention, and successful treatment of these infections is an ongoing struggle. In the UK, approximately one in 11 inpatients at any one time has an infection contracted in hospital (The National Audit Office, 2000). Internationally, figures suggest a similar trend, with prevalence rates ranging from 4 to 10% (The National Audit Office, 2004). HAIs can often lead to complications with existing illnesses, may cause anxiety and discomfort, and can even lead to death of the patient (Nosocomial Infection National Surveillance Scheme, 2000). Furthermore, infected patients remain in hospital on average 2.5 times longer than uninfected patients (Plowman, 2000), resulting in increased costs for the health service and/or the patient. One study by Plowman *et al.* (2001) found that infected patients incurred hospital costs on average 2.9 times higher than uninfected patients, equivalent to an additional £3,154 per patient. The same study also estimated that 320,994 patients per year acquire one or more infections in hospital in the UK, thereby incurring a total cost to the health sector of around £920 million pounds per annum. Likewise, approximately 2 million patients acquire HAIs each year in the United States, resulting in 90,000 deaths and adding an estimated $5 billion per year to the cost of patient care (Burke, 2003). In a census carried out in the UK by the National Audit Office in 2000, it was calculated that an average of 15 % of HAIs are potentially avoidable, and that if such a reduction was achieved it could save the health service as much as £150 million a year.

Current guidelines to reduce the incidence of HAIs focus on four different areas: (i) hospital environmental hygiene, (ii) hand hygiene, (iii) the use of personal protective equipment and (iv) the use and disposal of sharps (Department of Health, 2001). While each of these areas is important and requires individual consideration, only the first is relevant to the research described here and, therefore, only this will be discussed in detail.

1.1.1 Role of the inanimate environment in the transmission of HAIs

Although it was previously believed that the inanimate environment played little or no role in the transmission of infectious disease, this notion is now being reconsidered (Cozad & Jones, 2003). The Centres for Disease Control and Prevention (CDC) have listed contact transmission, direct from body to surface or indirectly via contaminated inanimate objects, as one of the main routes of microorganism transmission (CDC, 1996). Likewise, the Department of Health (2001) has stated that "good hospital hygiene is an integral and important component of a strategy for preventing hospital-acquired infections". In fact, there is mounting evidence that healthcare workers are nearly as likely to contaminate their gloves or hands after touching environmental surfaces as when they touch patients directly (Hayden *et al.*, 2006). These findings arose from a study by Hayden *et al.* (2006), who investigated how improved environmental cleaning might affect the spread of Vancomycin-resistant enterococci (VRE) in a medical intensive care unit both with and without the promotion of hand hygiene adherence. Interestingly, they discovered that despite only modest rates of hand hygiene compliance during the study, healthcare worker hand contamination decreased significantly during the environmental cleaning intervention and remained low throughout the rest of the

study. In addition, they documented a significant reduction in VRE cross-transmission which coincided with the implementation of the improved cleaning regime.

A separate study by Hardy *et al.* (2007) also illustrates transmission of pathogens from the environment. By using pulsed-field gel electrophoresis, these authors determined that strains isolated from patients were indistinguishable from strains isolated from the patients' immediate environment on 35.7 % occasions.

1.1.2 Environmental surfaces as microbial reservoirs

Environmental surface contamination can contribute to both endemic and epidemic spread of microbes (Figure 1.1) by acting as a reservoir from which personnel and patients can soil their hands (Rutala & Weber, 2004a). Many studies involving environmental surface sampling within hospitals have identified reservoirs of various nosocomial pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) (Rutala *et al.*, 1983; Oie *et al.*, 2002; 2005; Sexton *et al.*, 2006; Wilson *et al.*, 2007), VRE (Noskin *et al.*, 2000), *Acinetobacter* spp. (Getchell-White *et al.*, 1989) and *Pseudomonas aeruginosa* (Panagea *et al.*, 2005). The problem is that not only do these reservoirs exist but they can also persist for long periods of time due to the remarkable ability of some microorganisms to survive on surfaces for days, weeks and even months. Recently, a comprehensive literature review summarising all of the research available on the persistence of different nosocomial pathogens was published (Kramer *et al.*, 2006).

While hand hygiene is considered to be one of the most effective infection control strategies for interrupting transmission of organisms around the hospital, compliance levels are generally accepted to be poor among healthcare workers. In fact, 34 separate studies have reported that healthcare worker compliance with the Centres for Disease Control and Prevention guidelines for hand hygiene is on average only 40 % (range: 5 – 81 %; Boyce & Pittet, 2002). Such low compliance levels undermine the reliability and success of hand hygiene as a strategy for interrupting the spread of microorganisms and reinforce the importance of keeping environmental surface contamination to a minimum. In addition, low hand hygiene compliance will also increase the likelihood of environmental contamination by healthcare workers.

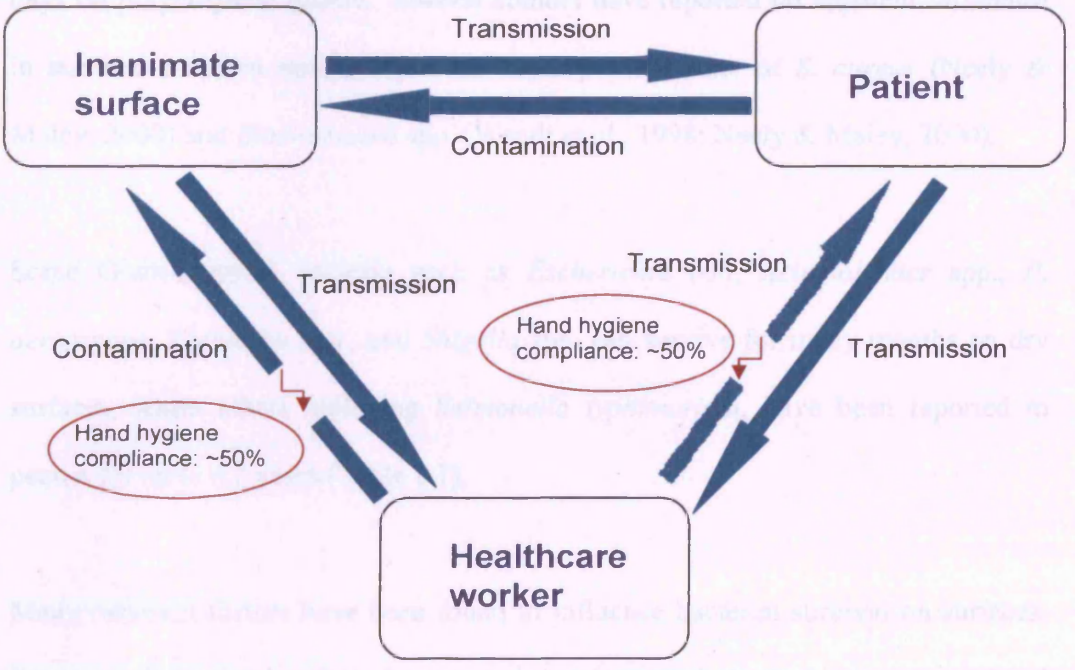


Figure 1.1 Transmission pathways between healthcare workers, patients and inanimate surfaces in the hospital environment.

1.1.2.1 Survival of bacteria on inanimate surfaces

Of the microbes commonly found in hospitals, bacteria are perhaps the most troublesome and this is due, in part, to their remarkable ability to persist for months or even years on dry surfaces (Table 1.1). Overall, Gram-negative bacteria have been reported to persist longer than Gram-positive bacteria (Hirai, 1991). With respect to Gram-positive bacteria, enterococci are among the most successful and can survive for prolonged periods under dry-conditions (Wendt *et al.*, 1998). Many investigators have studied the survival of enterococci (including VRE) and found that they can persist from 5 days to 4 months on a variety of different dry surfaces (Table 1.1). One of these studies, carried out by Neely and Maley (2000), also monitored the survival of staphylococci and found that their viability nearly equalled that of the enterococci with a range between 1 day (on all fabrics and plastics) and more than 90 days on polyethylene plastic. Several authors have reported no apparent difference in survival between multiresistant and susceptible strains of *S. aureus* (Neely & Maley, 2000) and *Enterococcus* spp. (Wendt *et al.*, 1998; Neely & Maley, 2000).

Some Gram-negative bacteria such as *Escherichia coli*, *Acinetobacter* spp., *P. aeruginosa, Klebsiella* spp., and *Shigella* spp. can survive for many months on dry surfaces, while others including *Salmonella typhimurium*, have been reported to persist for up to 4.2 years (Table 1.1).

Many different factors have been found to influence bacterial survival on surfaces. For example, several authors have noted that increased survival time is correlated with increased inoculum size (Neely & Maley 2000; Neely, 2000). Despite this general trend, however, even just a few hundred bacteria could survive for days on

most fabrics (Neely & Maley, 2000). Low temperatures of between 4 and 6 °C have also been shown to increase persistence of many bacteria including *E. coli* (Williams *et al.*, 2005), *Salmonella enterica* subtype *Typhimurium* (Helke & Wong, 1994) and MRSA (Noyce *et al.*, 2006). Other factors which appear to enhance survival include increased humidity (Helke & Wong, 1994; Jawad *et al.*, 1996; Williams *et al.*, 2005) and the presence of organic material such as serum (Hirai, 1991). Finally, there is some debate as to whether the type of test material influences bacterial persistence in the environment. While some authors have found no correlation between survival times and particular surfaces (Bale *et al.*, 1993; Wendt *et al.*, 1997), others have reported increased survival on plastic versus fabrics, on polyester when compared with cotton (Neely, 2000; Neely & Maley, 2000), and on wood relative to steel (Williams *et al.*, 2005).

Table 1.1. Persistence of a selection of clinically important bacteria on dry inanimate surfaces (adapted from Kramer *et al.*, 2006).

Bacterium	Duration of persistence (range)	Reference(s)
Acinetobacter spp.	3 days to > 4 months	Getchell-White *et al.*, 1989; Jawad *et al.*, 1996; Wendt *et al.*, 1997; Jawad *et al.*, 1998; Neely, 2000; Webster *et al.*, 2000
Clostridium difficile (spores)	5 months	Mulligan *et al.*, 1980; Kim *et al.*, 1981; McFarland & Stamm, 1986
Escherichia coli	1.5 hours – 16 months	Mitscherlich & Marth, 1984; Scott & Bloomfield, 1990; Kampf *et al.*, 1998; Neely, 2000; Wilks *et al.*, 2005; Williams *et al.*, 2005
Enterococcus spp. including VRE and VSE	5 days – 4 months	Bale *et al.*, 1993; Noskin *et al.*, 1995; Bonilla *et al.*, 1996; Wendt *et al.*, 1998; Neely & Maley, 2000, Lemmen *et al.*, 2004
Klebsiella spp.	2 hours to > 30 months	Mitscherlich & Marth, 1984; Scott & Bloomfield, 1990; Neely, 2000
Mycobacterium tuberculosis	1 day – 4 months	Smith, 1942; Mitscherlich and Marth, 1984
Pseudomonas aeruginosa	6 hours – 16 months	Scott & Bloomfield, 1990; Kampf *et al.*, 1998; Neely, 2000; Panagea *et al.*, 2005
Salmonella typhimurium	10 days – 4.2 years	Helke & Wong, 1994
Shigella spp.	2 days – 5 months	Mitscherlich & Marth, 1984; Islam *et al.*, 2001
Staphylococcus aureus including MRSA	7 days – 7 months	Scott & Bloomfield, 1990; Jawad *et al.*, 1996; Wagenvoort & Penders, 1997; Kampf *et al.*, 1998; Neely & Maley, 2000; Wagenvoort *et al.*, 2000; Kusumaningrum *et al.*, 2003
Streptococcus pyogenes	3 days – 6.5 months	Mitscherlich & Marth, 1984
Vibrio cholerae	1 – 7 days	Mitscherlich & Marth, 1984; Barua, 1970

1.1.2.2 Survival of viruses on inanimate surfaces

While bacteria such as MRSA and *C. difficile* receive a great deal of attention in the field of infection control, dated estimates have indicated that viruses may cause

between 5 and 32 % of all nosocomial infections in the United States (Valenti *et al.*, 1980). Accordingly, the issues surrounding their effective eradication from the hospital environment also need to be addressed. Like bacteria, some viruses can contaminate the environment, persist after drying (Table 1.2), and become re-aerosolised after sweeping (Hota, 2004). In a study by Bean *et al.* (1982), both influenza A and B viruses were cultured from experimentally-contaminated non-porous surfaces up to 48 hours after inoculation and from cloth, paper and tissues for up to 8-12 hours after inoculation. Unfortunately, such robustness is not unique to influenza viruses. Other investigators have studied the parainfluenza virus and documented survival times of up to 10 hours on nonporous surfaces and 4 hours on clothing (Brady *et al.*, 1990). Similarly the SARS-associated coronavirus was recovered from fomites 24 - 72 hours after contamination (Duan *et al.*, 2003; WHO, 2003).

As seen with the viability studies on staphylococci, Brady *et al.* (1990) found that survival increased as the concentration of virus in the inoculum increased. Likewise, low temperature is again associated with longer persistence for most viruses (Mahl *et al.*, 1975; Mbithi *et al.*, 1991; Abad *et al.*, 2001).

Table 1.2 Persistence of a selection of clinically relevant viruses and fungi on dry inanimate surfaces (adapted from Kramer *et al.*, 2006).

Organism	Duration of persistence (range)	References
Viruses		
SARS associated virus	72 – 96 hours	Duan *et al.*, 2003
Hepatitis A virus	2 hours – 60 days	Mbithi *et al.*, 1991; Abad *et al.*, 1994; Abad *et al.*, 2001
Hepatitis B virus	> 1 week	Bond *et al.*, 1981
Human Immunodeficiency virus	> 7 days	Barré-Sinoussi *et al.*, 1985; Tjotta *et al.*, 1991; van Bueren *et al.*, 1994
Influenza virus	1 – 2 days	Bean *et al.*, 1982; Brady *et al.*, 1990; Pirtle & Beran, 1991
Norovirus and feline calicivirus	8 hours – 28 days	Doultree *et al.*, 1999; Clay *et al.*, 2006
Rhinovirus	2 hours – 7 days	Reed, 1975; Sattar *et al.*, 1987
Rotavirus	6 – 60 days	Sattar *et al.*, 1986; Ansari *et al.*, 1991; Abad *et al.*, 1994; Abad *et al.*, 2001
Fungi		
Candida albicans	1 – 14 days	Rangel-Frausto *et al.*, 1994; Neely & Orloff, 2001; Traoré *et al.*, 2002
Candida parapsilosis	2 to > 30 days	Neely & Orloff, 2001; Traoré *et al.*, 2002
Aspergillus spp.	1 to > 30 days	Neely & Orloff, 2001

1.1.2.3 Survival of fungi on inanimate surfaces

The environment is also implicated as a reservoir for fungi such as *Candida* spp. (Table 1.2), which constitute the fourth most common nosocomial pathogen in intensive care units (Pfaller, 1995) accounting for 8-15 % and 9.3 % of nosocomial septicaemias in the US and Europe respectively (Flanagan & Barnes, 1998). One

study found that when experimentally dried onto glass and stainless steel surfaces, *C. albicans* and *C. parapsilosis* could survive for 3 and 14 days respectively (Traoré *et al.*, 2002). In an epidemiologic study investigating nosocomial *C. parapsilosis* infections, Sanchez *et al.* (1993) used restriction enzyme analysis (REA) to show that a culture taken from a new intensive care unit prior to occupation grew a strain type that was identical to cultures subsequently taken from the wound of a patient hospitalised in that room. Likewise, Romano *et al.* (1994) discovered that the REA profiles of *C. albicans* isolates taken from 5 patients were identical to those isolated from a nurse's hands and from the emergency kit desk. More recently, Vazquez *et al.* (1998) used molecular typing to show that environmental isolates of *Candida glabrata* were identical to those carried by bone marrow transplant patients.

In contrast to the large number of studies carried out on characterising the persistence of bacteria and viruses, very little work has been carried out with respect to the environmental factors that influence the survival of yeasts on surfaces. One study by Blaschke-Hellmessen *et al.* (1985) reported that the presence of serum, a low temperature and high humidity increased survival times for fungi. These researchers also reported that yeast species which are commonly found in the environment, such as *Rhodotorula* spp., were more successful at resisting desiccation than those more often found on mucous membranes, such as *C. albicans*. In contrast, when comparing the survival of different strains of *Aspergillus* spp., Neely and Orloff (2001) found that the longest-lived strain for each of the four species tested was a patient isolate and not the environmental isolate. The study conducted by Neely and Orloff also compared the length of fungal survival on various different types of surfaces, including 5 fabrics and 2 types of plastic and discovered that the fungi

survived longer on the synthetic fabrics (polyester and spandex) and plastics than on the fabrics with some natural fibre content (cotton, terry, and blends).

1.1.2.4 Contact transmission studies

The long term survival of microbes on surfaces increases the chance of contact transmission between surfaces and staff who can then spread these pathogens throughout the hospital. However, the presence and survival of microbes on surfaces does not mean that these microbes can or will be readily transmitted to patients and staff. Good evidence that contact transmission does occur comes from a study by Boyce *et al.* (1997) who found that 42 % of personnel with no direct patient contact contaminated their gloves with MRSA by touching contaminated surfaces. Similarly, Oie *et al.* (2002) found 7 % of door handles of non-MRSA patient rooms to be contaminated with MRSA, and French *et al.* (2004) detected 4 MRSA types on 43 % of beds in an open ward not being used to nurse MRSA patients.

Other researchers have shown the feasibility of horizontal spread of bacteria by performing contamination experiments. For example, Scott and Bloomfield (1990) demonstrated a 40 % and 39 % transfer efficiency of *E. coli* and *S. aureus* respectively from a laminate surface to fingers up to 2 hours after experimental contamination. Likewise, Rusin *et al.* (2002) reported efficient transfer of *Micrococcus luteus* and *Serratia rubidea* from various different fomites to hands. Interestingly, while Scott and Bloomfield found no difference between the transmission rates of Gram-positive and Gram-negative bacteria, Rusin and co-workers showed that the Gram-positive bacterium *Micrococcus luteus* was more readily transferred than *Serratia rubidea*. Clearly, more research needs to be carried

out in order to establish whether it is organism specificity or Gram-classification that plays a role in transmission efficiency. There is also considerable debate regarding the effect that the type of surface might have on the transfer of bacteria. Rusin *et al.* (2002) reported more efficient transfer rates from non-porous surfaces such as telephone receivers and faucets than from porous ones, hypothesising that the deep recesses present in porous fomites such as sponges and dishcloths allow bacteria to evade contact and therefore transmission. Montville and Schaffner (2003) meanwhile investigated the relationship between inoculum size and transmission and discovered that higher microbial loads led to lower rates of transfer, possibly due to improved attachment to the donor surface. These authors believe that varying inoculum sizes may have been a reason for the difference in transfer rates seen in the study by Rusin *et al.* (2002).

With respect to viruses, efficient transfer between surfaces and hands has also been shown. Rheinbaben *et al.* (2000) experimentally contaminated a door handle with bacteriophage φX174 and found that 14 persons could subsequently become contaminated after touching the door handle. Barker *et al.* (2004) produced similar findings when using Norovirus as the test organism. Following experimental contamination of melamine surfaces with Norovirus, clean fingers were used to demonstrate secondary contamination from this initial site to 3 other sites. Results showed that Norovirus was transferred to 4 out of 10 door handles, 5 out of 10 telephone receivers and 3 out of 10 taps. In a parallel experiment, Barker *et al.* (2004) also showed that contaminated fingers could transfer the virus to 7 clean melamine surfaces when touched sequentially. Other investigators studied the potential for dissemination of influenza viruses via environmental surfaces and reported that they

could be transferred from nonporous surfaces to hands for 24 hours after inoculation and from tissues to hands for 15 minutes after inoculation (Bean *et al.*, 1982).

Rangel-Frausto *et al.* (1994) performed a similar study using *Candida* spp. By experimentally inoculating the hands of volunteers and plastic surfaces, they demonstrated that transmission to and from inanimate surfaces occurred 90 % of the time. Furthermore, they found that transmission from one hand to a second hand occurred in 69 % of experiments and from a first to a third hand, 38 % of the time. Clearly the hospital environment provides a reservoir from which both direct and secondary transmission can occur, thus allowing the gradual dissemination of microbes throughout the hospital.

In an intervention study carried out by Rampling and associates (2001), it was demonstrated that a prolonged outbreak of epidemic methicillin-resistant *S. aureus* (E-MRSA-16) could not be controlled until the microbe was eliminated from the environment. Their longitudinal study spanned 27 months (from January 1998 to March 2000), during which time they continuously sampled patients and the environment and used molecular typing to confirm that these isolates were indistinguishable. During the last 6 months of the study an intervention was put in place that increased cleaning time by 57 hours per week, with emphasis being placed on removal of dust by vacuum cleaning. Following the intervention, there was a steep drop in the acquisition rate of E-MRSA-16 (from 30 patients to 3 patients) thus implicating the dusty ward as a reservoir of MRSA and supporting the cause for a clean hospital environment.

Another problem is that certain environmental reservoirs of nosocomial pathogens may go unnoticed. Keyboards and keyboard covers, for example, have been found to harbour reservoirs of *Acinetobacter baumannii* (Neely *et al.*, 1999), MRSA (Bures *et al.*, 2000; Devine *et al.*, 2001) as well as many other potential pathogens (Rutala *et al.*, 2006). While transmission of pathogens from keyboards (and other surfaces) could easily be prevented by good hand hygiene, as previously discussed, the reality is that compliance rates are universally poor. One study investigating the role of computer keyboards in the spread of MRSA reported that keyboards were touched 34.5 times/hour after patient or environmental contact but that hand hygiene was only performed 3.2 times/hour, a compliance rate of just 9.3 % (Wilson *et al.*, 2006). Moreover, since keyboards are frequently excluded from routine cleaning protocols, they can increase the risk of nosocomial cross-transmission.

All of these data help support the idea that microbes can be acquired from the hospital environment via direct and indirect contact and emphasises the need for good disinfection practices to interrupt such transmission.

1.2 Hospital Disinfection

1.2.1 Current surface decontamination processes

Fundamental to the discussion of current decontamination strategies is the classification scheme set forth by Spaulding (1968) nearly 40 years ago. Spaulding assessed the degree of risk of infection posed by instruments and items used for patient care and accordingly separated these items into 3 categories: critical, semicritical, and noncritical. Critical items are those associated with a high risk of

infection when contamination with any microorganism has occurred (Rutala & Weber, 2004a). Examples of such items include surgical instruments, urinary catheters, and implants. Semicritical items are those which come into contact with mucous membranes or non-intact skin and include instruments such as anaesthesia equipment and some endoscopes. Finally, noncritical items are those that come into contact with intact skin but not mucous membranes (Rutala & Weber, 2004a). This category encompasses the wide range of inanimate environmental surfaces found within a patient's immediate surroundings, including everything from blood-pressure cuffs and crutches to bedrails, patient furniture, and floors. For the purposes of this investigation, only the cleaning strategies relating to this last group will be discussed in detail.

Three levels of intensity exist when referring to the cleaning of hospital equipment and surfaces: cleaning by detergent, disinfection by disinfectants, and sterilization. Cleaning by detergents involves using mechanical methods in combination with detergent solutions to remove organic materials such as visible soil, blood, or protein substances (Exner *et al.*, 2004). Disinfection by disinfectants kills the majority of microbes, with the exception of spores, using chemical methods. In general, a disinfectant is expected to achieve at least a 5-$\log_{10}$ reduction of pathogenic bacteria during a time frame of between 5 and 10 minutes (Mazzola *et al.*, 2003). Low level disinfectants include chlorine-based products, phenolics, quaternary ammonium compounds (QACs), and alcoholic solutions. These compounds are generally safe, easy to use, and fast-acting but do not exhibit broad spectrum activity. While all of these chemicals can destroy vegetative bacteria, they vary in their virucidal, fungicidal, and mycobactericidal activities and are largely ineffective against spores,

although some sporostatic and sporicidal activity has been documented for QACs and chlorine-based products respectively (McDonnell & Russell, 1999). In contrast, high-level chemical disinfectants such as hydrogen peroxide, glutaraldehyde, ortho-phthalaldehyde, and peracetic acid destroy all microorganisms including bacterial spores. However, despite this broad spectrum activity, these compounds are more difficult to use and when deciding which to use, the advantages and disadvantages of each chemical must be considered (Table 1.3). In addition, there are solutions known as detergent-disinfectants which can remove dirt as well as kill infectious particles. Sterilization eliminates all microbial life on the object or surface using heat, pressure or chemical agents. Although it is universally accepted that sterilization is required for critical items and that disinfection is needed for semicritical items, the debate over the level of decontamination necessary for noncritical items continues. While a solution containing a combination of detergent and disinfectant is generally used for surface disinfection in the USA and some European countries, in the UK it is common for a detergent alone to be used (Rutala & Weber, 2001).

Table 1.3. Summary of the activities, advantages, and disadvantages of some of the most commonly used high-level disinfectants (adapted from Rutala & Weber, 2004b).

Disinfectant	Advantages	Disadvantages
Glutaraldehyde	Numerous studies published of use Relatively inexpensive Excellent compatibility with materials	Respiratory irritation from vapour Pungent and irritating odour Relatively slow mycobactericidal activity Coagulates blood and fixes tissue to surfaces Allergic contact dermatitis
Hydrogen peroxide	No activation required May enhance removal of organic material and organisms No disposal issues No odour or irritation issues Does not coagulate blood or fix tissues to surfaces Inactivates *Cryptosporidium* Published studies of use	Concerns regarding compatibility with materials and both cosmetic and functional damage Serious eye damage with contact
Peracetic acid	Rapid sterilisation cycle Environmentally friendly by-products May enhance removal of organic material and endotoxin No adverse health effect to operators Compatible with many materials and instruments Does not coagulate blood or fix tissues to surfaces Rapidly sporicidal	Used for immersible instruments only Only a small number of instruments can be treated in one cycle Expensive Serious eye and skin damage with contact Point of use system; no sterile storage
Ortho-phthalaldehyde	Fast-acting No activation required Odour not significant Claim of excellent compatibility with materials Claim of not coagulating blood or fixing tissues	Limited clinical use More expensive than glutaraldehyde Eye irritation with contact Slow sporicidal activity Repeated exposure may result in hypersensitivity in some patients with bladder cancer

In addition to these more traditional methods of chemical disinfection, vapour-phase sterilization systems using ethylene oxide and formaldehyde also exist but are rarely used for surface disinfection due to safety concerns, complexity of equipment, and long processing times (Cheney & Collins, 1995; McDonnell & Russell, 1999). More recently, a novel gaseous decontamination strategy involving vapourised hydrogen peroxide has been tested and found to be effective against MRSA (French *et al.*, 2004; Jeanes *et al.*, 2005), *Clostridium botulinum* spores (Johnston *et al.*, 2005) and *Mycobacterium tuberculosis* (Kahnert *et al.*, 2005). Once again, however, there are practical problems associated with this method; the entire decontamination process takes an average of 5 hours, several pieces of equipment are required, and monitoring of hydrogen peroxide vapour concentrations is necessary (French *et al.*, 2004). Furthermore, with some of these systems, silver and phosphoric ions can remain on surfaces following fumigation and consequently routine cleaning is still necessary to remove these ions (Gerald McDonnell, personal communication, 2006). Perhaps the biggest disadvantage with this method (and other forms of chemical disinfection) is that, following the decontamination process, patient rooms can become quickly re-contaminated. A study by Hardy *et al.* (2007) found that admission of MRSA-colonised patients to rooms previously decontaminated with hydrogen peroxide vapour resulted in recontamination of the room within 24 hours.

Another potential disinfection strategy involves the use of germicidal ultraviolet (UV) lamps. Prolonged exposure to UV light inactivates microbes by inducing cross-links and mutations in their DNA and has been found to be lethal to a wide range of microbes

including bacteria, bacterial spores, moulds, mould spores, yeast, viruses, and algae. In a recent study, Andersen *et al.* (2006) evaluated the efficacy of UV C light (wavelength 200 – 280 nm) as a means of surface disinfection. UV disinfection has several advantages over other methods: no manual labour is required, only a short exposure time is needed, and it leaves no residue (Andersen *et al.*, 2006). Despite this, however, UV light disinfection is not widely utilised for surface cleaning. This is because there are occupational health risks associated with UV light exposure and, in addition, the depth of penetration of the light is often inadequate for efficient disinfection (Kühn *et al.*, 2003).

One of the more novel approaches being developed for surface disinfection is coating materials such as glass and tiles with titanium dioxide which can then be activated by UV A (320 – 400 nm) light in the presence of water and oxygen to produce highly reactive OH-radicals capable of killing microorganisms. Several authors have demonstrated the successful inactivation of both bacteria and fungi using these films (Sunada *et al.*, 1998; Ohko *et al.*, 2000; Kühn *et al.*, 2003; Page *et al.*, 2007).

1.2.2 Problems with current disinfection practices

Although current disinfection practices have been shown in many studies (Sattar *et al.*, 1993; 1994; Denton *et al.*, 2005; Hayden *et al.*, 2006) to be successful at reducing microbial load and interrupting transmission, they are also plagued with problems. Detergent solutions, for example, often become contaminated during use, thus allowing the spread of microbes throughout the hospital via mop heads and cleaning cloths

(Rutala & Weber, 2004a). This principle was illustrated by Dharan *et al.* (1999) who analysed the efficacy of QACs, a detergent solution, and an active oxygen-based (AOB) compound for cleaning and disinfection of hospital ward floors. Their results showed that while AOB was successful in eliminating the majority of organisms (with the exception of fungi and *Bacillus* spp.), QACs failed to reduce the total bacterial count and at times the counts were higher than before disinfection. Likewise, the detergent solution was introducing bacteria into the patients' environment. For both QACs and the detergent solution, the mops being used as well as the solutions were becoming seeded with Gram-negative non-fermentative bacilli such as *P. aeruginosa*, thus aiding the mechanical spread of these organisms. More recently, Exner *et al.* (2004) developed an experimental model to characterise the cleaning and disinfection properties of detergents and disinfectants and used this model to independently confirm the dissemination of bacteria when using surfactants, glycol derivatives, QACs, and alkylamines. The same process is known to cause the spread of viruses around the hospital environment because when an ineffective cleaning product is used with a good surfactant, virus particles can be dislodged from surfaces without being killed (Sattar, 2004).

Another issue concerning current hospital cleaning protocols is that the widespread use of disinfectants in low concentrations is imposing a selective pressure which subsequently leads to the emergence and dissemination of disinfectant-resistant organisms. Related to this is the more recently recognised problem that such organisms can foster cross-resistance to antibiotics (Aiello & Larson, 2004; Russell, 2000). For

example, Chaunchuen *et al.* (2001) found that exposure of *P. aeruginosa* to triclosan led to the selection of multi-drug resistant strains at high frequencies. In particular, they found that the minimum inhibitory concentration (MIC) of ciprofloxacin increased 94-fold after exposure to triclosan. In another study, Sidhu *et al.* (2002) documented a genetic linkage between resistance to the QAC benzalkonium chloride (BC) and penicillin resistance in staphylococci. In fact, they observed that BC-resistant isolates were more frequently resistant to a wide range of antibiotics than the BC-sensitive organisms. Nomura *et al.* (2004) discovered a similar link between glutaraldehyde and antibiotic resistance in *Mycobacterium chelonae*. Of the 18 isolates examined, 9 were glutaraldehyde-tolerant and all of these isolates were resistant to 2 or 3 classes of antibiotics.

In addition to being found within hospitals, isolates with increased resistance to disinfectants and antibiotics have also been detected in the environment (Kummerer, 2004). This may be yet another consequence of disinfectant-based cleaning methods since products used for surface cleaning inevitably reach wastewaters (Kummerer, 2001).

Since conventional cleaning protocols rely on regular cleaning, any lapse in staff compliance will inevitably cause problems for infection control. Consequently, even if strict protocols that are proven to be effective at eliminating microbes are drawn up, there is no guarantee that these procedures will be followed. Furthermore, there is the issue of staff education. Many cleaning personnel are unaware of the reasoning behind certain procedures, thus potentially reducing the likelihood that they will adhere to

guidelines. For example, housekeeping staff often uphold the belief that disinfectants kill all bacteria (including spores) and therefore find it difficult to understand the need to keep mops clean and dry when not in use (Dharan *et al.*, 1999). It is therefore imperative that there is good communication with cleaning staff as well as effective management and education. In fact, in a study of cleaning behaviours, it was shown that when housekeeping staff were given constructive feedback, it led to improved environmental cleaning and a 3-fold reduction in environmental VRE contamination (Hota *et al.*, 2003).

Finally, as previously mentioned for hydrogen peroxide vapour disinfection, there are health concerns related to the residues left behind on surfaces after cleaning. Both patients and staff are at risk from coming into contact with these residues and allergic reactions such as contact dermatitis and asthma have been reported for a variety of different disinfectants (Bernstein *et al.*, 1994; Burge & Richardson, 1994; Di Stefano *et al.*, 1999; Medina-Ramon *et al.*, 2006).

1.3 Light as an antimicrobial agent

When reviewing current disinfection strategies, it becomes evident that the most commonly used disinfection and sterilization practices utilise heat or chemicals. However, as mentioned previously, UV C light disinfection (wavelength 200 – 280 nm) has also proved to be successful in eliminating microbial contamination from surfaces. The application of light as a means of surface disinfection has evolved from the field of phototherapy which uses light alone in the treatment of disease.

Phototherapy can be traced back thousands of years to the ancient civilisations of Egypt, India, and China where it was used to treat skin diseases such as psoriasis and vitiligo (Epstein, 1990). Likewise, in ancient Greece whole-body sun exposure, known as heliotherapy, was hailed for its therapeutic benefits in curing various diseases and soon developed into a fashionable pastime (Ackroyd *et al.*, 2001). At the turn of the last century, the field of phototherapy was further advanced by Danish physician, Niels Finsen, who discovered that the application of red light in the treatment of smallpox prevented suppuration of the pustules (Finsen, 1901). Finsen later went on to develop a carbon arc lamp which he used to successfully treat cutaneous tuberculosis, an achievement that earned him a Nobel Prize in 1903.

1.3.1 UV light

The observed antimicrobial effect exerted by UV C light occurs because light with a short wavelength (less than 300 nm) is readily absorbed by microbial proteins and nucleic acids and once absorbed, it can induce cell killing by mutagenic effects such as DNA dimerization (Wilson, 1993). While the mutagenic potential of UV light renders it inappropriate for the treatment of infectious diseases in humans, it is being widely investigated for disinfection purposes. In addition to environmental surface disinfection, UV irradiation has also been applied in the water industry (Abbaszadegan *et al.*, 1997; Oppenheimer *et al.*, 1997) and to decontaminate the surfaces of food (Wallner-Pendleton *et al.*, 1994; Kuo *et al.*, 1997). More recently, Devine *et al.* (2001) have shown that

special UV-generating beakers can efficiently kill both planktonic and surface-associated bacteria, yeasts, and viruses.

1.3.2 Visible light

While longer wavelength visible light (400 – 700 nm) is not inherently antimicrobial, some bacteria contain compounds which absorb light within the visible spectrum. As will be explained in detail in section 1.4.1, the absorption of light by these compounds (known as endogenous photosensitisers) in the presence of oxygen generates cytotoxic species (e.g. singlet oxygen and free radicals) which mediate cell death. This process is known as endogenous lethal photosensitisation.

1.3.2.1 Incoherent Light

Most natural and artificial light sources produce light beams comprising waves that are not in phase which is known as incoherent light. One of the earliest reports of incoherent light being used for endogenous lethal photosensitisation of microorganisms was the killing of *Propionibacterium acnes* using blue light (Kjeldstad & Johnsson, 1986). Since then, the successful killing of *P. acnes*, which is implicated in the pathophysiology of acne vulgaris, has been repeatedly reported and is now known to be caused by the production of porphyrins by this bacterium (Papageorgiou *et al.*, 2000; Elman *et al.*, 2003; Goldberg & Russell, 2006). Similarly, Hamblin *et al.* (2005) have demonstrated that due to the build-up of endogenous porphyrins, *Helicobacter pylori* can be killed when exposed to an endoscopically delivered blue light. Other researchers have used various incoherent light sources, some of which are commonly used in

restorative dentistry, to kill oral black-pigmented bacteria such as *Porphyromonas gingivalis* which are often associated with periodontal disease (Feuerstein *et al.*, 2004; Feuerstein *et al.*, 2005; Soukos *et al.*, 2005).

1.3.2.2 Laser Light

In contrast to incoherent light sources such as incandescent, fluorescent and halogen light bulbs, lasers generate a light beam that consists of parallel waves which all have the same frequency. One advantage of laser light sources is that the light beam they produce has a greater energy output due to the amplification caused by its stimulated emission (Wilson, 1993) and consequently shorter exposure times can be employed to kill microbes. This characteristic makes them desirable candidates for clinical applications. As with the incoherent light sources, the majority of work investigating endogenous lethal photosensitisation using lasers has focussed on the inactivation of oral black pigmented bacteria (Dederich *et al.*, 1990; Cobb *et al.*, 1992; Henry *et al.*, 1995; Henry *et al.*, 1996; Ando *et al.*, 1996; König *et al.*, 2000). Interestingly, a recent randomized controlled study by Ambrosini *et al.* (2005) found that the use of a yttrium aluminium laser during the initial treatment of adult periodontitis conferred no additional advantage.

1.4 Exogenous lethal photosensitisation of microbes

Photosensitising compounds can, of course, also be added to bacterial suspensions. Such exogenous lethal photosensitisation not only improves the killing of pigmented

bacteria, but can also render those microorganisms that are devoid of photosensitisers susceptible to light-induced killing.

The concept of cell death resulting from the interaction of light and chemicals was first described approximately 100 years ago by Oscar Raab, a medical student in Munich whose research into the effects of acridine on malaria-causing protozoa led him to discover that the combination of acridine red and light had a lethal effect on paramecia (Raab, 1900). As is often the case with scientific breakthroughs, this was a chance finding that occurred because the experiment was conducted during a thunderstorm when lighting conditions were abnormal. In subsequent experiments, Raab went on to demonstrate that the lethal effect exerted by the combination of acridine and light was far greater than that produced by either light or acridine alone. This led him to the conclusion that it was not the light itself but rather some product of fluorescence that caused the *in vitro* toxicity. Furthermore, Raab hypothesised that this lethal activity was induced by the transfer of energy from the light to the chemical, as occurs in plants following the absorption of light by chlorophyll (Ackroyd *et al.*, 2001). Soon afterwards, fellow researchers von Tappeiner and Jodblauer solved another piece of the puzzle by demonstrating that oxygen was an essential component in photosensitisation reactions (von Tappeiner & Jodblauer, 1904). In 1907, their work eventually led them to coin the term 'photodynamic action', and thus the field of Photodynamic Therapy, or PDT, was born (von Tappeiner & Jodblauer, 1907).

1.4.1 Mechanism of action: Photophysics and Photochemistry

In addition to the wealth of medical research regarding the various applications of PDT for the treatment of diseases, a large body of scientific literature describing the mechanism of action behind the process of photosensitisation has also accumulated. Indeed, the photophysics and photochemistry behind the photosensitised production of singlet oxygen ($^1O_2$) and other cytotoxic species are now well understood.

The production of cytotoxic species is the ultimate outcome of photodynamic action but several important steps involving light absorption and subsequent energy transfer must first take place. In the ground state, photosensitiser molecules have two electrons with opposite spins in the low energy molecular orbital (Castano *et al.*, 2004). Upon irradiation, this initially stable ground state photosensitiser molecule absorbs a photon of ultraviolet or visible radiation causing one of these electrons to be transferred to a high-energy orbital while maintaining its spin, thus resulting in an excited singlet state (Castano *et al.*, 2004). This unstable molecule is known as the excited singlet state and is very short-lived, possessing a lifetime of just 4 μs in aqueous solution (Kohen *et al.*, 1995), an estimate which is thought to be a magnitude shorter in the cellular environment where reactive substrates are plentiful (Ouédraogo & Redmond, 2003). The excited singlet state can be further converted to the longer-lived triplet state (Kochevar & Redmond, 2000) which may in turn react with neighbouring molecules via one of two mechanisms (Figure 1.2).

The type I reaction involves electron transfer from the triplet state with the participation of a substrate to produce radical ions which can then react with oxygen to produce superoxide (Hamblin & Hasan, 2004). Although superoxide itself is relatively inert in biological systems and is unable to cause much in the way of oxidative damage, it takes part in two further reactions. Firstly, the superoxide molecule undergoes dismutation, whereby it reacts with itself to produce hydrogen peroxide and oxygen (Castano *et al.*, 2004). In the second reaction, known as the Fenton reaction, the superoxide molecule acts as a reducing agent by donating an electron to metal ions, which in turn catalyse the conversion of hydrogen peroxide into the cytotoxic hydroxyl radical (Castano *et al.*, 2004).

In contrast, the type II mechanism involves direct energy transfer (via an electron spin exchange) from the triplet state to ground state molecular oxygen to form excited-state $^1O_2$ while the photosensitiser returns to the singlet state (Nyman & Hynninen, 2004). Collectively, hydroxyl radicals, superoxide, and $^1O_2$ are known as reactive oxygen species.

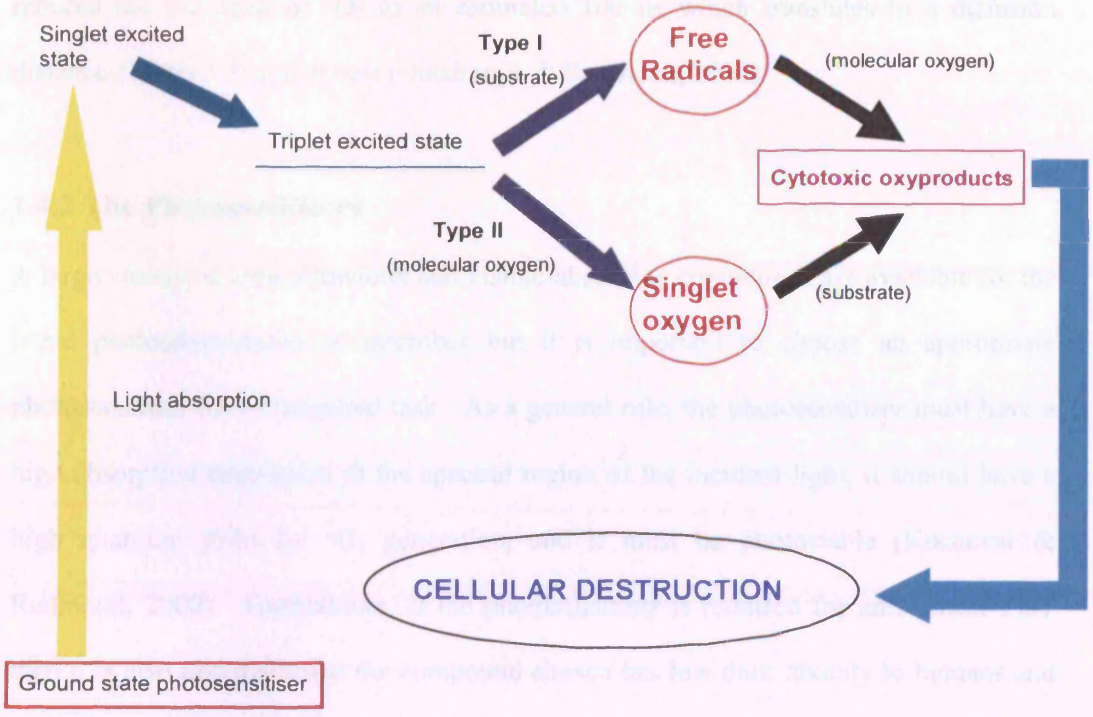


Figure 1.2 Schematic diagram of the photosensitisation pathways.

Importantly, the $^1O_2$ species are generated without transformation of the photosensitiser, thus allowing each single photosensitiser molecule to produce many times its own concentration of $^1O_2$ (MacRobert *et al.*, 1989). Over time, however, the photosensitiser will be degraded by light through a process known as photobleaching which can result from both type I and type II reactions. Crucially, photosensitiser molecules do not have to be taken up by the bacterium or even adsorbed to the bacterial surface in order to exert an effect. This is due to the ability of $^1O_2$ to diffuse from its site of generation. Based on its short life time, (~ 4 μs in solution) it has been estimated that a $^1O_2$ molecule can travel a distance of between 100 nm (Kochevar & Redmond, 2000; O'Riordan *et al.*, 2005) and 300 nm (Stenstrøm *et al.*, 1980) in solution. However, as previously mentioned, the abundance of reactive substrates within the cellular environment further

reduces the life span of $^1O_2$ to an estimated 100 ns which translates to a diffusion distance of only ~75 nm at best (Ouédraogo & Redmond, 2003).

1.4.2 The Photosensitisers

A large variety of both ultraviolet and visible-absorbing compounds are available for the lethal photosensitisation of microbes but it is important to choose an appropriate photosensitiser for the required task. As a general rule, the photosensitiser must have a high absorption coefficient in the spectral region of the incident light, it should have a high quantum yield for $^1O_2$ generation, and it must be photostable (Kochevar & Redmond, 2000). Furthermore, if the photosensitiser is required for anti-cancer PDT then it is also important that the compound chosen has low dark toxicity to humans and that it absorbs light in the red or far-red wavelengths in order to penetrate tissues (Castano *et al.*, 2004). With respect to the use of photosensitisers for antimicrobial applications, the following features are also desirable (Jori *et al.*, 2006):

- Broad spectrum of action against bacteria, fungi, yeasts, viruses, and parasitic protozoa.
- Efficacy independent of the antibiotic resistance patterns of the target organisms.
- Ability to cause extensive reduction in pathogen populations without causing damage to the host tissues.
- Lack of selection of photoresistant strains after multiple treatments.
- Small probability to promote the onset of mutagenicity.
- Availability of formulations allowing specific delivery of the photosensitiser to the infected area.

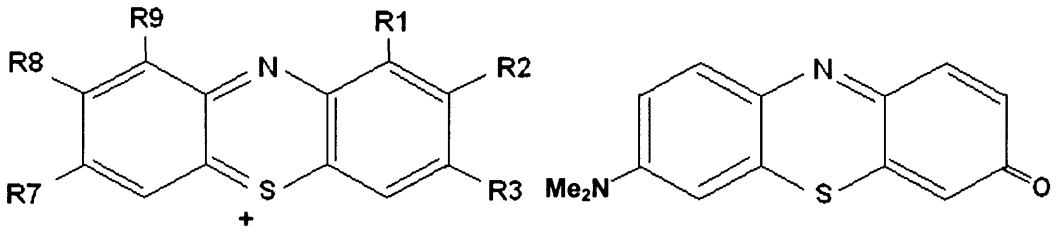
- Low cost of both the photosensitiser and the activating light source.

While currently available photosensitisers may not meet all of these criteria, many certainly come close, and any shortcomings can often be overcome by chemical manipulation. For example, regarding the third point listed above, photosensitisers can be conjugated to monoclonal antibodies or bacteriophages specifically targeted against certain bacteria, thereby minimising the destruction of human tissue surrounding the infection. Several different groups have successfully demonstrated this technique both *in vitro* (Bhatti *et al.*, 2000; Embleton *et al.*, 2002; 2004; 2005) and *in vivo* (Berthiaume *et al.*, 1994). Similar to this, cationic side chains can be conjugated to photosensitisers. The resulting polycationic entity is a macromolecule and will therefore be taken up by mammalian cells by the time-dependent process of endocytosis, thus incurring temporal selectivity for bacteria (Hamblin & Hasan, 2004). Soukos *et al.* (1997; 1998) have demonstrated that these polycations successfully kill bacteria while epithelial cells remain unharmed. Broad spectrum activity of anionic photosensitisers can also be achieved by this form of chemical modification; this will be discussed in a subsequent section.

To date several groups of compounds have been studied in detail for the lethal photosensitisation of microbes, these include: phenothiaziniums, acridines, cyanines, xanthenes, porphyrins, phthalocyanines, chlorins, psoralens and fullerenes.

1.4.2.1 Phenothiaziniums

Phenothiaziniums are blue dyes (λ_{max} = 600-660 nm) with a planar tricyclic heterochromatic ring structure (Wainwright & Giddens, 2003) (Figure 1.3).



Methylene violet

	R1	R2	R3	R7	R8	R9
TBO	H	Me	NH_2	NMe_2	H	H
MB	H	H	NMe_2	NMe_2	H	H
DMMB	Me	H	NMe_2	NMe_2	H	Me
NMB	H	Me	NHEt	NHEt	Me	H
MVOMe	H	H	OMe	NMe_2	H	H
AA	H	H	NH_2	NMe_2	H	H
AB	H	H	NHMe	NMe_2	H	H
AC	H	H	NH_2	NHMe	H	H
Th	H	H	NH_2	NH_2	H	H

Figure 1.3 Structures of the most commonly used phenothiazine-derived photosensitisers. TBO = toluidine blue O, MB = methylene blue, DMMB = dimethyl-methylene blue, NMB = new methylene blue, MVOMe = O-methylated derivative of methylene violet, AA = Azure A, AB = Azure B, AC = Azure C, Th = thionin. Me = methyl group ($-CH_3$), Et = ethyl group ($-CH_2-CH_3$).

Toluidine Blue O (TBO) and Methylene blue (MB) are among the most well studied of these dyes. MB in particular has been widely used in histology for over 100 years and both dyes have been used in surgical identification at reasonably high concentrations (% w/v) without causing human toxicity (Wainwright, 1998). This lack of human toxicity, in combination with the high singlet oxygen yields produced by the phenothiaziniums, has fostered a keen interest in the development of these dyes as photo-activated antimicrobial agents. A vast body of scientific literature now exists describing the antibacterial, antiviral, and antifungal activity of the phenothiaziniums with TBO and MB again being the most thoroughly studied. In terms of practical applications, MB has already been widely utilised by several European transfusion services in the photo-decontamination of blood plasma since 1992 (Wainwright, 2002) while TBO and MB are being developed for use in dentistry, since they have been shown to successfully eliminate cariogenic and periodontopathogenic bacteria from disease lesions (Williams *et al.*, 2004; 2006; Wilson, 2004;).

1.4.2.2 Acridines

Like the phenothiaziniums, the acridines are also cationic dyes with a simple tricyclic skeleton (Figure 1.4) but they differ in that they are red/orange dyes, absorbing towards the blue end of the spectrum (400 – 500 nm). Shortly after Raab's initial reports on the photo-inactivation of paramecia using acridine, Browning demonstrated the bactericidal activity of proflavine and acriflavine which later led to the application of these dyes as wound antiseptics during World War I (Wainwright, 2002). Since then, the aminoacridines have taken on new roles as biological stains and more recently, as

molecular probes (Wainwright, 1998) but there has been little research into their potential as antimicrobial agents. One study by Wainwright *et al.* (1997) has reported phototoxicity of proflavine and acridine orange against a range of Gram-negative and Gram-positive bacteria while others have demonstrated the inactivation of yeasts using acridine orange (Ito & Kobayashi, 1977; Ito, 1980).

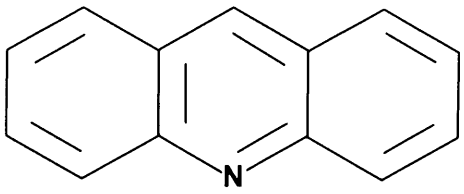


Figure 1.4 Basic structure of an acridine compound.

1.4.2.3 Cyanines

The use of acridines as wound antibacterials in the First World War led directly to the development of another class of photosensitisers for the same purpose: the cyanines (Browning *et al.*, 1924)(Figure 1.5). Despite the proven antimicrobial activity of the cyanines, however, very few studies have been carried out in this field. One group of researchers performed a detailed investigation which reported cyanine-mediated phototoxicity against a wide range of bacteria and fungi (bd El-Aal & Younis, 2004) while others have described the photoinactivation of blood-borne enveloped viruses by merocyanine 540 (Günther *et al.*, 1992). One possible reason for this paucity of research is the discovery that plasma and serum components may inactivate cyanines (Anderson *et al.*, 1996) thus limiting the clinical applications of these dyes.

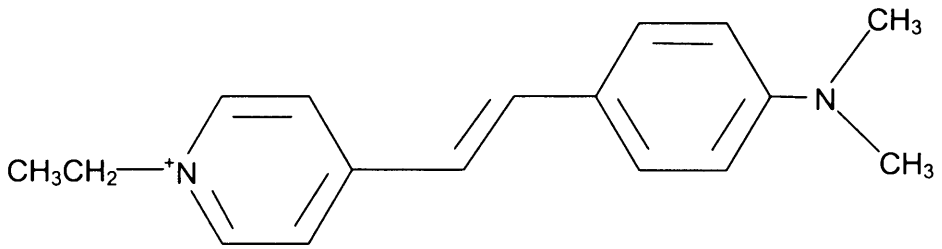
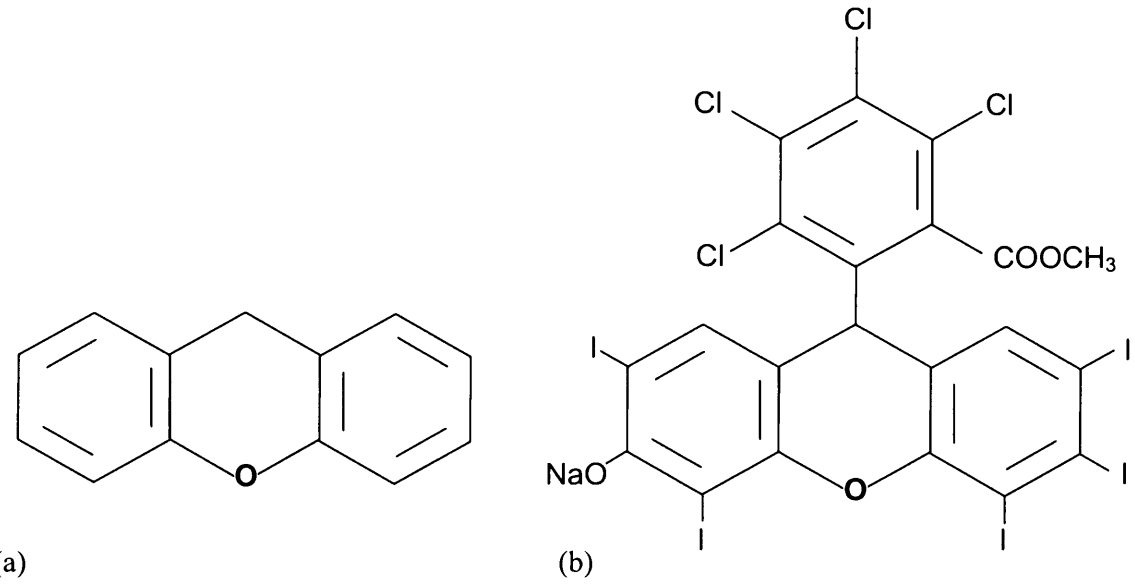


Figure 1.5 Basic structure of a cyanine dye.

1.4.2.4 Xanthenes

The xanthenes (Figure 1.6a), in particular eosin and erythrosine, were among the first photosensitisers investigated for antimicrobial PDT (Huber, 1905). They are positively charged cyclic compounds which absorb light in the visible region (500 – 550 nm). The best studied xanthene, Rose Bengal (RB, figure 1.6b), is one of the most widely utilised of all the photosensitisers (Paczkowski *et al.*, 1985), with applications as a biological dye, food colouring agent, and in cosmetics (O'Neil *et al.*, Eds., Merck Index, 2001). Likewise, erythrosine has been adopted in dentistry as a plaque-disclosing agent (Metcalf *et al.*, 2006). Xanthenes have been reported to have activity against both Gram-positive and Gram-negative bacteria (Bezman *et al.*, 1978; Demidova and Hamblin, 2005; Wang *et al.*, 2006), fungi (Ito and Kobayashi, 1979; Demidova and Hamblin, 2005; Wang *et al.*, 2006) and enveloped viruses (Lenard *et al.*, 1993). Importantly, erythrosine has also been shown to be effective at killing *Streptococcus mutans* in biofilms, thus illustrating its clinical potential (Wood *et al.*, 2006).



(a) (b)

Figure 1.6 (a) Basic structure of a xanthene dye and (b) the structure of rose bengal.

1.4.2.5 Porphyrins/chlorins

From a medical stand point, the porphyrins (Figure 1.7) are by far the most successful class of photosensitisers, with applications in cancer photochemotherapy and ophthalmology. In fact, two of the porphyrins, a benzoporphyrin derivative known as Vertoporfin (Visudyne®) and a Haematoporphyrin derivative (Photofrin®) have already been licensed for use in the USA. The use of porphyrins for antimicrobial chemotherapy has been extensively investigated by Nitzan and colleagues in Israel and Bertoloni *et al.* in Italy. Both research groups have reported porphyrin-mediated inactivation of the Gram-positive bacteria *S. aureus* (Nitzan *et al.*, 1983; 1989; Malik *et al.*, 1990a, b; Nir *et al.*, 1990; Bertoloni *et al.*, 2000) and *Streptococcus faecalis* (Nitzan *et al.*, 1987; Bertoloni *et al.*, 1984; Malik *et al.*, 1990b). Additional studies revealed the susceptibility of the yeast *Candida albicans* to haematoporphyrin-induced photosensitisation (Bertoloni *et al.*, 1984; 1989). Interestingly, both groups repeatedly

reported that Gram-negative bacteria were resistant to treatment with porphyrins (Malik *et al.*, 1982; Nitzan *et al.*, 1983; 1987; Bertoloni *et al.*, 1984). This has been attributed to the inability of the dye to penetrate both the inner and outer membranes of these bacteria and can be overcome by the addition of a membrane disorganising peptide (Nitzan *et al.*, 1992).

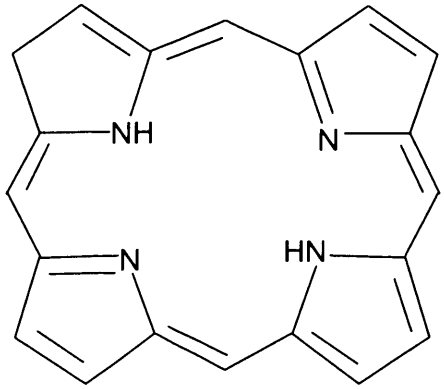


Figure 1.7 Basic structure of a porphyrin.

Chlorins are closely related to porphyrins and have been similarly studied as virucidal agents *in vitro* (Grandadam *et al.*, 1995). Hamblin *et al.* have also investigated the activity of various chlorin (e6) conjugates against a range of bacteria as well as against a yeast (Hamblin *et al.*, 2002a; Demidova and Hamblin, 2005; Tegos *et al.*, 2006).

1.4.2.6 Phthalocyanines

The phthalocyanines (Figure 1.8) are synthetic porphyrins which absorb at around 675 nm and are often chelated with a variety of metal ions including aluminium and zinc to

increase their phototoxicity (Hsi *et al.*, 1999). They are very efficient producers of singlet oxygen, giving higher yields than those of photosensitisers such as MB (Griffiths *et al.*, 1997). As with the porphyrins, initial interest in the phthalocyanines surrounded the development of these dyes as an anti-cancer treatment (Spikes, 1986; Brasseur *et al.*, 1999; Barge *et al.*, 2004) but their potential in the field of antimicrobial PDT was soon realised. During the 1990s they were extensively studied for the purposes of blood product disinfection (Horowitz *et al.*, 1992; Rywkin *et al.*, 1994; Ben-Hur *et al.*, 1995; Moor *et al.*, 1999) and for the inactivation of oral bacteria in the treatment of dental caries (Burns *et al.*, 1994; Wilson *et al.*, 1995; 1996). Other studies have examined the bactericidal activity of cationic water-soluble zinc phthalocyanine (Bertoloni *et al.*, 1992; Minnock *et al.*, 1996; Griffiths *et al.*, 1997; Soncin *et al.*, 2002; Scalise & Durantini, 2005).

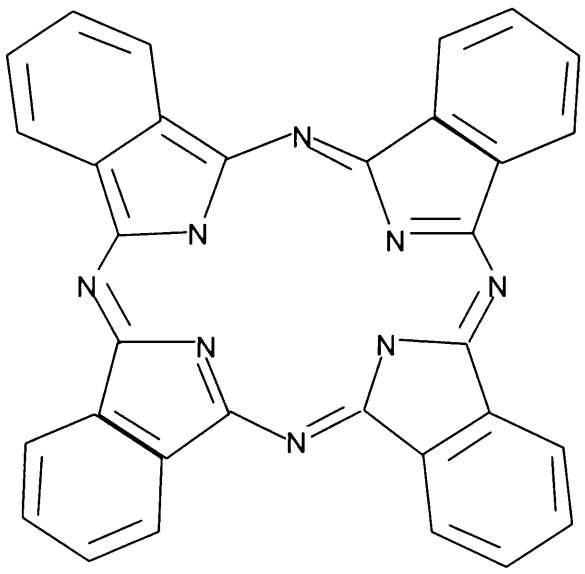


Figure 1.8 Basic structure of a phthalocyanine.

1.4.2.7 Psoralens

Psoralens (Figure 1.9) are naturally-occurring photosensitisers which absorb UV light (normally, UVA, 320-400 nm). In Asia, they have been used for thousands of years in the treatment of various skin disorders (Wainwright, 1998). As with the majority of other classes of photosensitisers, the psoralens are now being investigated for the disinfection of blood products. In particular, the psoralen amotosalen has been shown to successfully inactivate many different pathogens in plasma (Jauvin *et al.*, 2005; Singh *et al.*, 2006) and platelet concentrates (Lin *et al.*, 2004; Jauvin *et al.*, 2005; Pinna *et al.*, 2005). Amotosalen has even undergone phase III clinical trials and is now being developed and marketed as 3 different pathogen-inactivation systems: the INTERCEPT™ Platelet System, the INTERCEPT™ Plasma System, and the Allogeneic Cellular immunotherapies (ACIT) system (No authors listed, *BioDrugs*, 2003).

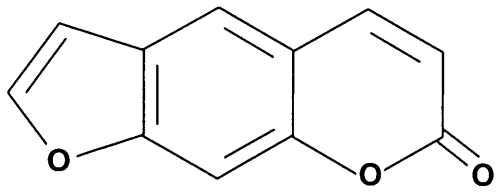


Figure 1.9 Basic structure of a psoralen compound.

1.4.2.8 Fullerenes

Discovered in 1985 by Kroto *et al.*, the fullerenes (originally called buckminsterfullerenes) are a new addition to the vast arsenal of photosensitisers. They

have a condensed aromatic ring organisation, with 60 carbon atoms arranged in a football-shaped structure. This tight configuration of the carbon atoms ultimately leads to significant absorption of visible light (Tegos *et al.*, 2005). One of the main problems with fullerenes, and perhaps one of the reasons they have not been studied more, is their insolubility in biologically-compatible solvents. This can, however, be overcome by chemically modifying the molecules (Da Ros *et al.*, 1996; Foley *et al.*, 2002). During the past decade, the fullerenes have been shown to inactivate viruses (Kasermann & Kempf, 1997) as well as bacteria and fungi (Tegos *et al.*, 2005), thus encouraging further testing of these compounds for antimicrobial applications.

1.4.3 Sites of Action

A number of different studies have focussed on elucidating which specific sites within microbes act as targets for lethal photosensitisation. To date, three main targets have been identified: the cell membrane (or viral envelope), essential enzymes, and nucleic acids. These targets vary based on both the photosensitiser used and the microorganism being studied. Each of these sites of action will now be discussed within the context of three major groups of hospital-associated pathogens: bacteria, viruses, and fungi.

1.4.3.1 Bacteria

Since Mathews and Sistrom (1959) first showed that *Sarcina lutea* could be killed in the presence of TBO upon exposure to visible light, a tremendous amount of research has been carried out describing both the successful inactivation of a variety of bacteria using

many classes of photosensitiser and the different factors that can influence the level of bactericidal activity.

1.4.3.1.1 Membrane Damage

Given the short diffusion distance of singlet oxygen, it is perhaps not surprising that the cell membrane has been repeatedly investigated as a target for lethal photosensitisation of bacteria. In fact, loss of membrane integrity mediated by lipid peroxidation and/or protein-protein crosslinking has been reported as the primary target of lethal photosensitisation in many different species of bacteria (Bhatti *et al.*, 1998; Bertoloni *et al.*, 2000; Kömerik *et al.*, 2000; Nitzan & Ashkenazi, 2001; Bhatti *et al.*, 2002; Romanova *et al.*, 2003). As seen in figure 1.5, the lipid bilayer plasma membrane is a fundamental structural component and consequently, any decrease in membrane integrity or fluidity will have detrimental effects on the microbial cell. Integral membrane proteins are embedded in the plasma membrane of bacteria and these proteins are stabilised by the electrostatic interactions between the lipid head groups and charged amino acids and between the fatty acyl chains and the membrane-spanning fragments (Bhatti *et al.*, 2002). Lipid peroxidation disrupts these interactions, thus causing loss of membrane activity. However, while lipid peroxidation is generally accepted to be the cause of photosensitiser-mediated membrane damage, especially with respect to mammalian cell lines, Bhatti *et al.* (2002) have suggested that the decrease in membrane fluidity in bacteria may also be a consequence of protein-protein crosslinking. This hypothesis is supported by work carried out by Bertoloni and co-workers (1987; 2000), which described modifications in the electrophoretic patterns of cytoplasmic membrane

proteins of MRSA strains following exposure to light and haematoporphyrin when compared with untreated cells. Likewise, polarization experiments conducted by Ehrenberg *et al.* (1993) also suggest that damage to protein sites, probably in the membrane, were responsible for leakage of potassium ions and subsequent cell death. Dahl *et al.* (1989) suggest that total singlet oxygen toxicity is actually the sum of the $^1O_2$ reaching the inner membrane and the lethal effects mediated by reaction of $^1O_2$ with the outer membrane which generates reactive secondary products. Whatever the exact mechanism, it is clear that cytotoxic species mediate alterations in the cell membrane, thus allowing leakage of intracellular material. Furthermore, since the cytoplasmic membrane is the site of energy generation, anabolic, and catabolic metabolism and active transport of solutes, any loss of function of the membrane inevitably leads to cell death.

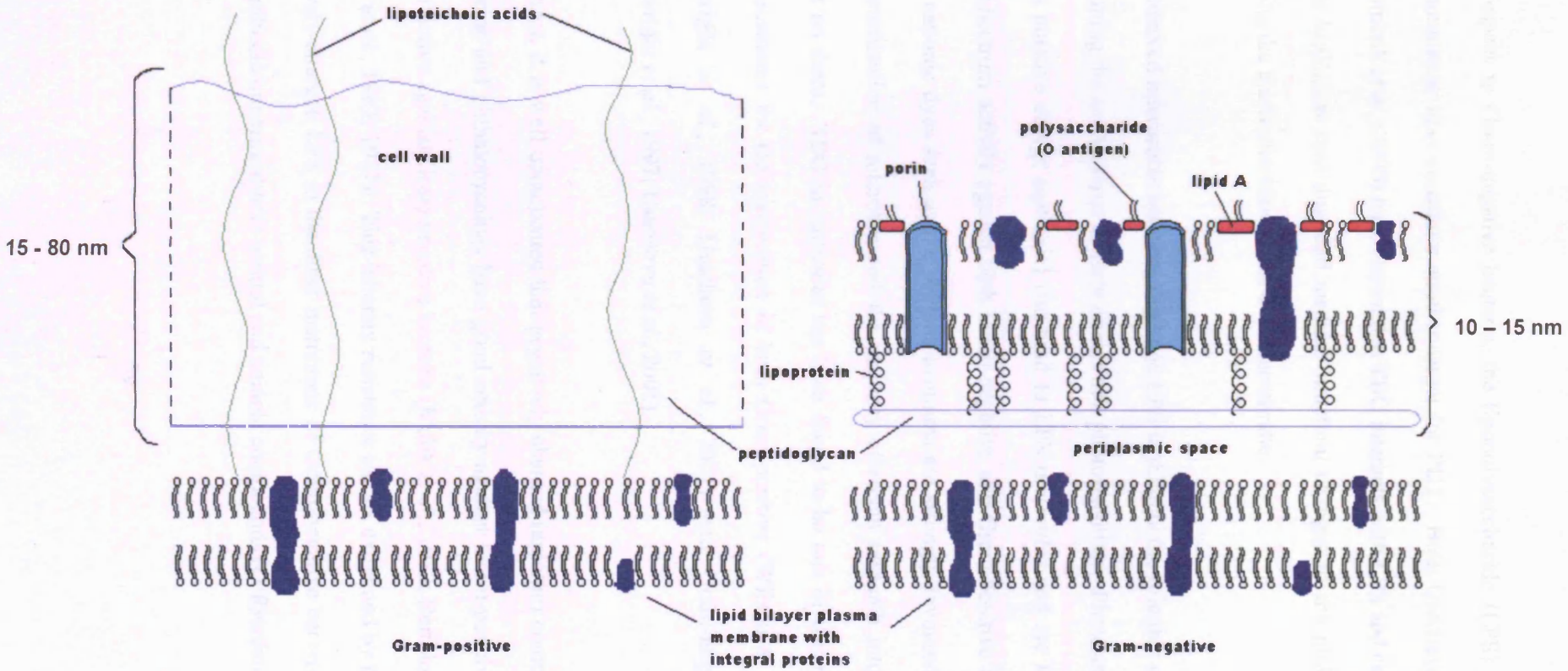


Figure 1.10 The cell-wall structures of Gram-positive and Gram-negative bacteria (adapted from Mims, 1998).

With respect to Gram-negative bacteria, the lipopolysaccharide (LPS) present in the outer membrane may constitute another target for PDT. Both Usacheva *et al.* (2003) and Kömerik *et al.* (2000) have shown that TBO interacts with LPS and can dramatically alter its biological functions and mediate structural changes to the molecule, thereby hindering the barrier function of the outer membrane.

This observed interaction between TBO and LPS highlights the role that charge plays in determining the antibacterial activity relates of a photosensitiser. Photosensitisers which carry a positive charge (cationic) can bind to LPS molecules and are known to have broad spectrum activity against both Gram-negative and Gram-positive bacteria. As a result, cationic dyes such as the phenothiaziniums are strongly favoured for the lethal photosensitisation of microbes and the majority of recent research into the field has centred on them. TBO in particular has been found to be one of the most successful photosensitisers for the inactivation of both Gram-positive (Wilson & Yianni, 1995; Wainwright *et al.*, 1998; Usacheva *et al.*, 2001) and Gram-negative bacteria (Wainwright *et al.*, 1997; Usacheva *et al.*, 2001).

In contrast, it is well documented that negatively charged (anionic) compounds such as porphyrins and phthalocyanines have good activity against Gram-positive bacteria but are ineffective against Gram-negative bacteria (Malik *et al.*, 1982; Bertoloni *et al.*, 1984; Nitzan *et al.*, 1983; 1987). This inherent resistance can be explained by the presence of negatively-charged LPS in the outer membrane of Gram-negative bacteria which make the membrane impermeable to neutral and anionic compounds (O'Riordan *et al.*, 2005).

There are, however, solutions to overcome the problem and achieve broad-spectrum effective killing with anionic compounds. One approach is to use membrane permeabilising agents such as polymyxin nonapeptide (Malik *et al.*, 1992; Nitzan *et al.*, 1992; 1995) or ethylenediaminetetraacetic acid (EDTA) (Bertoloni *et al.*, 1990) which disorganise the structure of the outer membrane and allow the anionic photosensitiser molecules that are normally excluded from the cell to penetrate and cause fatal damage. Another technique that has proven successful is to attach a cationic polypeptide to the anionic photosensitiser molecule, thus facilitating binding to the LPS (Soukos *et al.*, 1997; 1998; Rovaldi *et al.*, 2000; Hamblin *et al.*, 2002a). Likewise, as previously mentioned, anionic photosensitisers can also be attached to antibodies that target specific structures of Gram-negative bacteria.

1.4.3.1.2 Protein/enzyme inactivation

While membrane damage appears to be the main target of lethal photosensitisation in bacteria, photosensitisation can also induce the inactivation of essential enzymes via protein cross-linking and oxidation. The phenothiazinium dyes have long been known to photo-oxidise various amino acids including tryptophan, tyrosine, histidine, methionine, and cysteine via both type I and type II mechanisms (Tuite & Kelly, 1993). Gantchev and van Lier (1995) studied the effect that phthalocyanine-mediated photoinactivation had on the activity of catalase. They discovered that both forms of the enzyme (in solution and cell-bound) were susceptible to photodynamic inactivation mediated by $^1O_2$ and free radicals. Oxidation of amino acids, destruction of haeme,

formation of intersubunit crosslinks, and protein aggregation were responsible for loss of catalase activity. Since catalase protects cells against oxidative stress caused by the accumulation of H_2O_2, loss of this protection has lethal consequences. Packer *et al.* (2000) demonstrated the inactivation of extracellular proteolytic enzymes in *Porphyromonas gingivalis* upon irradiation with red light in the presence of TBO. They postulated that the documented loss of activity might result from the oxidation of active-site thiol groups. The inactivation of extracellular proteases has also been reported in the Gram-negative bacterium *P. aeruginosa* (Kömerik *et al.*, 2000).

1.4.3.1.3 DNA damage

Hussein and coworkers (2006) recently conducted a study evaluating the phototoxicity that phenothiazinium-based photosensitisers have on *E. coli* membranes. In contrast to the work carried out on *E. coli* by Romanova *et al.* (2003), they discovered that *E. coli* membranes are either not targeted or are not the key site of photodynamic action for these dyes. Instead, they hypothesised that the dyes probably act on intracellular targets, most likely DNA. In fact, several other investigations have also provided evidence that DNA is the major site of photodynamic inactivation in bacteria (Phoenix & Harris, 2003; Sayed *et al.*, 2005).

A study exploring the effects of porphyrin-induced phototoxicity on *S. aureus* and *E. coli* documented damage to both chromosomal and plasmid DNA and showed that this damage was directly correlated with the antibacterial effect exerted by the porphyrins (Nir *et al.*, 1991).

Photosensitiser-induced DNA damage can be mediated by oxidative damage, strand breaks or cross-linking (Castano *et al.*, 2005). While both type I and type II mechanisms are involved in DNA damage, several studies have shown that the $^1O_2$ produced via the type II pathway is responsible for the majority of damage (Epe *et al.*, 1989; Piette, 1991). Other authors have demonstrated that, in fact, the type of mechanism responsible for DNA damage is dependent on which photosensitiser is used (Harris *et al.*, 2004; Sayed *et al.*, 2005). In contrast, some investigators have noted a distinct lack of DNA interaction and strand break formation following exposure of bacteria to pure singlet oxygen (Nieuwint *et al.*, 1985; Dahl *et al.*, 1987; 1988). These authors suggest that because unsaturated fatty acids and certain proteins react with $^1O_2$ much more rapidly than double-stranded DNA, it would be unlikely that critical DNA damage would arise from a $^1O_2$-mediated attack (Dahl *et al.*, 1987).

1.4.3.1.4 Factors affecting lethal photosensitisation of bacteria

Many environmental factors can affect the antibacterial efficacy of lethal photosensitisation. For example, Nitzan *et al.* (1998) found that the type of growth medium as well as the type and concentration of protein present in the medium influenced the kills obtained, with the high protein brain-heart infusion leading to reduced kills compared with low protein nutrient broth. Likewise, the presence of saliva, serum or blood in the medium has been investigated and found to decrease the efficacy of photosensitised killing (Wilson *et al.*, 1993; Wilson & Pratten, 1995; Bhatti *et al.*, 1997; Kömerik & Wilson, 2002). Also of significance was the finding that the bactericidal effect induced by photosensitisation is reduced at acid pHs but is unaffected

by the growth phase of the organism (Kömerik & Wilson, 2002). This last result is in contrast to the findings of Bhatti *et al.* (1997), who reported that cells in the logarithmic phase of growth were more susceptible to photodynamic action than those in the stationary phase. Finally, several researchers have found that bacteria remain susceptible to lethal photosenitisation even when they are present in a biofilm (Dobson & Wilson, 1992; Wilson *et al.*, 1996; O'Neill *et al.*, 2002; Zanin *et al.*, 2005; 2006).

1.4.3.1.5 Bacterial Spores

Recently, Demidova and Hamblin (2005) provided the first report of the successful inactivation of bacterial spores by lethal photosensitisation. These authors described photodynamic killing of several different types of *Bacillus* spores using red light and phenothiazinium dyes. This is of particular importance since spores are notoriously difficult to kill using conventional cleaning and disinfection protocols and therefore cause a significant problem for hospitals worldwide. The exact mechanism and targets of photosensitisation in spores have not yet been identified

1.4.3.2 Viruses

1.4.3.2.1 Viral envelope damage

One of the main factors governing the success of photodynamic inactivation of viruses is the structure of the virus, and in particular the presence of a viral envelope (Figure 1.6). It has been repeatedly reported that enveloped viruses are more susceptible to lethal photosensitisation than nonenveloped viruses, thus implicating the viral envelope as a

major target of photoinactivation. Specifically, photosensitisers such as merocyanine 540, hypericin and RB can induce cross-linking of viral membrane proteins (O'Brien *et al.*, 1992; Lenard & Vanderoef, 1993; Lenard *et al.*, 1993; Smetana *et al.*, 1998). It is thought that damage to the viral envelope inhibits adsorption and/or penetration of the virus thus destroying its infectivity. In contrast, Moor *et al.* (1997) were unable to find significant damage to the viral proteins of vesicular stomatitis virus (VSV) following treatment with aluminium phthalocyanine.

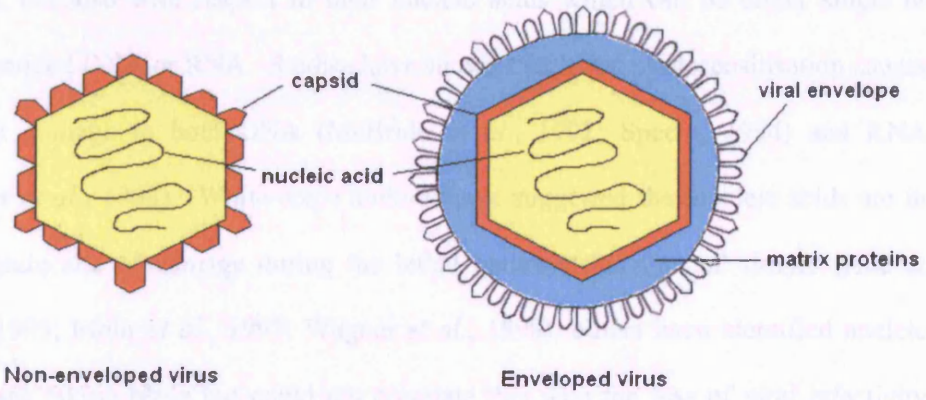


Figure 1.11 The general structure of an enveloped and non-enveloped virus.

1.4.3.2.2 Protein/enzyme inactivation

With respect to enzyme inactivation in viruses, the enzymes responsible for carrying out replication and transcription of nucleic acids appear to be targeted. Researchers studying the photodynamic inactivation of vesicular stomatitis virus have documented

the rapid decrease of RNA polymerase activity upon irradiation with aluminium phthalocyanine (Moor *et al.*, 1997) while others have reported reduced reverse transcriptase activity in HIV following treatment with dihaematoporphyrin (Matthews *et al.*, 1991). Investigators studying HIV have suggested that cross-linking of the proteins that make up the viral capsid also takes place during the photosensitisation process (Corash, 1996).

1.4.3.2.3 Nucleic acid damage

Viruses show tremendous variation not only in terms of their capsid and envelope structures, but also with respect to their nucleic acids which can be either single or double-stranded DNA or RNA. Studies have shown that lethal photosensitisation causes significant damage to both DNA (McBride *et al.*, 1992; Specht, 1994) and RNA (Schneider *et al.*, 1993). While some authors have suggested that nucleic acids are in fact the main site of damage during the lethal photosensitisation of viruses (Abe & Wagner, 1995; Mohr *et al.*, 1997; Wagner *et al.*, 1998), others have identified nucleic acid damage taking place but could not correlate this with the loss of viral infectivity (Specht, 1994).

Unlike merocyanine, hypericin and RB, the phenothiazines associate strongly with both DNA and RNA and are therefore believed to mediate virus inactivation via nucleic acid damage. MB for example is known to cause nucleic acid damage via a series of different mechanisms including generating alkali-labile sites (Ohuigin *et al.*, 1987), base modifications and sites of base loss (Cadet *et al.*, 1983; Floyd *et al.*, 1989; Epe *et al.*,

1993), single-strand breaks (Epe *et al.*, 1988; Schneider *et al.*, 1990), and crosslinks of DNA with proteins (Villanueva *et al.*, 1993). Single-stranded DNA and RNA are particularly vulnerable to phenothiazine-mediated nucleic acid modification since the host repair processes in these viruses are less efficient (Specht, 1994).

Regarding the exact sites of damage within both DNA and RNA, it has now been well established that $^1O_2$ preferentially targets guanosine (Simon & Van Vunakis, 1962; Floyd *et al.*, 1989; Schneider *et al.*, 1993; Ravanat *et al.*, 2000; Kawanishi *et al.*, 2001; Sayed *et al.*, 2005) although other nucleosides are also involved (Sayed *et al.*, 2005). The main reaction product formed from $^1O_2$-mediated photo-oxidation of guanosine is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (Ravanat *et al.*, 2000). Mispairing between 8-oxodG and adenine then occurs, causing G → C transversions and a high frequency of mutations which in turn leads to cell death (Phoenix & Harris, 2003).

1.4.3.2.4 Factors affecting the lethal photosensitisation of viruses

In common with photosensitiser-mediated bactericidal activity, the molecular structure, charge, and relative hydrophobicity of photosensitisers also play an important role in determining virucidal activity. In contrast to the positively-charged compounds like MB and Neutral Red which intercalate into viral nucleic acids, the anionic phthalocyanines act against the viral envelope (Wainwright, 2004). The well documented resistance of intracellular viruses to photoinactivation by certain phenothiazines (such as MB) is also thought to be a result of the permanently charged nature of the dyes since this charge limits their permeability across the plasma membrane of mammalian cells. This concept

was illustrated by a study carried out on VSV which demonstrated that the uncharged phenothiazine dye methylene violet (MV) was capable of inactivating both intracellular and extracellular VSV (Skripchenko *et al.*, 1997). Likewise, it has been suggested that the hydrophobic (or amphiphilic) nature of certain photosensitisers is responsible for the preferential partitioning of these dyes into viral membranes which, upon irradiation, leads to membrane damage and subsequent loss of adherence (O'Brien *et al.*, 1992; Wagner *et al.*, 1998).

As previously mentioned, one of the most significant and best developed applications of lethal photosensitisation is in the area of blood disinfection and consequently, the effect that blood components such as plasma have on virus inactivation must be considered. Plasma is known to quench both $^1O_2$ and other reactive species and, in addition, plasma proteins may also compete with virus particles for binding to photosensitiser molecules. While Wagner *et al.* (1993) did not find any reduction in the inactivation, other investigators observed that inactivation of viruses by both chemically-generated $^1O_2$ and MB in combination with light was suppressed by human plasma and human albumin (Müller-Breitkreutz *et al.*, 1995). This difference in results can be explained by variations in light dose since Müller-Breitkreutz *et al.* (1995) noted that the observed inhibition of virus inactivation could be overcome with stronger illumination.

In a separate study, Abe *et al.* (1997) investigated the inactivation of bacteriophage M13 by MB photosensitisation and found that the bacteriophage was more rapidly inactivated at higher temperatures although dye penetration into the virus particles remained

unchanged with incubation temperature. In addition, these authors also evaluated the effects of pH on virus inactivation and showed that the bacteriophage was more rapidly inactivated in basic solutions and more slowly inactivated in acidic conditions.

1.4.3.3 Fungi

Overall, fungi have been the least well-studied group of organisms with respect to photosensitisation. The study of photosensitised yeast inactivation began in the 1960s when Macmillan *et al.* (1966), reported the susceptibility of *Saccharomyces cerevisiae* to irradiation with a continuous gas wave laser in the presence of TBO. Since then a handful of studies have demonstrated similar findings. Once again, TBO has been the photosensitiser of choice in the majority of these investigations (Paardekooper *et al.*, 1992, Wilson & Mia, 1993, Jackson *et al.*, 1999) but other dyes including acridine orange (Iwamoto *et al.*, 1984) and haematoporphyrin (Ito, 1981; Stenstrom *et al.*, 1980; Bliss *et al.*, 2004) have also been found to induce lethal photosensitisation of yeast cells.

1.4.3.3.1 Membrane damage

Bearing in mind the limited diffusion distance of $^1O_2$, the large size and complex structure of yeast cells strongly implicate the cell membrane as the main target of lethal photosensitisation. In fact, several different research groups have reported this to be the case (Ito & Kobayashi, 1976; Bertoloni *et al.*, 1989; Paardekooper *et al.*, 1992). One suggestion is that the mode of inactivation in yeast cells (Figure 1.7) appears to involve several phases. Taking TBO as an example, the dye is known to bind specifically to

periplasmically localised polyphosphates (Tijssen *et al.*, 1981) and upon illumination a subtle change in the membrane properties allows a small number of TBO molecules to enter the cell. Once inside, the $^1O_2$ produced by the photo-activated TBO inactivates intracellular enzymes (see next section) which in turn can lead to an inhibition of energy metabolism (Paardekooper *et al.*, 1995). In addition, the inactivation of membrane carrier proteins inhibits the transport of sugars, amino acids, and phosphate which also contributes to the loss of viability (Paardekooper *et al.*, 1993). Finally, following the loss of viability, extensive damage to the plasma membrane causes an all-or-none loss of barrier function accompanied by an efflux of potassium ions (Paardekooper *et al.*, 1992).

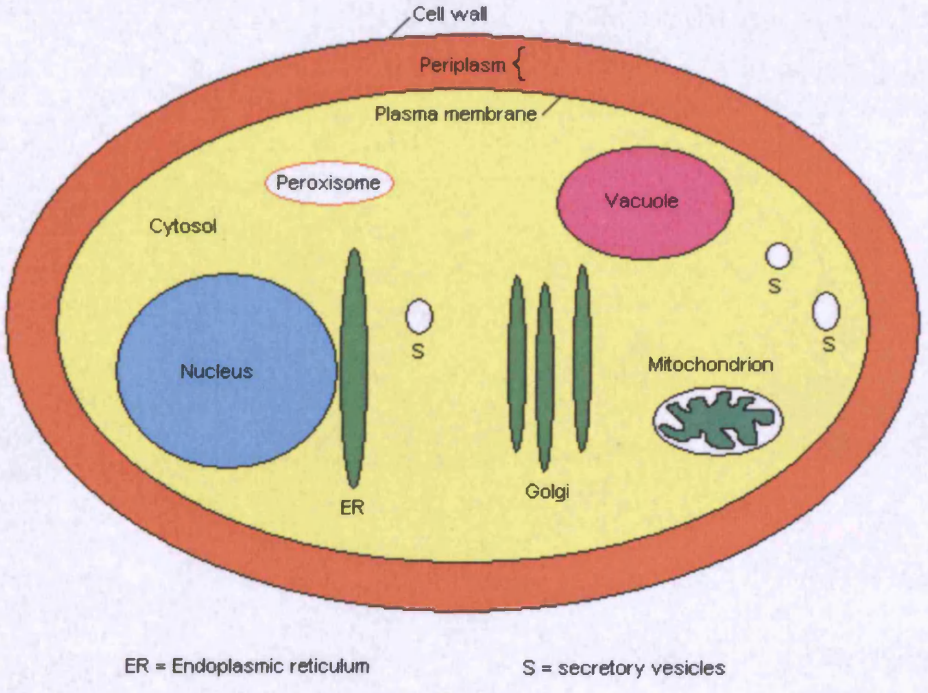


Figure 1.12 The structure of a yeast cell.

1.4.3.3.2 Enzyme/protein inactivation

Paardekooper and co-workers (1995) have demonstrated the rapid inactivation of the intracellular enzymes alcohol dehydrogenase (ADH) and cytochrome C oxidase following photosensitisation of yeast cells. Hexokinase and glyceraldehyde-3-phosphate dehydrogenase activities were also affected at a slower rate. Inactivation of these enzymes adversely affected the essential processes of fermentation, oxidative phosphorylation, and glycolysis, thus contributing to the loss of viability.

1.4.3.3.3 DNA damage

Photosensitiser-induced DNA damage is less well characterised in yeast cells. Damage to nucleic acids has been reported in several species of yeast including *Saccharomyces cervisiae* (Carré *et al.*, 1999; Padua *et al.*, 1999) and *Kluyveromyces marxianus* (Paardekooper *et al.*, 1997), but it is not thought to be a major cause of photosensitiser-mediated cell death in yeasts.

1.4.3.3.4 Factors affecting the lethal photosensitisation of fungi

Unlike with Gram-negative bacteria, the response of yeast cells to photosensitisation is less strictly controlled by the charge and structure of the photosensitiser, as evidenced by the accumulation of anionic photosensitisers in *Candida* spp. (Bertoloni *et al.*, 1992). Temperature does however appear to affect photosensitisation in yeasts as demonstrated by Bertoloni *et al.* (1989) who found that the photosensitivity of *C. albicans* cells increased with increasing temperature. Other authors reported that addition of foetal calf

serum to the growth medium enhanced leaching of Photofrin from yeast cells, thereby reducing their photosensitivity (Chabrier-Roselló *et al.*, 2005).

1.4.3.4 The multi-site attack

As discussed above, there is considerable debate as to the identity of the primary site of photodynamic action and whether there is in fact just one major target. Some photosensitisers appear to attack several different sites at the same time (Bhatti *et al.*, 1998; Ouédraogo & Redmond, 2003) or in succession (Bertoloni *et al.*, 1989; 2000), while others can show different sites of action in different bacterial species, as is the case with TBO and MB (Wainwright, 1998). As it turns out, the discussion surrounding the various potential sites targeted by lethal photosensitisation illustrates one of the greatest strengths of this emerging alternative antimicrobial approach: the multi-site attack.

1.4.4 Resistance?

During the course of the 20th century, it soon became evident that microbes were able to rapidly evolve mechanisms to enable them to evade the actions of antimicrobial drugs. This is especially true of conventional antimicrobials which generally work on only one specific site of action. In contrast, because the $^1O_2$ produced during lethal photosensitisation is capable of instigating a multi-site attack against the cell membrane, intracellular proteins and DNA, the possibility of adequate resistance mechanisms simultaneously evolving is reduced. Furthermore, redox reactions (Type I) of the

photosensitiser with water in the cellular environment (e.g. the cytoplasm) may also take place, thus producing additional reactive oxygen species (Wainwright, 2005).

Another upshot of the multi-site mode of action is that it should render the present-day conventional drug-resistance profiles of microbes irrelevant. In fact, a number of studies have provided evidence of the efficacy of lethal photosensitisation against resistant strains such as MRSA (Wilson & Yanni, 1995; Wainwright *et al.*, 1998) and vancomycin-resistant *Enterococcus faecium* (Wainwright *et al.*, 1999). It is believed that many of the mechanisms bacteria employ to evade the action of antibiotics would be of little use against photosensitisers. This idea can be explained by the nature of resistance mechanisms. Very broadly, antibiotic resistance mechanisms can be classified into 3 main categories: altered target, altered uptake, and drug inactivation (Mims *et al.*, 1998). Clearly, altering the target of the drug is a very sensible line of defence but since reactive oxygen species can have many different targets at the same time, this would prove a difficult task. Evidence rejecting the efficiency of altered uptake mechanisms (e.g. efflux pumps) comes from the polymer based PDT research described here (Decraene *et al.*, 2006) and elsewhere (Wilson, 2003; Bonnett *et al.*, 2006; Wainwright *et al.*, 2006) which illustrate that the photosensitiser does not need to enter the cells to exert a phototoxic effect. Lastly drug-inactivation by enzymes should be less successful against photosensitisers than conventional antibiotics since they themselves are susceptible to damage by $^1O_2$. Likewise, it should be remembered that antioxidant enzymes such as superoxide dismutase and catalase are also inactivated by $^1O_2$.

It would, however, be naïve to assume that resistance to lethal photosensitisation is an impossibility since microbes have repeatedly shown us how rapidly they can evolve.

1.5 Applications of antimicrobial PDT

PDT initially evolved as a treatment for cancer in the 1970s when Diamond *et al.* demonstrated that the tumour-localising and phototoxic properties of porphyrins could be harnessed together and utilised to successfully treat cancer (Diamond *et al.*, 1972; Dougherty *et al.*, 1975). The use of this new form of therapy to treat microbial infections has been slow to catch on. Although Raab reported his findings describing the lethal activity of acridine and light against paramecia more than a century ago, the subsequent discovery of antibiotics meant that early interest in using PDT to combat microbial infections was largely forgotten. During recent years, however, the continued increase in hospital-acquired infections and antibiotic resistance has emphasised the need to develop novel disinfectants and antimicrobials. To this end, the field of PDT has been re-embraced by the scientific and medical communities and is proving to be a promising alternative to conventional antibiotics and antiseptics. One successful application has developed in the field of dentistry, where the Periowave^{TM} disinfection system uses MB in combination with a specially designed hand-held diode laser for the disinfection of periodontal pockets (Andersen *et al.*, 2007). Staying with oral applications for lethal photosensitisation, TBO and MB are also showing potential as treatments for oral candidiasis (Teichert *et al.*, 2002; Donnelly *et al.*, 2007). Additionally, it is hoped that antimicrobial PDT will be of use in the treatment of wound infections and several studies have shown the successful treatment of wound and soft

tissue infections in mice (Hamblin *et al.*, 2002b; Hamblin *et al.*, 2003; Gad *et al.*, 2004; Lambrechts *et al.*, 2005). Interestingly, the eradication of *S. aureus* in burn wounds has also been demonstrated in guinea pigs in the absence of light using a deuteroporphyrin-hemin complex (Orenstein *et al.*, 1998), thus overcoming the problems surrounding light penetration. Recently, a publication has described the photosensitised killing of mycobacteria both *in vivo* and *in vitro* thus illustrating the potential for PDT to be used in the treatment of cutaneous and pulmonary mycobacterial infections (O'Riordan *et al.*, 2006).

1.6 Photosensitisers for disinfection

In addition to being used as a treatment for various microbial diseases in humans, photosensitisers also show potential as environmental disinfectants. One area in which photosensitiser-mediated disinfection is proving successful is in the treatment of microbiologically-polluted water. Magaraggia *et al.* (2006) have demonstrated the disinfection of fungal contaminated waters from fish-farming ponds using visible light in combination with porphyrins while other authors have disinfected wastewater using various photosensitisers either as a solution (Alouini & Jemli, 2001; Jemli *et al.*, 2002) or immobilized in a polymer (Bonnett *et al.*, 2006).

Medically, there has also been tremendous interest in utilising photosensitisers for the disinfection of blood products. As mentioned previously, MB has already been widely utilised by several European transfusion services in the photo-decontamination of blood plasma since the early 1990s (Wainwright, 2002), and many other photosensitisers are

being investigated as potential candidates for this purpose (Ben-Hur *et al.*, 1995; Margolis-Nunno *et al.*, 1997; Casteel *et al.*, 2004).

Lastly, it is also envisaged that aqueous photosensitiser solutions could be applied to floors, walls, or other environmental surfaces and activated by ambient light (Wainwright & Crossley, 2004). Such solutions may have applications in both the food industry and the hospital environment.

1.7 Aims and objectives

The majority of research into the lethal photosensitisation of microbes up to this point has used low-power laser light to activate the photosensitisers, while the use of incandescent or fluorescent white light sources has been largely overlooked. Likewise, the investigation of possible applications of antimicrobial PDT is limited to some extent and has mainly involved the treatment of topical infections. This introduction has provided a summary of the problems associated with hospital-acquired infections and contamination of environmental surfaces and has highlighted the problems associated with current cleaning protocols. Lethal photosensitisation may offer an alternative approach to traditional surface disinfection. It is envisaged that incorporating one or more photosensitisers into a material that could be applied to surfaces would provide a self-disinfecting coating which could be continuously activated by the ambient light present in the hospital environment.

The overall aim of this study was to develop a coating containing one or more photosensitisers that would be capable of killing representative microbes when irradiated with white light using light energy doses similar to those found under normal hospital conditions. To achieve this aim, the project had four main objectives:

1. To select appropriate photosensitisers for incorporation into a coating by evaluating their ability to generate $^1O_2$ as well as their microbicidal activity in aqueous suspension.

2. To incorporate the most-promising photosensitisers selected in (1) into cellulose acetate and assess the antimicrobial efficacy of the resulting coatings.

3. To establish the types and numbers of bacteria present on surfaces in the dental clinic setting prior to testing the coatings in this environment.

4. To evaluate in a clinical setting the antimicrobial effectiveness of the most promising coatings selected in (2).

CHAPTER 2

Materials and Methods

2.1 Light source and photosensitisers

2.1.1 Selection of light source

Several NHS trusts within London were consulted (informal contact via telephone) about the types of lamps they used in the different areas of their hospitals, the idea being that a lamp with similar qualities would be used for all experiments. The variables enquired about were as follows:

1.) The type of lamps used (e.g. halogen, fluorescent, incandescent).

2.) The colour rendering temperature of the lamps (measured in degrees K, e.g. cool or warm white light).

3.) The power output (W) of the lamps.

In addition, the guidelines set out in the CIBSE lighting guide LG2: hospitals and healthcare buildings (1989) were also consulted.

2.1.2 Light source and incubator

The light source selected (based on the information obtained from 2.1.1) and used throughout the study was a General Electric® 28W Biax 2D (replaced by the Starcoat 2D T5) compact fluorescent lamp (GE Lighting Ltd., Enfield, UK) which emits light across the visible spectrum (Figure 2.1). This lamp was fitted into a refrigerated incubator (Uni-profile Qualicool 260, LTE Scientific Ltd., Oldham, UK or LMS Series 1 model 303; LMS Ltd., Sevenoaks, UK) which kept the temperature at a constant 22°C. The light intensity was measured using a digital luxmeter (Hagner instruments Ltd., Bosham, UK). For all experiments, the lamp was positioned 45 cm above the samples.

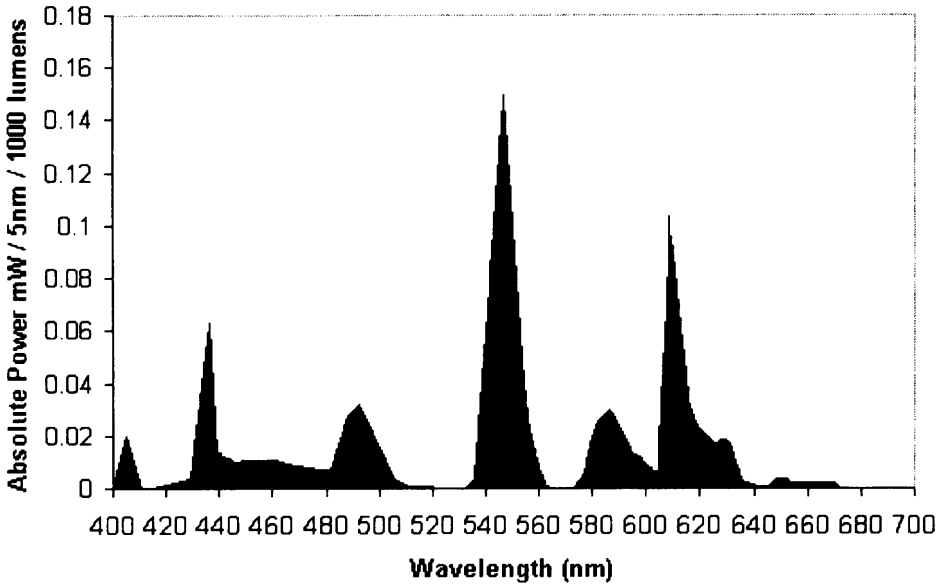


Figure 2.1 Emission spectrum of General Electric® 28W Biax 2D compact fluorescent lamp.

2.1.3 Selection of photosensitisers

Initially, photosensitisers were selected on the basis of their absorption spectra. In order to harness the maximum amount of light energy from the fluorescent lamp, it was envisaged that a combination of photosensitisers would be used; one of which absorbed light at the blue end of the spectrum, and another which absorbed light towards the red end of the spectrum. Both the Sigma Dye Book (Green, 1991) and Conn's Biological Stains (Horobin & Kiernan, Eds., 2002) were consulted during the photosensitiser selection process.

2.1.4 Photosensitisers

A large number of dyes were initially tested for photodynamic activity; a list of the dyes and photosensitisers used in this study is given in table 2.1. All photosensitisers were purchased from Sigma (Poole, UK) and were weighed out on a Sartorius balance (Sartorius Instruments Ltd., Surrey, UK). For all experiments carried out in solution, stock solutions of photosensitisers were made up in sterile distilled (dH_2O) at a concentration of 100 μg/mL and stored in the dark at room temperature for up to 1 week. A sonicator (IKA Labortechnik, Staufen, Germany) was used to help methylene violet Bernthsen (MV) and neutral red (NR) dissolve. Further dilutions were prepared in phosphate buffered saline (PBS) (Oxoid Ltd., Basingstoke, UK) as necessary.

Table 2.1 Dyes screened for photodynamic activity.

Dye	Molecular Formula	Absorption maximum (nm)	Dye Content (%)
Methylene blue	$C_{16}H_{18}ClN_3 \bullet 3H_2O$	664	du
Toluidine blue O	$C_{15}H_{16}ClN_3S$	632	89
Methylene violet Bernthsen	$C_{14}H_{12}N_2OS$	560	du
Rose bengal	$C_{20}H_2Cl_4I_4Na_2O_5$	549	90
Rhodamine B	$C_{28}H_{31}ClN_2O_3$	543 *	90
Neutral red	$C_{15}H_{17}ClN_4$	540 *	60
Erythrosine B	$C_{20}H_6I_4Na_2O_5$	526	92
Acridine orange	$C_{34}H_{40}Cl_4N_6Zn$	489	du

* Absorption maximum in ethanol; for all other dyes, the maxima are in water (information was provided by Sigma). du = data unavailable.

2.2 Preparation of photosensitiser-containing coatings

The photosensitisers incorporated into coatings were toluidine blue O (TBO), methylene blue (MB), MV, and rose bengal (RB). Stock solutions of all photosensitisers were made up in acetone ('AnalaR', VWR International Ltd., Lutterworth, UK) at a concentration of 100 μg/mL. A sonicator was used to help TBO dissolve. The coatings were made up of 50 mg/mL cellulose acetate (Sigma) dissolved in acetone. Aliquots of the stock solutions of photosensitisers were added to the cellulose acetate mixture to give final concentrations ranging from 10 to 150 μM. Cellulose acetate solutions were vortexed and left to dissolve for up to 5 hours.

2.2.1 Coatings in glass bijou bottles

Solutions were vortexed again and aliquots (450 μL) of each cellulose acetate mixture were transferred to a flat-bottomed glass bijou (diameter = 18 mm) by pipetting using solvent-safe tips (Sigma, Poole, UK). The acetone was allowed to evaporate overnight.

2.2.2 Coatings in glass Petri dishes

2.2.2.1 Coatings in small glass Petri dishes

TBO and RB were added to solutions of cellulose acetate in acetone (50 mg/mL) at a concentration of 25 μM each. Once completely dissolved, these cellulose acetate/photosensitiser solutions (final volume, 3.5 mLs) were poured into small (diameter = 50 mm) glass DUROPLAN® Petri dishes (Sigma) and the acetone was left to evaporate for 48 hours.

2.2.2.2 Coatings in large glass Petri dishes

For the larger 25 μM TBO/RB coatings, 9 mL solutions of cellulose acetate were made up in large thick-walled glass bijou bottles. Each bijou was repeatedly vortexed during a 6 hour period, and once the cellulose acetate had completely dissolved, the solutions were poured into large (diameter = 86 mm) resistance glass Petri dishes (Fisher Scientific UK, Loughborough, UK). The acetone was left to evaporate for at least 48 hours.

All coatings were prepared between 2 and 7 days before an experiment and were stored at room temperature in the dark until used.

2.2.3 Measurement of thickness of coatings

When necessary, the coatings were removed from the glass bijou or Petri dishes and their thickness was measured using a Starrett micrometer (No. 436.1, 0 – 25 mm; Starrett, Athol, Massachusetts, USA). For the coatings in bijou bottles, the average thickness was calculated from 3 measurements taken from different areas of the coating while for the coatings in Petri dishes, the average thickness was calculated from 5 different measurements. For each type of coating, 4 separate samples were measured to determine the average thickness.

2.3 Singlet oxygen production

2.3.1 Singlet oxygen production of photosensitisers in solution

The following protocol, adapted from Fischer *et al.* (1998), was used to quantify the singlet oxygen yield produced by each of the photosensitisers. The assay measures the absorbance of uric acid (UA) at 293 nm before and after irradiation; any decrease in the absorbance is directly proportional to the singlet oxygen yield produced. A fresh stock solution of uric acid (Sigma) was made up in PBS at a concentration of 100 μg/mL on each day the assay was to be carried out. Each photosensitiser was further diluted as appropriate in 1600 μL of PBS and this was then added to 400 μL of the UA solution to give 2 mL aliquots with a final photosensitiser concentration of 10 μM and a UA concentration of 20 μg/mL. Some experiments were carried out with a UA concentration of 40 μg/mL. The samples were thoroughly mixed and then 1 mL was transferred to special UV range cuvettes (Jencons-PLS, East Grinstead, UK). The absorbance at 293 nm (A_{293}) of each sample was measured using an Ultrospec 2000 UV/Visible spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Following irradiation under the 28W compact fluorescent lamp for 4 hours (see section 2.1.2), the remaining 1 mL of each sample was removed and the A_{293} was again measured. The difference between the initial A_{293} at time 0 and the A_{293} after 4 hours was then calculated and the average change in A_{293} (calculated from triplicate measurements taken during 2 separate experiments) was compared for each photosensitiser. To evaluate whether the photosensitisers were becoming photobleached during irradiation, separate absorbance measurements of the photosensitisers at their absorption maxima (see table 2.1) were also taken.

2.3.2 Singlet oxygen production of photosensitiser-containing coatings

Coatings were made up in glass Petri dishes as described in section 2.2.2.1. Five mLs of 20 μg/mL UA solution was added to each dish. Two photosensitiser-free and 2 photosensitiser-containing coatings were used in each experiment and experiments were carried out twice. Duplicate 1 mL volumes were removed before and after of irradiation, and their A_{293} was measured. Absorbance measurements at the absorption maximum of each photosensitiser present in the coating were also taken.

2.3.3 Absorption spectra

The absorption spectra of all photosensitisers in dH_2O and in coatings were determined using a UNICAM UV 500 UV/Visible spectrophotometer (ThermoSpectronic, Rochester, NY, USA) over the range 250 to 800 nm.

2.4 Lethal photosensitisation experiments in solution

2.4.1 Target organisms

The organisms used in this series of experiments were: *Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 10418, *Candida albicans* (clinical isolate), and Bacteriophage ΦX174 (host organism: *E. coli* ATCC 13706). All bacteria were maintained by weekly subculture on nutrient agar (Oxoid), while *C. albicans* was subcultured weekly on Sabouraud dextrose agar (Oxoid). The phage was propagated and titred according to ATCC guidelines, and the resulting stock solution was stored at 4°C. For experimental purposes, bacteria were grown aerobically in nutrient broth (Oxoid) while *C. albicans* was grown in Sabouraud dextrose liquid medium (Oxoid); all

were incubated at 37°C for 16 hours, centrifuged for 1 min at 16,110 x g in a microcentrifuge (Eppendorf 5415D, Cambridge, UK) and resuspended in an equal volume of PBS. *S. aureus* and *E. coli* NCTC 10418 were diluted in PBS to an optical density of 0.3 at 600 nm, which corresponded to approximately 10^8 cfu/mL. *C. albicans* was diluted in PBS to an optical density of 0.7 at 600 nm, which corresponded to approximately 10^6 cfu/mL. For the bacteriophage, a 1 in 10 dilution of the stock solution in PBS was used to achieve a final count of approximately 10^8 pfu/mL.

2.4.2 Photosensitisers

The photosensitisers used were TBO, MB, and MV. Using stock solutions (100 μg/mL of photosensitiser in dH_2O), the photosensitisers were diluted to 20 μM in PBS. A sonicator was used to help MV dissolve.

2.4.3 Lethal Photosensitisation

Four aliquots (100μL) of a suspension of each organism in PBS were transferred to separate wells of a microtitre plate (Sarstedt, Leicester, UK). An equal volume of photosensitiser was added to 2 of the wells to give a final concentration of 10 μM. PBS (100 μL) was added to the remaining 2 wells of the plate. This plate was then placed under the white light (see section 2.1.2) so that both the photosensitiser-containing (L+S+) and photosensitiser-free (L+S-) suspensions were irradiated for 2 or 6 hours. A second microtitre plate was prepared in an identical manner but was wrapped in aluminium foil before being placed in the incubator alongside the irradiated plate. The photosensitser-containing wells of this second plate acted as controls to determine the

microbicidal effect of the photosensitiser alone (L-S+) while the photosensitiser-free wells enabled calculation of the initial concentration of organisms in the suspension (L-S-). Following irradiation, each sample was serially diluted in PBS and each dilution was plated out in duplicate (50 μL) onto nutrient agar (*S. aureus, E. coli* NCTC 10418 and ATCC 13706) or Sabouraud dextrose agar (*C. albicans*). For the bacteriophage, 30 μL aliquots of each dilution were added to 300 μL of the host organism, *E. coli* ATCC 13706 (mid-exponential phase: 0.5 mL of overnight culture inoculated in 10 mL of nutrient broth and grown to an optical density of around 0.7 at 600nm) in polypropylene phage tubes (Barloworld Scientific, Stone, UK). Following incubation of the tubes at room temperature for 30 minutes, 3 mL of 0.5% nutrient agar (kept at 42 - 45°C) was added to each. Tubes were inverted and then poured evenly onto pre-warmed nutrient agar plates. All organisms were grown aerobically overnight at 37°C and survivors were enumerated by viable counting. Experiments were carried out at least twice. The minimum detection limit for all organisms was 10 cfu/mL per well.

2.5 Lethal photosensitisation experiments using coatings

2.5.1 Target organisms

The target organisms used for kill experiments with the coatings were: *S. aureus* NCTC 6571, MRSA-16 NCTC 13143, *E. coli* NCTC 10418, *C. albicans* (clinical isolate, obtained from Dr Jonathan Pratten), *P. aeruginosa* PAO1 (laboratory strain, acquired from Dr. Derren Ready), and *Clostridium difficile* 630 (laboratory strain, obtained from Dr. Adam Roberts). MRSA-16 was grown in the same manner as *S. aureus* NCTC 6571, as described in section 2.4.1. *C. difficile* was subcultured every 5 days onto brain heart

infusion (BHI) agar (Oxoid) supplemented with 5 % horse blood, Clostridium supplement (Oxoid), and 10 μg/mL tetracycline. For experimental purposes, *C. difficile* was grown overnight in BHI (Oxoid) at 37°C in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, UK). All other organisms were grown as described in section 2.4.1 except that for the purposes of these experiments, the microbial suspensions used were a 1:1000 dilution of an overnight culture.

2.5.2 Photosensitisers and coatings

The photosensitisers which were incorporated into coatings were TBO, MB, MV, and RB. The coatings were made up in glass bijou bottles as described earlier (section 2.2.1).

2.5.3 Lethal Photosensitisation in PBS

Aliquots (250 μL) of the microbial suspensions were placed on 4 coatings containing the photosensitiser(s) in glass bijou bottles. Two of these (L+S+) were exposed to the fluorescent lamp (see section 2.1.2) while the other two were kept in the dark (L-S+). In addition, microbial suspensions were also inoculated onto 4 control coatings (containing no photosensitiser), two of which were exposed to light (L+S-) and two of which were kept in the dark (L-S-). Following incubation for either 6 or 16 hours, duplicate 25 μL aliquots were removed from each sample and plated out. 1800 μL of sterile PBS was added to each bijou and all samples were vortexed for 5 s. Samples were then serially diluted in PBS and duplicate 25 μL aliquots were plated out onto nutrient agar (*S. aureus,* MRSA-16, *P. aeruginosa, E. coli* NCTC 10418 and ATCC 13706), BHI agar supplemented with 5 % horse blood, Clostridium supplement (Oxoid) and 10 μg/mL

tetracycline (*C. difficile*), or Sabouraud dextrose agar (*C. albicans*). All plates were incubated aerobically as described previously (except for *C. difficile* which was incubated anaerobically) and survivors were enumerated by viable counting. Experiments were carried out at least twice. For all organisms, the minimum detection limit was 20 cfu/mL per coating.

For each experiment, 1 mL of PBS was placed onto a photosensitiser-containing coating and following irradiation, the absorbance of this solution at the absorption maxima of the photosensitisers was measured to assess whether there had been any leaching of the photosensitisers out of the coating.

2.6 Nebuliser experiments on coatings

2.6.1 Target organisms

The target organisms for this series of experiments were *S. aureus* NCTC 6571 and *P. aeruginosa* PAO1. Both organisms were grown in nutrient broth overnight at 37°C. A 1 mL aliquot was centrifuged at 16,110 x g in an Eppendorf 5415D microcentrifuge for 1 min and resuspended in sterile PBS.

2.6.2 Nebuliser experiments in PBS

The coatings used were those prepared in small glass Petri dishes (section 2.2.2.1). Four photosensitiser-containing coatings and photosensitiser-free coatings were used for each experiment.

Bacterial suspensions were diluted 1:1000 or 1:50 (both in PBS) for *S. aureus* and *P. aeruginosa* respectively. Three mL of each of these suspensions was transferred to a sterilised Pari Tia nebulizer (Pari Aerosol Research Institute, Munich, Germany) which was connected to a Pari turboboy-N compressor (PARI Medical Ltd., West Byfleet, UK). For each of the 8 Petri dishes, the lid was removed, the coating was sprayed twice with the nebuliser at a distance of approximately 5 cm and then the lid was immediately placed back on. In addition, two control nutrient agar plates were also sprayed twice; one before the coatings were inoculated and one after. The agar plates were then incubated aerobically at 37°C for 24 hours. Coatings were kept in the dark for 5 minutes to allow bacteria to settle. Two photosensitiser-free coatings (L+S-) and 2 TBO/RB (L+S+) coatings were placed under the fluorescent lamp mounted in the refrigerated incubator (see section 2.1.2) for either 6 hours (for *S. aureus*) or 1 hour (for *P. aeruginosa*). It should be noted that experiments with *P. aeruginosa* using a 6 hour irradiation time (and subsequent experiments using 2 and 4 hours irradiations) were performed initially but the poor survival of *P. aeruginosa* (less than 10 cfu visible per coating) on control (L-S-) coatings necessitated shortening the irradiation time to 1 hour. The remaining two TBO/RB coatings (L-S+) as well as the other 2 photosensitiser-free coatings (L-S-) were kept in the dark for the same length of time.

Following irradiation or dark incubation for the controls, 3 mL of 0.5 % nutrient agar was added to each Petri dish and allowed to set. The Petri dishes were then wrapped in foil and incubated aerobically at 37°C for 24 hours. Survivors were enumerated by

viable counting. For each organism, the experiment was repeated at least 3 times. The minimum detection limit for all nebuliser experiments was 255 cfu/m^2 per coating.

2.7 Identification of environmental isolates

2.7.1 Biochemical Tests

Bacteria were initially characterized by determining their atmospheric growth requirement, Gram-stain, haemolysis, catalase and oxidase reaction.

2.7.2 16S PCR

All isolates were further identified to species level using partial 16S ribosomal RNA (rRNA) gene sequencing (Lane, 1985). The following global primers were used for 16S rDNA sequencing reactions: 27F [5'–AGAGTTTGATCMTGGCTCAG–3'], 1492R [5'–TACGGYTACCTTGTTACGACTT–3'], and 357F [5'–CTCCTACGGGAGGCAGCAG–3'] (Sigma-Genosys, Poole, UK). For each isolate, a distinctly separated colony was picked and suspended in 50 µL of molecular grade water (Sigma, Poole, UK). To this, 50 µL of mastermix [22.7 µL of dH_20, 10 µL of 10 X buffer (Bioline, London, UK), 10 µL of dNTPs (Bioline), 5 µL of $MgCl_2$ (Bioline), 1 µL each of primers 27F and 1492R (10µM), and 0.3 µL of BioTaq (Bioline)] was added. Reaction mixtures were subjected to the following thermal cycling conditions: 94°C for 5 mins, 29 cycles of 94°C for 60 s, 54°C for 60 s, and 72°C for 90 s, with a final step extension step of 72°C for 5 min (Biometra T3000 Thermocycler; Thistle Scientific Ltd., Glasgow, UK).

2.7.3 Agarose Gel Electrophoresis

Amplification products were confirmed by agarose (Bioline) gel electrophoresis. Gels were made up with 1 x TAE (Eppendorf) according to the volume required. A standard concentration of 1% agarose was used. The mixture was heated in a glass conical flask in a microwave (Sharp Compact) until all of the agarose had dissolved. The liquid was then cooled and 0.5 μg/ml of Ethidium Bromide was added while the mixture was still liquid. The agarose was poured into the appropriate gel tray and allowed to cool and set.

Five μL of each PCR product were mixed with 1 μL of 5 x loading buffer (Bioline) and loaded into the gel wells. Five μL of Hyperladder 1 (Bioline) was loaded in left hand lane of the gel. The gels were run at an approximately 100 Volts for an appropriate length of time depending on the size of the gel being run. The gel was exposed to UV light in an Alpha Imager (Alpha Innotech Corporation, distributed through Flowgen) to visualise the DNA.

2.7.4 DNA purification

In order to remove any excess components such as primers, nucleotides, DNA polymerase, and salts, the PCR products were cleaned up using a QIAquick® PCR purification kit (QIAGEN, Crawley, UK). Each sample (100 μL) was mixed with 500 μL of binding buffer PB, added to the QIAquick® spin column, and then centrifuged for 1 min at 16,110 x g in a benchtop microcentrifuge (Eppendorf 5415D). The flow-through was discarded, 750 μL of wash buffer PE was added, and the sample was again spun for 1 min at 16,110 x g. The flow-through was again poured off and the sample

was spun again for 1 min (16,110 x g). The column was transferred to a fresh eppendorf tube, 30 µL of Elution Buffer was applied to the centre of the column and each tube was incubated at room temperature for 1 min (16,110 x g). DNA was eluted by a final 1 min centrifugation step. All samples were run on a gel to confirm the presence of DNA before sequencing.

2.7.5 Sequencing reactions

For the sequence PCR reactions, 2 µL of the 16S PCR product was added to 2 µL of sterile dH_20, 1 µL of the 357F primer, and 2 µL BigDye (PE Applied Biosystems, Warrington, UK) DNA sequencing kit (diluted 1 in 4 in 5 x SEQ buffer; PE Applied Biosystems) to give a final reaction volume of 7 µL. The cycling parameters were 99 cycles of 95°C for 10 s, 50°C for 5 s and 60°C for 4 min (Biometra T3000 Thermocycler).

DNA was then precipitated via ethanol precipitation, which involved adding 13 µL dH_20, 2 µL 3M sodium acetate, and 50 µL 95% ETOH (-20°C) to each sequence PCR reaction. Samples were incubated on ice for 20 min, transferred to a 1.5 mL eppendorf and centrifuged (Eppendorf 5402) at 15,800 x g for 25 min at 4°C. The liquid was removed by pipetting and 250 µL of 70% ETOH (-20°C) was added. The samples were centrifuged again at 15,800 x g for 15 min at 4°C. The ethanol was removed by pipetting and the samples were left at room temperature until all the ethanol had evaporated. Once dry, samples were resuspended in 15 µL of Template Suppressor Reaction buffer (PE Applied Biosystems), vortexed, and heated at 95°C for 2 min. The

samples were then vortexed again, and transferred to Genetic Analyzer sequence tubes (PE Applied Biosystems). Finally, samples were placed on the sequencer (ABI PRISM® 310 Genetic Analyzer, PE Applied Biosystems) by Dr. Adam Roberts and Miss Tracey Moss (Eastman Dental Institute, London, UK) for sequence analysis.

Sequences were analysed using the CHROMAS programme (v.1.43) and were then entered into BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and RDP II (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) databases to obtain sequence matches.

2.7.6 Identification of staphylococcal and streptococcal isolates

Due to a high degree of 16S rRNA gene homology, the staphylococci and streptococci were further differentiated biochemically using the API Staph ID test (BioMérieux UK Ltd, Basingstoke, UK) and API Strep ID test. Results were interpreted using the API LAB ID computer software and tests were performed according to the manufacturer's instructions.

2.8 Statistical analysis

For all lethal photosensitisation experiments, the Mann-Whitney *U* test was used to compare the number of survivors from treated samples (L+S+) with the number of survivors from control samples (L-S-). L+S+ samples were also compared to the other control samples (L+S- and L-S+). $P < 0.05$ was considered statistically significant. All

statistical analyses were carried out using the SPSS statistical package (version 12.0, SPSS Inc., Chicago, IL, USA).

CHAPTER 3

Selection of suitable photosensitisers based on their absorption spectra and singlet oxygen yields

3.1 Introduction

Since von Tappeiner and Jodblauer's (1904) discovery that oxygen is a critical component of the photodynamic process, there has been a great deal of research and debate surrounding the toxicity of free radicals and singlet oxygen. Although oxygen radicals produced via the type I pathway are known to be microbicidal (Martin & Logsdon, 1987a,b,c) and do play a role in photosensitiser-mediated phototoxicity, it is widely believed that the $^1O_2$ generated via the type II pathway is the key mediator of the lethal effects caused by photosensitisation (MacRobert *et al.*, 1989). Consequently, the $^1O_2$ production efficiency of the photosensitiser should always be one of the key criteria used when selecting photosensitisers for any application that involves the killing of microbes. Furthermore, one of the main factors determining the amount of $^1O_2$ generated by a photosensitiser is the rate of absorption of photons and this in turn is governed in part by the overlap between the emission spectrum of the light source and the absorption spectrum of the photosensitiser (Kochevar & Redmond, 2000). This part of the project had three main aims:

1.) To select an appropriate light source that would mimic the ambient lighting conditions in hospitals as closely as possible.

2.) To select a range of photosensitisers which have absorption spectra that complement the chosen light source as closely as possible.

3.) To screen and compare these compounds for their $^1O_2$ producing ability as well as their levels of bleaching over both short and prolonged periods of time.

3.2 Materials and Methods

3.2.1 Selection of light source and photosensitisers

This has been described in Chapter 2, sections 2.1.1 to 2.1.3.

3.2.2 Preparation of photosensitising agents

This has been described in Chapter 2, section 2.1.4.

3.2.3 Singlet Oxygen Production

The general protocol for the singlet oxygen assay used throughout this study has been described in Chapter 2, section 2.3.1. Slight modifications were made for several of the experiments performed and these will be described in detail here.

3.2.3.1 Preliminary screening of suitable dyes

This has been described in Chapter 2, section 2.3.1. The photosensitisers tested were those listed in table 2.1. All dyes tested were used at a final concentration of 10 μM unless otherwise stated.

3.2.3.2 Four hour time course experiment

A four hour time course experiment was carried out on 5 of the dyes (TBO, MB, MV, RB, and Erythrosine B (ErB)). Double the UA concentration (40 μg/mL) was used in order to provide a longer time frame in which to study the kinetics before the UA is depleted. Five mL of the UA and photosensitiser solution was irradiated for 4 hours and

samples were removed at 0, 0.5, 1, 2, and 4 hours and their absorbance determined at 293 nm (A_{293}). In order to illustrate the kinetics of $^1O_2$, the change in A_{293} at each time point was converted to the relative change in UA content using the following equation:

Eq. 1 Relative ΔUA content = $1 - (A_{293(t0)} - A_{293(tx)}) / A_{293(t0)})$

Where ΔUA content is the change in uric acid content, $A_{293(t0)}$ is the absorbance at time 0 and $A_{293(tx)}$ is the absorbance at time point x.

3.2.3.3 Twenty-four hour experiment

A longer, 24 hour experiment was also carried out to see whether the photosensitisers could maintain their $^1O_2$ production throughout 24 hours of continuous irradiation. Triplicate 800 μL aliquots of each photosensitiser (final concentration = 10 μM) as well as a PBS control were transferred to wells of a 24-well tissue culture plate (Sarstedt, Leicester, UK). Five separate 24-well plates were prepared in this way, each irradiated for either 0, 1, 2, 6, or 24 hours. One hour before each of these times points, 200 μL of 100 μg/mL UA solution was added to give a final UA concentration of 20 μg/mL. For the initial measurement (time 0), the UA was added and the A_{293} measured directly afterwards. Samples were removed from the other plates at 1, 2, 6, 24 hours and the A_{293} measured. The difference between the initial A_{293} at time 0 and the A_{293} at each time point was then calculated and compared for each photosensitiser. To evaluate photobleaching, the absorbance of each solution at the absorption maximum of the individual photosensitiser (see table 2.1) was also measured at each time point.

3.2.3.4 Light/dark experiments

Finally, an assay to evaluate singlet oxygen production following alternating cycles of irradiation and incubation in the dark was performed. The experiment was carried out as described in the previous section except that the time points were as follows:

1.) Time 0.

2.) After 1 hour of irradiation.

3.) After 16 hours of irradiation.

4.) After 16 hours of irradiation followed by 8 hours in the dark.

5.) After 16 hours of irradiation followed by 8 hours in the dark and another 16 hour irradiation.

6.) After 2 complete cycles of 16 hours of irradiation and 8 hours of incubation in the dark.

For time points #1 and #2 above, the UA was added immediately and the A_{293} and absorbance at the absorption maxima for each photosensitiser were measured straight away and after 1 hour of irradiation respectively. For time points #3 and #5, the UA was added 1 hour before the end of the irradiation (i.e. after 15 hours of illumination), with absorbance measurements being taken after the final hour of irradiation. Finally for time points #4 and #6, the UA was added following completion of the dark incubation step and then the samples were irradiated for an additional hour, after which the absorbance measurements were recorded. The average change in A_{293} was again calculated and used to compare the $^1O_2$ generation of each photosensitiser while the relative change in

absorbance at the absorbance maxima were expressed as the relative change in absorbance using the following equation:

Eq. 2 Relative $\Delta A_{Max} = 1 - (A_{Max(t0)} - A_{Max(tx)}) / A_{Max(t0)})$

Where ΔA_{Max} is the change in absorbance at the photosensitiser absorption maximum, $A_{Max(t0)}$ is the absorbance measured at the photosensitiser's absorption maximum at time 0, and $A_{Max(tx)}$ absorbance measured at the photosensitiser's absorption maximum at time point x.

3.2.4 Statistical analyses

For all singlet oxygen experiments, the Mann-Whitney *U* test was used to compare the change in absorbance at A_{293} of the photosensitiser solutions with that of the control solution (PBS and UA). The Mann-Whitney *U* test was also used to compare the change in absorbance at A_{293} and the change in absorbance at absorption maxima between each of the photosensitisers. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using the SPSS statistical package (version 16.0, SPSS Inc., Chicago, IL, USA).

3.3 Results

3.3.1 Selection of light source and photosensitisers

In order to ensure that the coatings being developed could be activated by the ambient light present in hospitals, selecting an appropriate experimental light source was of

critical importance. Consequently, the initial stage of the project was focused on choosing both an appropriate light source and suitable photosensitisers for incorporation into the coatings. Consultation with various London NHS trusts revealed that the most commonly used light bulbs in hospital wards, ward corridors, and operating rooms were 2 ft 20 W and 4 ft 40 W fluorescent luminaries. The light rendering properties of the luminaries varied from hospital to hospital but generally warm (3000K) white light was used in the wards and cool (4000K) white light was used in operating theatres. In line with this, a smaller (in order to fit in the refrigerated incubator) 28 W compact fluorescent lamp with similar light rendering properties (cool temperature, 4000K) and spectral power distribution was chosen for the study. The emission profile of the lamp shows prominent peaks at 435, 495, 545, 588 and 610 nm (Figure 3.1). For experimental purposes, the lamp was installed in the incubator at a distance of 40 cm from the shelf on which the test coatings were placed - the light intensity at the surface of the test coatings was approximately 3700 lux.

The first criteria used to screen and select potential candidate photosensitisers was their absorption spectra. In order to harness the maximum amount of light energy from the fluorescent lamp, the emission spectrum of the lamp was compared with the absorption spectra of a variety of different compounds (Figure 3.1). This revealed that the best way to capture the highest proportion of the radiant energy emitted by the lamp would be to use a combination of blue (red light absorbing) and red (blue light absorbing) photosensitisers.

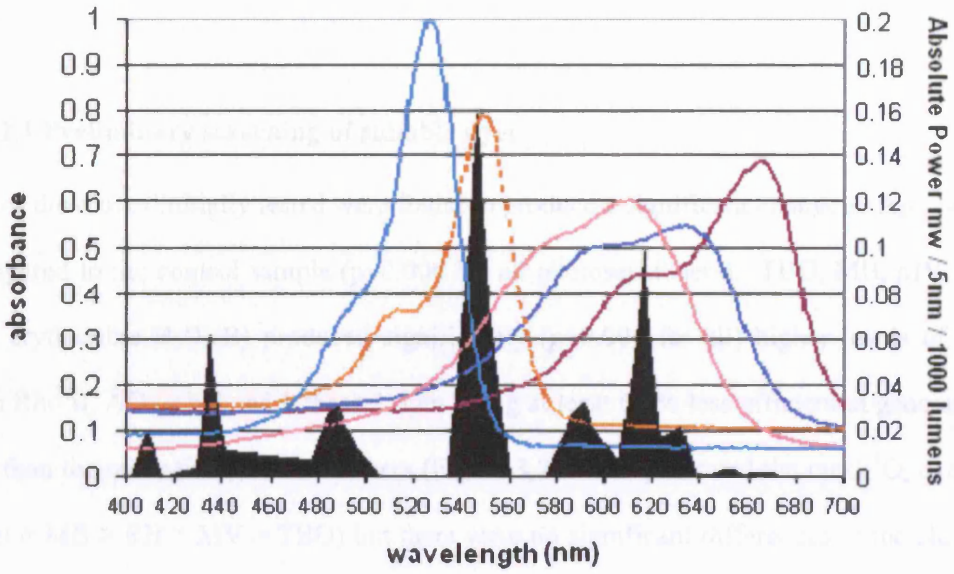


Figure 3.1 Overlap of the emissions spectrum of the 28 W compact fluorescent lamp (shown in black) and the absorption spectra of different photosensitisers when dissolved in dH_2O. The photosensitisers shown are toluidine blue (—), methylene blue (—), rose bengal (—), erythrosine B (—), and methylene violet (—).

3.3.2 Singlet Oxygen Production

Once suitable dyes had been chosen on the basis of their absorption spectra, the next step was to evaluate the photodynamic activity of these compounds. This was done using a uric acid (UA) assay. UA is a known scavenger of $^1O_2$ and the test uses a decrease of UA absorbance at 293 nm after irradiation of a solution containing UA and a photosensitiser as a fast assessment of the photosensitiser's $^1O_2$ producing capability (Fischer *et al.*, 1998). UA has 2 absorbance maxima in the UV range of the spectrum, the first at 220 nm and the second at 293 nm, but shows no absorbance in the wavelength range from 330 – 700 nm, where all photosensitisers absorb (Fischer *et al.*,

1998). Consequently, the mixing of UA with photosensitisers in solution does not cause any complications with regards to absorbance measurements.

3.3.2.1 Preliminary screening of suitable dyes

All of the 8 dyes initially tested were found to produce a significant change in A_{293} when compared to the control sample ($p=0.004$ for all photosensitisers). TBO, MB, MV, RB and erythrosine B (ErB) produced significantly ($p=0.004$ for all) higher levels of $^1O_2$ than Rho B, AO or NR, with these 3 dyes being at least 66 % less efficient at generating $^1O_2$ than the other five photosensitisers (Figure 3.2). ErB generated the most $^1O_2$ overall (ErB > MB > RB > MV > TBO) but there were no significant differences in the change in A_{293} among these 5 dyes following 4 hours of irradiation. Based on these results, only ErB, MB, RB, MV and TBO were selected for further study.

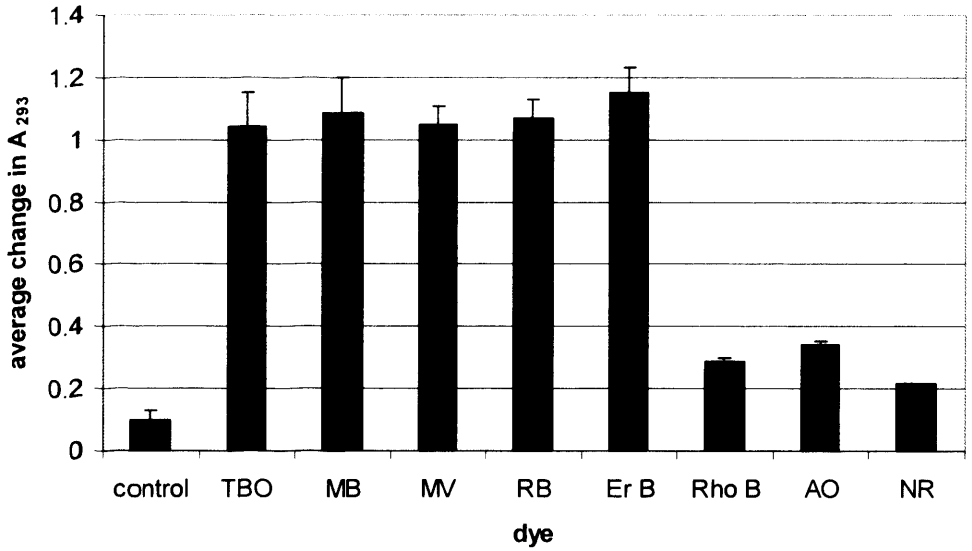


Figure 3.2 Singlet oxygen production of a selection of dyes following irradiation with a compact fluorescent lamp for 4 hours. Control = PBS; TBO = toluidine blue O; MB = methylene blue; RB = rose bengal; Er B = erythrosine B; Rho B = rhodamine B; AO = acridine orange; NR = neutral red. Bars represent mean values and error bars represent standard deviations (n = 6).

3.3.2.2 Four hour time course experiment

To provide insight into the overall kinetics and rate of $^1O_2$ generation, a four hour time-course experiment was performed on the photosensitisers selected from the previous experiment (Figure 3.3). All of the photosensitisers tested generated $^1O_2$, with the most rapid producers being the red dyes (RB, Er B, and MV). All of the photosensitisers produced $^1O_2$ immediately upon irradiation except for TBO, which had a slight delay in its rate of production. Maximum $^1O_2$ production for all photosensitisers was achieved after 2 hours of exposure, by which time all of the uric acid was depleted.

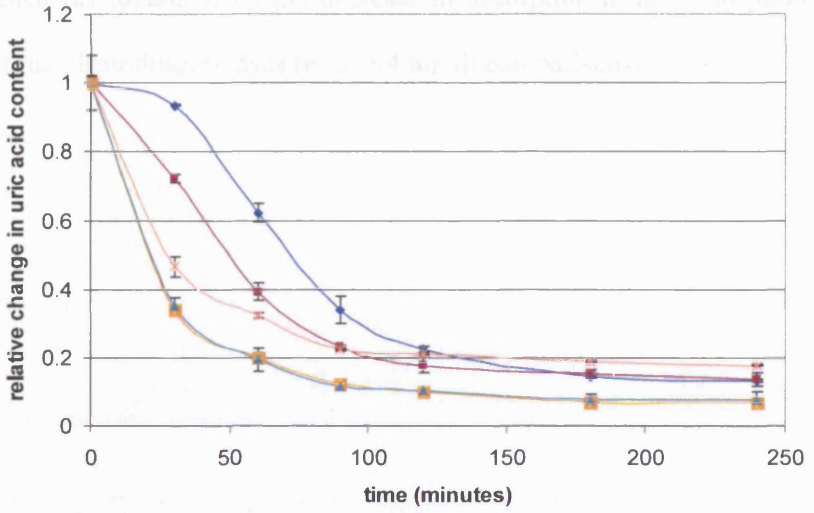


Figure 3.3 Relative change in uric acid content of TBO (◆), MB (■), RB (■), ErB (▲), and MV (✱) over the course of 4 hours. Measurements were taken at 30, 60, 90, and 120 minutes. Error bars represent standard deviations (n = 6).

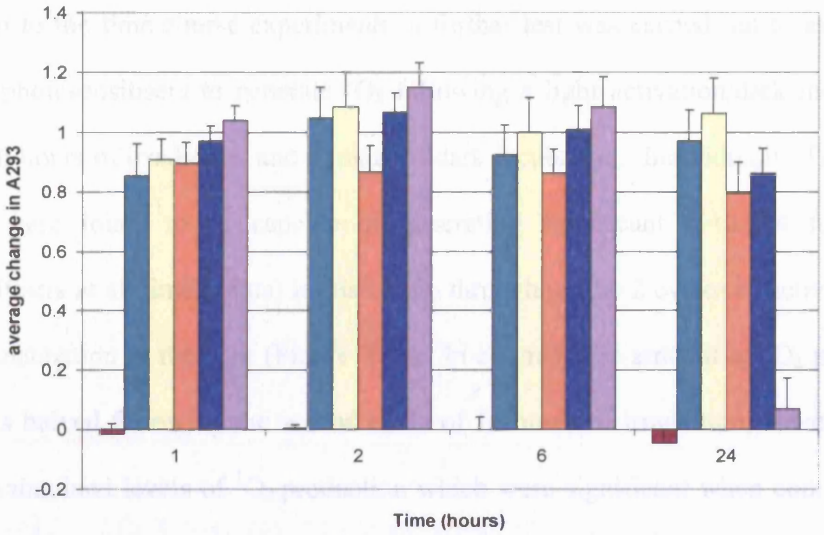
3.3.2.3 Twenty four hour experiment

In order to test for photo-bleaching and to evaluate whether $^1O_2$ generation is ongoing under conditions of continuous light exposure, a 24 hour experiment was carried out. With the exception of Er B, all photosensitisers maintained high $^1O_2$ producing ability throughout the 24 hours of light exposure (Figure 3.4a). After the first hour of irradiation, the change in A_{293} generated by Er B was significantly greater than that produced by TBO ($p=0.004$), MB ($p=0.006$) and MV ($p=0.004$) but interestingly the activity drops away completely after 24 hours of irradiation.

While all the compounds became slightly photobleached over time, Er B and RB were almost completely bleached, with their absorbance falling below 0.1 after 24 hours

(Figure 3.4b). At all time points, both Er B and RB were significantly more photobleached (as measured by the decrease in absorption at the absorption maxima) than any of the phenothiazine dyes ($p=0.004$ for all comparisons).

(a)



(b)

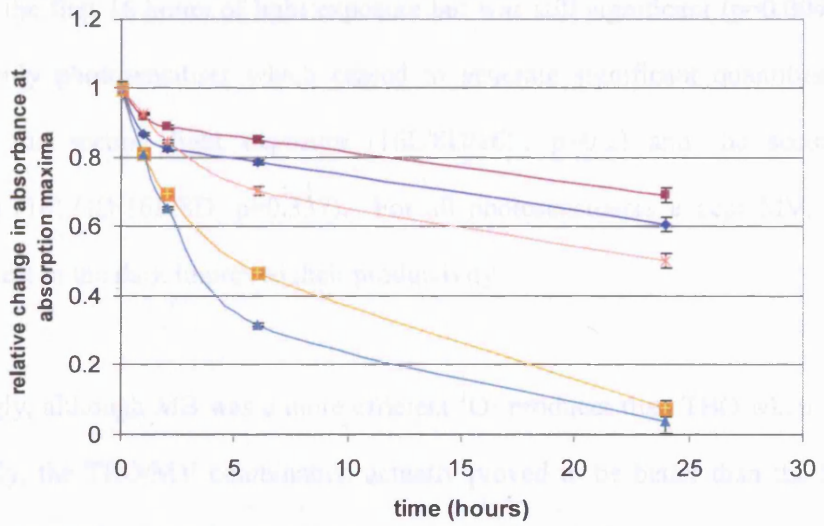


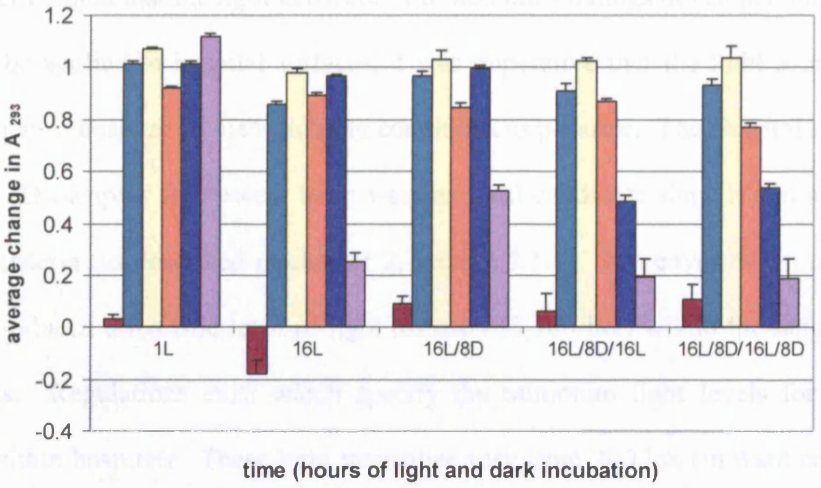
Figure 3.4 Singlet oxygen production and photobleaching of selected photosensitisers. (a) The average change in absorbance at 293nm of Blank (■), TBO (■), MB (□), MV (■), RB (■), and ErB (■) and (b) The relative change in absorbance at the absorption maxima of TBO (◆), MB (■), RB (■), ErB (▲), and MV (●) during 24 hours of light exposure. Measurements were taken at 1, 2, 6 and 24 hours. Bars represent mean values and error bars represent standard deviations (n = 6).

3.3.2.4 Light/dark experiments

In addition to the time course experiments, a further test was carried out to assess the ability of photosensitisers to generate $^1O_2$ following a light activation/dark incubation cycle of 16 hours of irradiation and 8 hours of dark incubation. Individually, TBO, MB and MV were found to be capable of generating significant ($p=0.004$ for all 3 photosensitisers at all time points) levels of $^1O_2$ throughout the 2 cycles of activation by light and incubation in the dark (Figure 3.5a). In contrast, the amount of $^1O_2$ produced by RB was halved following the second cycle of 16 hours of irradiation. Nevertheless, RB still maintained levels of $^1O_2$ production which were significant when compared to the control ($p=0.004$). The amount of $^1O_2$ produced by Er B decreased by around 75 % after only the first 16 hours of light exposure but was still significant ($p=0.004$). Er B was the only photosensitiser which ceased to generate significant quantities of $^1O_2$ following the second light exposure (16L/8D/16L; $p=0.2$) and the second dark incubation (16L/8D/16L/8D; $p=0.337$). For all photosensitisers except MV, the first period of rest in the dark improved their productivity.

Interestingly, although MB was a more efficient $^1O_2$ producer than TBO when assessed individually, the TBO/MV combination actually proved to be better than the MB/MV combination at all time points (Figure 3.5b). The difference between the two combinations was greatest after the second cycle of light and dark incubation but was not significant ($p=0.109$).

(a)



(b)

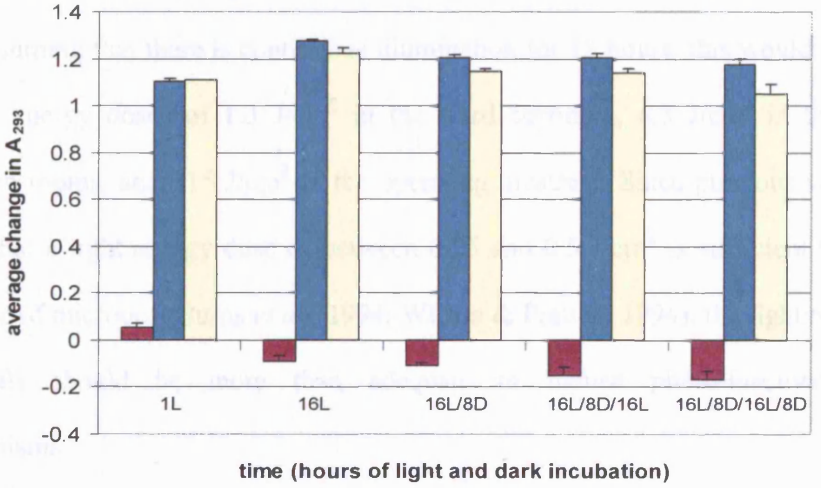


Figure 3.5 Singlet oxygen production of selected photosensitisers following cycles of light and dark incubation. **(a)** The average change in absorbance at 293 nm of individual photosensitisers following cycles of light and dark incubation. Blank (■), TBO (■), MB (□), MV (■), RB (■), and ErB (■). **(b)** The average change in absorbance at 293 nm of 2 combinations of photosensitisers following cycles of light and dark incubation. Blank (■), TBO/MV (■), MB/MV (□). 1L = 1 hour light, 16L = 16 hours light, 8D = 8 hours dark. All other abbreviations are the same as in figure 3.2.

3.4 Discussion

Since it is envisaged that the light-activated antimicrobial coatings developed during this study will be applied to hospital surfaces, it was imperative that the light source used was as similar to hospital ambient lighting conditions as possible. The General Electric® 28W Biax 2D compact fluorescent lamp was the ideal candidate since it met all of the necessary criteria (as described in chapter 2, section 2.1.1). Moreover, when installed, the lamp produced a realistic level of light intensity (3,700 lux) within the range found in hospitals. Regulations exist which specify the minimum light levels for various locations within hospitals. These light intensities vary from 200 lux (in ward corridors) to as high as 1,000 and 50,000 lux in Accident and Emergency examination rooms and operating theatres respectively (Chartered Institution of Building Services Engineers, 1989). Assuming that there is continuous illumination for 12 hours, this would produce daily light energy doses of 1.3 J/cm^2 in the ward corridors, 6.3 J/cm^2 in the A&E examination rooms, and 315 J/cm^2 in the operating theatres. Since previous work has indicated that a light energy dose of between 0.25 and 0.5 J/cm^2 is sufficient to kill a broad range of microbes (Burns *et al.*, 1994; Wilson & Pratten, 1994), the lighting levels in hospitals should be more than adequate to induce photo-inactivation of microorganisms.

The phenothiaziniums have been thoroughly studied and found to be extremely effective at killing a variety of bacteria, viruses, and yeasts. TBO and MB are the two best characterised phenothiazine photosensitisers and therefore were the first obvious candidates for use in this study. Since the absorption spectra of both of these dyes

overlap with two of the emission peaks from the light source (588 and 610 nm), the other compounds chosen were those that had absorption spectra which would complement TBO and MB and overlap with the remaining light emission peaks (435, 495, 545 nm).

While the uric acid test developed by Fischer *et al.*, (1998) is a simple, reliable assay that allows for meaningful comparison of the relative singlet oxygen production efficiencies of photosensitisers, it should be noted that most studies researching $^1O_2$ production utilise a different assay. The most commonly used test relies on measuring the decolourisation of the singlet oxygen quenching agent 1,3-diphenylisobenzofuran (DPIBF) at 410 nm (O'Neill *et al.*, 2003; Phoenix *et al.*, 2003). However, because the initial photosensitiser selection to be tested included compounds such as acridine orange, which absorbs between 360 and 480 nm, it was of interest to use a $^1O_2$ scavenger such as uric acid which shows no absorbance in the visible spectrum (330 – 700 nm). The application of different assays make it difficult to make direct comparisons between the results reported here and the findings discussed elsewhere. Nevertheless, both assays generate relative rather than absolute figures and are used to give a simple and meaningful evaluation of the $^1O_2$ generating capacity of different photosensitisers.

While a large number of lethal photosensitisation studies have been carried out using red-light emitting lasers, only a few have used a white light source to demonstrate photosensitised killing of microbes (Wainwright *et al.*,1997; Wainwright *et al.*, 1998; Zeina *et al.*, 2001), and none appear to have conducted an in-depth analysis of the

kinetics of $^1O_2$ production using white light. Moreover, of the studies that exist, most examine just one group of photosensitisers. The purpose of this part of the investigation was to compare the $^1O_2$ yields produced by many different types of dyes. Of the dyes tested, the xanthene dyes (RB and Er B) and the phenothiazinium dyes (TBO, MB, and MV) were all efficient $^1O_2$ producers while the remaining dyes (Rho B, AO, and NR) only generated very low levels of $^1O_2$. The finding that NR only produces a relatively small amount of $^1O_2$ is consistently supported by the literature (Phoenix *et al.*, 2003; Sayed *et al.*, 2005). As in this study, Wainwright & Giddens (2003) also reported that, of these 3 particular phenothiazine dyes, MB produced the highest $^1O_2$ yield. In contrast, however, while these authors reported TBO to be a more efficient $^1O_2$ producer than MV, such conclusions could not be drawn from the results described here since the initial preliminary screening and the 24 hour experiment both revealed MV to generate slightly higher yields of $^1O_2$ than TBO. The reasons for such differences are unclear. It may simply be a result of the type of white light source used. Compared with TBO, MV has an absorption spectrum that is shifted to the left (towards the blue end of the spectrum) and consequently it is able to capture more of the light energy emitted by the compact fluorescent lamp (Figure 3.1).

It is interesting to note that, despite the initial findings that MV was a highly efficient $^1O_2$ producer, the longer exposure light/dark experiments (Figure 3.5a) revealed that following repeated cycles of irradiation and rest in the dark, the $^1O_2$ generating ability of MV had decreased so that it was lower than that of TBO. This may be explained by the higher loss of absorbance of MV incurred during 24 hours of irradiation compared to

that of TBO (Figure 3.4b). As mentioned, the rate of absorption of photons is one of the main factors influencing $^1O_2$ generation and consequently any reduction in absorbance will affect the dye's $^1O_2$ productivity. Such a reduction in absorbance can be attributed to "photobleaching", whereby the photosensitiser itself is degraded by the reactive oxygen species generated upon activation with light (Finlay *et al.*, 2004). This phenomenon has been reported for many classes of photosensitisers used in PDT (Bonnett & Martínez, 2002; Lasalle *et al.*, 2004; Dysart & Patterson, 2005). Photobleaching is manifested in the loss of absorption or emission intensity (Verhoeven, 1996) that results from light exposure. It can occur via two irreversible processes: photomodification, where a loss of absorbance takes place at some wavelengths but the chromophore remains in a modified form, and true photobleaching where chemical changes are inherent, resulting in small fragments which have no appreciable absorbance in the visible region thus causing the sample to become colourless (Bonnett & Martínez, 2001). While all of the compounds became slightly photobleached over time, ErB and RB were almost completely bleached with their absorbance dropping to 0 after 24 hours of continuous irradiation. This loss of absorbance and colour indicates that the irreversible process of true photobleaching is taking place. Wood *et al.* (2006) reported similar findings for erythrosine which became irreversibly photobleached following irradiation times of more than 15 minutes. Due to the limited nature of the photobleaching seen for the phenothiazine dyes during the investigations described here, further research would need to be carried out to study the extent and nature of the photobleaching taking place.

When exposed to 16 hours of light irradiation followed by 8 hours in the dark, the xanthene dyes again became photobleached but while ErB lost its activity immediately following the first 16 hour irradiation, RB was only affected after the second irradiation. These results demonstrate the tremendous variation that exists in the photostability of the different compounds. Perhaps more significant was the observation that for all dyes except MV, the first period of incubation in the dark improved their $^1O_2$ productivity. Alternating periods of irradiation with dark intervals is known as light fractionation and has been repeatedly shown to enhance PDT in cancer treatment (van Geel *et al.*, 1996; Tsutsui *et al.*, 2002; de Bruijn *et al.*, 2006; de Haas *et al.*, 2006). Metcalf *et al.* (2006) have shown that the use of intermittent light exposure may also be beneficial in the treatment of microbial infections. These authors demonstrated that light fractionation of *Streptococcus mutans* biofilms in combination with erythrosine can significantly increase cell killing when compared with continuous irradiation. Although this is a well-documented phenomenon, the precise reasons for why it occurs are not clearly understood. It has been suggested that replenishment of target molecules such as oxygen may take place during the dark incubations (de Bruijn *et al.*, 2006; Metcalf *et al.*, 2006). Another possibility is that the unbleached photosensitiser molecules themselves are redistributed and replenished during the resting periods.

Due to the time-consuming nature of microbial kill experiments, it was not feasible to directly test all of the many candidate photosensitisers against a range of organisms. The uric acid assay provided a quick and simple way to compare the $^1O_2$ generation and, therefore, the potential antimicrobial effectiveness of different compounds under identical

lighting and temperature conditions. Furthermore, by measuring the absorbance of the photosensitisers during these experiments, it was also possible to evaluate whether the compounds were prone to photobleaching. Taken together, these preliminary $^1O_2$ investigations have illustrated that, of the dyes tested, TBO, MB and MV are not only the most efficient $^1O_2$ producers but they also proved to be the most photostable during longer periods of illumination. As a result, these 3 photosensitisers were selected for further study, with the next stage of the investigation being to test the antimicrobial activity of this more limited range of dyes in aqueous suspensions.

CHAPTER 4

Lethal photosensitisation of microbes in photosensitiser solutions

4.1 Introduction

A vast body of scientific literature exists surrounding the efficacy of the phenothiaziniums as antimicrobial agents against a wide variety of microbes, including bacteria (Wilson *et al.*, 1993; Wainwright *et al.*,1997; Wainwright *et al.*, 1998; Usacheva *et al.*, 2001; Phoenix *et al.*, 2003), viruses (Mohr *et al.*, 1997; Skripchenko *et al.*, 1997; Wagner *et al.*, 1998), and fungi (Paardekooper *et al.*, 1992; Wilson & Mia, 1993; Jackson *et al.*, 1999). The results of these studies demonstrated that the phenothiaziniums are extremely efficient photosensitisers and ultimately led to the development of 2 of the phenothiazine dyes, TBO and MB, for commercial applications. Despite this, however, there have only been a handful of reports that have directly compared the activities of these dyes in the same study (Wilson *et al.*, 1993; Wainwright *et al.*, 1997). Both of these studies involved bacteria; there appears to be a lack of comprehensive investigations that examine the anti-viral and anti-fungal properties of the phenothiaziniums together with their antibacterial activity. Moreover, all of the studies were performed under different conditions, making meaningful comparisons difficult. In particular, the light source used was different in each case. For the most part, red light- emitting lasers were utilised, although a number of studies also used white light sources such as an Exal light box, having a wavelength output of 350-800 nm (Wainwright *et al.*, 1997; Wainwright *et al.*, 1998) or a slide projector equipped with a 250W-lamp producing white light in the range 400 - 700 nm (Zeina *et al.*, 2001). For the purposes of the research described here, a compact fluorescent lamp was used as the light source as it mimics hospital lighting. Consequently, since no data exist on the antimicrobial properties of the phenothiaziniums when activated by such a light source, it was necessary to perform basic kill experiments using solutions of the dyes to confirm their activity before incorporating them into a polymer. Furthermore, because the

overall aim of this research was to develop a light-activated surface coating for use in hospitals, it would be preferable to produce one that is active against the three major classes of hospital-associated microbes: bacteria, viruses and fungi. The aim of this part of the study was to compare the antimicrobial activity of the 3 photosensitisers which performed best in the singlet oxygen experiments (TBO, MB, and MV) against a Gram-positive bacterium, a Gram-negative bacterium, a bacteriophage and a yeast.

4.2 Materials and Methods

4.2.1 Target organisms

The organisms and culture conditions have been described in Chapter 2, section 2.4.1.

4.2.2 Photosensitisers

The photosensitisers used were TBO, MB and MV. For all experiments, the final concentration of each dye was 10 μM.

4.2.3 Lethal Photosensitisation

This has been described in Chapter 2, section 2.4.3.

4.2.4 Statistics

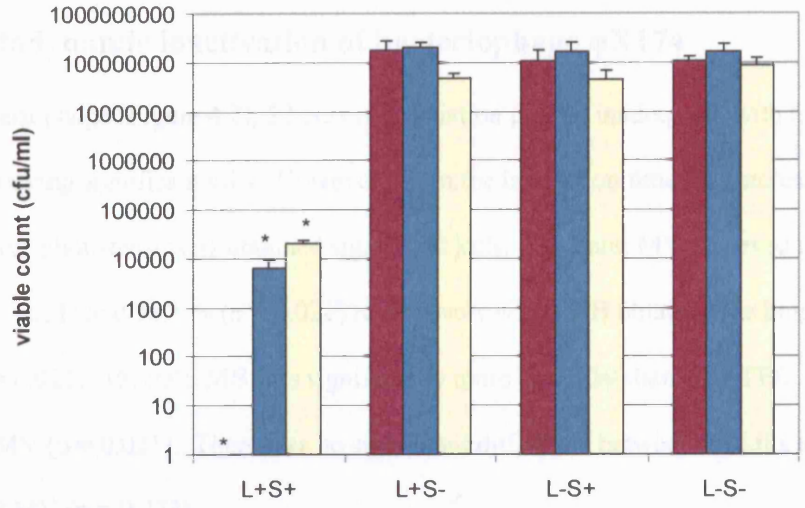
The Mann-Whitney *U* test was used as described in Chapter 2, section 2.8.

4.3 Results

4.3.1 Photodynamic inactivation of bacteria

The light intensity at the surface of the coating was 3700 ± 20 lux. The photobactericidal activities of TBO, MB, and MV are compared in figure 4.1. All three photosensitisers were able to achieve considerable reductions in the viable counts of both *S. aureus* and *E. coli* after irradiation for 2h. TBO was able to attain 100 % kills in each case ($p = 0.021$ for both) and was significantly more active than both MB ($p = 0.013$) and MV ($p = 0.014$). For MB, 99.9 % kills were achieved for both bacteria, although it was slightly more active against *E. coli*, achieving a 5 $\log_{10}$ reduction ($p = 0.014$) compared with a 4 $\log_{10}$ reduction for *S. aureus* ($p = 0.014$). In contrast, the activity of MV was higher against *S. aureus* than against *E. coli* with kills of 99.9% ($p = 0.021$) and 99.5 % ($p = 0.02$) respectively. MB was more effective than MV against both *S. aureus* ($p = 0.021$) and *E. coli* ($p = 0.02$). Overall, the order of photobactericidal efficacy observed against both bacteria was TBO>MB>MV. Exposure of the suspensions to either light or photosensitiser alone (L+S-, L-S+) showed no significant changes in the viable counts.

(a)



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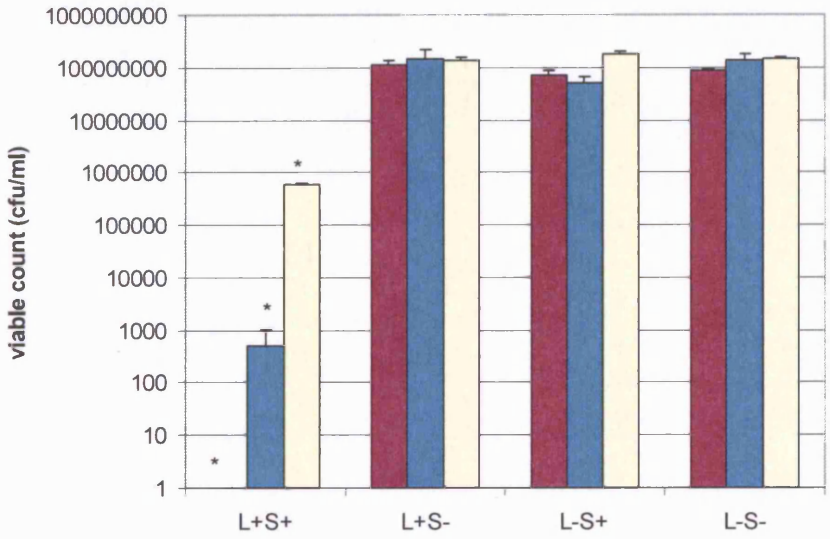
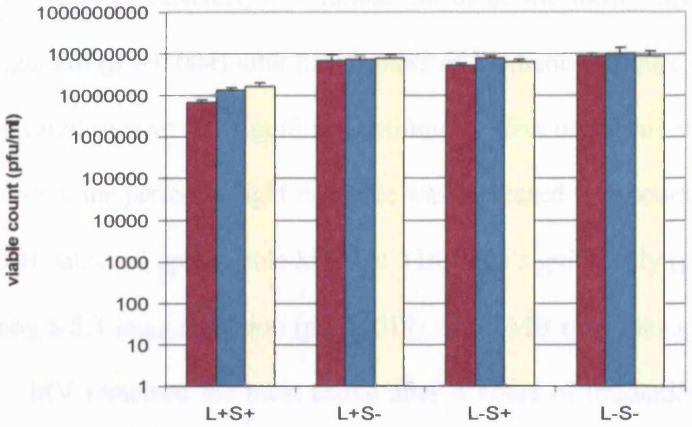


Figure 4.1 Lethal photosensitisation of bacteria in photosensitiser solutions. Viability of (a) *S. aureus*, and (b) *E. coli* in suspension when irradiated for 2 hours in the solutions of TBO (■), MB (■), and MV (□). In addition to the test suspensions in which bacteria were irradiated in the presence of 10 μM photosensitiser, 3 controls were also carried out: bacteria irradiated in the absence of photosensitiser (L+S-), bacteria kept in the dark in the presence of photosensitiser (L-S+), and bacteria kept in the dark in the absence of photosensitiser (L-S-). Columns represent mean values (n = 8) and bars represent standard deviations. cfu = colony-forming units. * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

4.3.2 Photodynamic inactivation of bacteriophage φX174

For the bacteriophage (Figure 4.2), 2 hours of irradiation proved inadequate, with none of the dyes achieving significant kills. However, when the irradiation time was increased to 6 hours, all three photosensitisers obtained significant kills. TBO and MV achieved kills of 98.3 % ($p = 0.021$) and 98.1 % ($p = 0.021$) respectively while MB obtained the biggest kill (99.7 %, $p = 0.021$). Overall, MB was significantly more virucidal than both TBO ($p = 0.043$) and MV ($p = 0.021$). There was no significant difference between the kills achieved by TBO and MV ($p = 0.773$).

(a)



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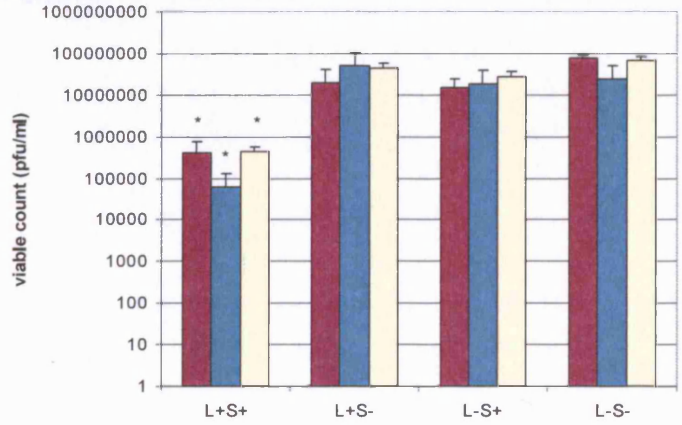
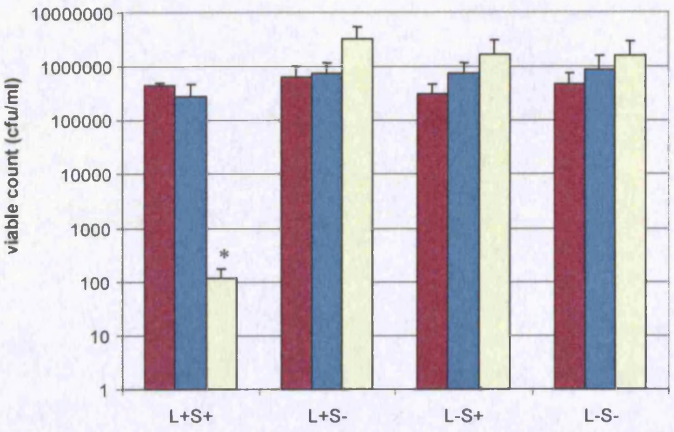


Figure 4.2 Viability of bacteriophage φX174 in suspension when irradiated for (a) 2 hours or (b) 6 hours in the presence of either TBO (■), MB (■), and MV (□). In addition to the test suspensions in which bacteria were irradiated in the presence of 10 μM photosensitiser, 3 controls were also carried out: suspensions irradiated in the absence of photosensitiser (L+S-), suspensions kept in the dark in the presence of photosensitiser (L-S+), and suspensions kept in the dark in the absence of photosensitiser (L-S-). Columns represent mean values (n = 8) and bars represent standard deviations. cfu = colony-forming units. * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

4.3.3 Photodynamic inactivation of *C. albicans*

In the case of the yeast *C. albicans*, MV turned out to be the most active, obtaining a significant 4.1 $\log_{10}$ kill ($p = 0.004$) after just 2 hours of irradiation (Figure 4.3a). Neither TBO nor MB appeared to have any significant antifungal effect using this short irradiation time. However, when the period of light exposure was increased to 6 hours (Figure 4.3b), both TBO and MB achieved appreciable kills but TBO was significantly ($p = 0.02$) more effective, obtaining a 5.3 $\log_{10}$ reduction ($p = 0.019$) while MB only managed a 1.1 $\log_{10}$ kill ($p = 0.021$). MV remained the most active after 6 hours of irradiation, achieving a complete (100%) kill which was significantly better than that achieved by MB ($p = 0.014$) but not TBO ($p = 0.131$).

(a)



(b)

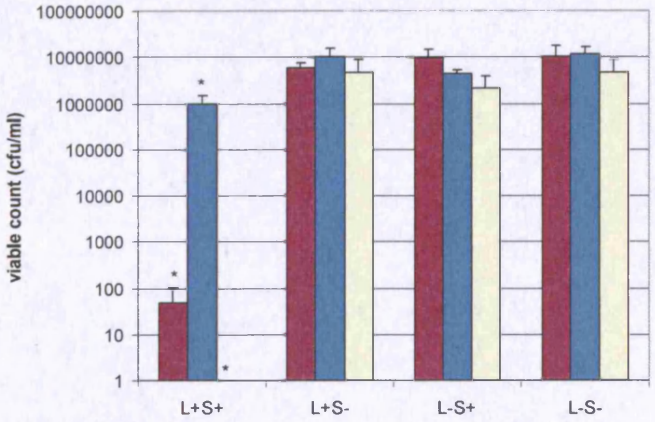


Figure 4.3 Viability of *C. albicans* following exposure to light from a 28-W fluorescent lamp for (a) 2 hours or (b) 6 hours in the presence of either TBO (■), MB (■), and MV (□). In addition to the test suspensions which were irradiated in the presence of 10 μM photosensitiser, 3 controls were also carried out: suspensions irradiated in the absence of photosensitiser (L+S-), suspensions kept in the dark in the presence of photosensitiser (L-S+), and suspensions kept in the dark in the absence of photosensitiser (L-S-). Columns represent mean values (n = 8) and bars represent standard deviations. cfu = colony-forming units. * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

4.4 Discussion

When examining the results from the uric acid experiments described in the previous chapter, MB was found to be a more effective singlet oxygen producer than TBO, a finding which has also been reported elsewhere (Pottier *et al.*, 1975; Wainwright *et al.*, 1998; O'Neill *et al.*, 2003). However, these results were not mirrored in the data obtained from the bacterial kill experiments performed in photosensitiser solutions which have demonstrated that TBO was the most bactericidal photosensitiser. It has been suggested that such discrepancies may be due to differences in cellular localisation of the photosensitisers (Wainwright *et al.*, 1997; Usacheva *et al.*, 2001). For example, if TBO binds to bacteria more efficiently than MB, then even though its cumulative $^1O_2$ production is lower, more of this $^1O_2$ will be reaching the critical targets, thereby conferring increased bactericidal activity. Indeed, in the study carried out by Usacheva *et al.* (2001), the partition coefficient, which measures the hydrophobicity of the dyes, was nearly 3 fold higher for TBO than for MB, thus suggesting that TBO should be more soluble in the hydrophobic region of the membrane, allowing greater interaction with the membrane. This finding that TBO is more effective against the majority of bacteria is supported by the literature (Wainwright *et al.*, 1997; Usacheva *et al.*, 2001, O'Neill *et al.*, 2003). However, in the case of *S. aureus* NCTC 6571, conflicting results were obtained by Wainwright *et al.* (1997) who found that TBO and MB had the same efficacy against this bacterium.

Gram-positive bacteria have generally been shown to be more susceptible to photodynamic inactivation than Gram-negative bacteria, irrespective of which photosensitiser is used (Usacheva *et al.*, 2001; Phoenix *et al.*, 2003). While the kills achieved here using TBO and MV support these findings, MB actually achieved marginally greater kills for *E. coli* than

for *S. aureus*. The reasons for this are unclear since other authors using MB have consistently reported greater kills against Gram-positive bacteria than against Gram-negative bacteria (Wainwright *et al.*, 1997; Usacheva *et al.*, 2001). It may simply be a consequence of the small data set and further investigations would need to be carried out to confirm whether the kills achieved against *E. coli* are significantly higher than those obtained against *S. aureus*. It is thought that the high susceptibility of Gram-positive bacteria is due to the relatively porous layer of peptidoglycan and lipoteichoic acid which surrounds their cytoplasmic membrane and allows photosensitiser molecules to cross (Hamblin & Hasan, 2004). Conversely, Gram-negative bacteria exhibit decreased susceptibility because their outer membrane provides an extra layer of protection by hindering the uptake of photosensitising molecules and intercepting photo-generated reactive oxygen species (Malik *et al.*, 1990a). The relatively large periplasmic space (~ 7nm) present in Gram-negative bacteria confers additional protection by making it more difficult for the $^1O_2$ to reach the plasma membrane and cause damage. Due to the negatively-charged surface of Gram-negative bacteria, cationic photosensitisers such as the phenothiazine dyes TBO or MB are necessary to exert a high bactericidal effect against them (Jori & Brown, 2004). Moreover, these dyes appear to interact readily with lipopolysaccharides (LPS). A study conducted by Usacheva *et al.* (2003), found that TBO interacted more significantly with LPS than MB and suggested that this may be one of the main factors influencing its greater photobactericidal activity against Gram-negative bacteria.

Due to the very different culture conditions and protocols required to grow viruses, research into lethal photosensitisation of microbes have tended to focus either on

bacteria and yeasts or on viruses; very rarely are all three studied alongside each other. The research described here provides a comprehensive study which investigates the photosensitiser-mediated inactivation of bacteria, a virus and a yeast under identical experimental conditions. Bacteriophage φX174 was chosen as the representative virus to be used in this study because not only is it safe and easy to work with, but it has also previously been used as a model virus in transmission studies and has a comparable stability to the most resistant human pathogenic viruses such as the poliovirus (Rheinbaben *et al.*, 2000). Furthermore, bacteriophage φX174 is a non-enveloped single-stranded DNA (ssDNA) virus and as previously discussed, non-enveloped viruses have been repeatedly shown to be less susceptible to lethal photosensitisation than enveloped viruses.

As expected, results revealed that bacteriophage φX174 was much less susceptible to photodynamic inactivation than both the Gram-positive and Gram-negative bacteria tested, with no significant kill seen after 2 hours of irradiation. Results following 6 hours of illumination were encouraging, however, with all 3 dyes achieving significant kills of more than 2 $\log_{10}$. Other authors have reported the sensitivity of the non-enveloped ssDNA virus parvovirus B19 following treatment with MB in combination with a fluorescent white light source (Mohr *et al.*, 1997). Likewise, Wagner *et al.* (1998) demonstrated the successful inactivation of the non-enveloped RNA bacteriophage R17. In contrast, Trannoy *et al.*, (2006) were unable to detect any inactivation of canine parvovirus using a positively-charged porphyrin chloride. The viral capsid has been suggested as one of the major sites affected during photosensitisation and consequently, the differences in susceptibility reported between various non-enveloped viruses may be

attributable to the different compositions of their capsids. This is particularly true of the nucleic-acid binding photosensitisers such as the phenothiaziniums since their activity will, at least in part, depend on the permeability of the viral capsid; viruses with tightly interdigitating viral capsid proteins may be less susceptible to treatment since access of the dye to nucleic acids would be limited (Skripchenko *et al.*, 2006).

One hypothesis for the enhanced resistance of non-enveloped viruses when compared with enveloped viruses is that the viral envelope is the major target of photoinactivation. However, the fact that non-enveloped viruses can still be inactivated by photosensitisers, albeit at higher concentrations and increased light doses, indicates that other structures such as the capsid and nucleic acids are also targeted. Since MB was found to be a highly effective virucidal agent in this study and elsewhere (Specht, 1994; Wagner *et al.*, 1994; Mohr *et al.*, 1997; Mohr *et al.*, 2004), this indicates that nucleic acid is a more likely site of action since MB and its derivatives are known to be extremely efficient nucleic acid intercalators (Wainwright, 2004). The hypothesis that nucleic acids are targeted during the lethal photosensitisation of bacteriophage φX174 is also supported by the fact that this virus has ssDNA which, as previously mentioned, is more vulnerable to damage.

Although the activity of photosensitisers against yeasts has not been as widely documented as for bacteria, several studies have shown that TBO is highly effective against the yeasts *C. albicans* (Wilson & Mia, 1993; Wilson & Mia, 1994; Jackson *et al.*, 1999; Demidova & Hamblin, 2005), *Saccharomyces cerevisiae* (Ito & Kobayashi, 1977; Böcking *et al.*, 2000) and *Kluyveromyces marxianus* (Paardekooper *et al.*, 1992; Paardekooper *et al.*, 1995). In

the present study, only MV in solution was able to achieve significant kills of *C. albicans* following 2 hours of irradiation with a 28W compact fluorescent lamp. Once again, it is difficult to compare the results presented here with those reported elsewhere in light of the different organisms and experimental conditions employed. However, the significant kills obtained in the other studies can most likely be attributed to the higher light energy doses used. As illustrated by Jackson *et al.* (1999), a direct correlation exists between the energy dose employed and the kill achieved, where the highest energy dose of 42 J used in their study achieved a reduction in the viable count of 3.3 $\log_{10}$. Since the studies carried out by Jackson *et al.* (1999) and Mia and Wilson (1993; 1994) employed a helium neon gas laser which has a power output of 35 mW and emits light at the specific wavelength of 632.8 nm (to coincide with the peak absorption of TBO), the energy dose achieved will inevitably be larger than that obtained using a white light source. Furthermore, it is possible that the white light could not penetrate the suspensions as easily as the laser light, thus inhibiting the photo-activation of TBO. It was therefore expected that much longer irradiation times would be needed in order to achieve similar kills when using a fluorescent lamp. This is supported by the results obtained following 6 hours of irradiation with the 28 W fluorescent lamp, where significant kills were shown using all photosensitisers. By increasing the irradiation time even further it is likely that even larger kills could be achieved for TBO and MB. Similarly, in the studies that did employ white light, much more powerful lamps such as a 150 W halogen lamp (Paardekooper *et al.*, 1992; Paardekooper *et al.*, 1995) or a 500 W Xenon arc lamp (Ito & Kobayashi, 1977) were used. It should be noted, however, that direct comparisons are difficult to make since the light fluence in these studies was measured in the radiometric unit of W/m^2 whereas in the research presented here, light intensity was measured in the photometric unit Lux. Conversion between the two units is

problematic since it is affected by the wavelength of light used, and white light encompasses a wide range of wavelengths.

The most striking observation to be made from the results presented here is the large discrepancy between the susceptibility of bacteria and that of the yeast *C. albicans* to photosensitised killing. While 100 % kills were obtained for both bacteria using TBO in combination with 2 hours of light exposure, no significant fungicidal effect could be seen using the same irradiation time. A similar trend was seen with MB. This finding that bacteria are more susceptible to PDT than yeasts is supported in the literature (Macmillan *et al.*, 1966; Wilson & Mia, 1993; Demidova & Hamblin, 2005). The increased resistance of yeasts to PDT can be attributed to their larger size and more complex eukaryotic cell structure. In general, *C. albicans* cells are known to be 10 - 50 times larger than *S. aureus* and *E. coli* cells and consequently the amount of $^1O_2$ necessary to kill a *Candida* cell is much larger than that needed to kill a bacterial cell (Demidova & Hamblin, 2005). The concentration of TBO used in the experiments described here was comparatively low (10 μM or 3 μg/mL) and it is likely that if this concentration was increased, greater kills could be achieved. Jackson *et al.* (1999) also failed to obtain any significant kills with such a low concentration but showed that the optimum concentration of TBO in their study was in fact 25 μg/mL.

In contrast to the other two photosensitisers, MV was able to achieve a significant kill against *C. albicans* after just 2 hours of illumination. This difference in efficacy can be explained by the variation in the chemical structures (see figure 1.3) exhibited by the dyes. It is thought that the neutral structure of MV allows for enhanced uptake by eukaryotic cells,

thereby improving the photodynamic effect. Support for this hypothesis comes from research by Carré *et al.* (1999), who demonstrated that neutral, amphiphilic porphyrin derivatives had increased activity against *S. cerevisiae*.

The results described here confirmed that all three of the selected dyes show broad-spectrum antimicrobial activity following irradiation with a 28 W compact fluorescent lamp and were therefore chosen for incorporation into a cellulose acetate polymer during the next stage of the research. TBO was found to be the most bactericidal overall (TBO > MB > MV) while MB was the most virucidal (MB > TBO > MV) and MV was the most fungicidal (MV > TBO > MB). Based on the overlap of absorption spectra of the 3 dyes and the emission spectrum of the light source (Figure 2.1), it was decided that the 2 combinations to be trialed as part of a coating would be TBO together with MV and MB together with MV.

CHAPTER 5

Lethal photosensitisation of microbes using photosensitiser-containing coatings

5.1 Introduction

The initial stages of this project demonstrated that not only did the chosen combination of light source (28 W compact fluorescent lamp) and photosensitisers (TBO, MB and MV) produce singlet oxygen, but also that photosensitiser solutions were effective at killing a range of different microorganisms. The next step was to show that these dyes could maintain their $^1O_2$ production and antimicrobial properties when immobilised in a cellulose acetate coating.

Previously, several groups have shown the efficacy of porphyrins, pthalocyanines and phenothiaziniums when incorporated in a polymer film (Bonnett *et al.*, 1993; Wilson, 2003; Wainwright *et al.*, 2006) or grafted to fibres (Bozja *et al.*, 2003). While these studies have demonstrated the antimicrobial activity of various photo-activated materials, in all cases the results were qualitative and only tested against bacteria. Furthermore, the light sources used in these studies were very different from those used in hospitals. Consequently, the main aim of this part of the research was to quantitatively test cellulose acetate coatings containing various combinations of photosensitisers against a range of different organisms including both Gram-positive and Gram-negative bacteria, a virus, and a yeast. It was important to ascertain whether an antimicrobial effect could be achieved using lighting conditions similar to those present in hospitals. Finally, it was also of interest to investigate the effects of certain parameters such as the presence of organic matter or the prolonged irradiation of coatings since such factors are commonplace in the hospital environment and could hinder the activity of the coatings.

5.2 Materials and Methods

5.2.1 Preparation of photosensitiser-containing coatings

This has been described in chapter 2, section 2.2.

5.2.2 Absorption spectra of coatings and solutions

This has been described in chapter 2, section 2.3.3.

5.2.3 Singlet oxygen production of photosensitiser-containing coatings

Coatings were made up in small glass Petri dishes as described in chapter 2, section 2.2.2.1. The uric acid assay was then carried out on these coatings as described in chapter 2, section 2.3.2. For the TBO/MV and MB/MV coatings, $^1O_2$ production was measured for two concentrations of photosensitiser (10 and 150 μM) after an initial 4 hours of irradiation. The coatings were then air-dried in the dark overnight and the experiment was repeated with $^1O_2$ production again being measured following a second 4 hour illumination. For the 25 μM TBO/RB coatings, the uric acid assay was carried out on coatings that had been irradiated for 6 or 16 hours, or for 6 hours following 7, 14, or 28 days of prolonged light exposure (1 day = 1 cycle of 16 hours of light and 8 hours of darkness).

5.2.4 Leaching experiments

In order to identify the ideal concentration of TBO that minimises leaching of TBO out of the coating, a range of different concentrations of TBO coatings were made up (10 – 100 μM) in glass bijoux as described in chapter 2, section 2.2.1. Each coating also contained 150 μM MV. One mL of PBS was placed on each coating and then all coatings were exposed to the fluorescent lamp for 6 hours. Following irradiation,

the leachates were removed and their absorbance (250 – 800 nm) was measured. The experiment was repeated 6 times, with a new coating each time.

5.2.5 Lethal photosensitisation

5.2.5.1 Target organisms

The organisms and culture conditions used were as described in chapter 2, section 2.5.1.

5.2.5.2 Lethal Photosensitisation in PBS

This has been described in chapter 2, section 2.5.3.

5.2.5.3 Lethal Photosensitisation of *S. aureus* in saliva

S. aureus NCTC 6571 was grown in nutrient broth overnight at 37°C. A 1 mL aliquot was centrifuged at 16,110 x g for 1 min in an Eppendorf 5415D microcentrifuge and resuspended in sterile PBS. This sample was further diluted down to 1:500 in PBS and then mixed 1:1 with fresh unsterilised whole human saliva (the author's). The experimental controls and procedures were as described in the previous section. Irradiation/dark incubation of the coatings was for 16 hours. All samples 5were serially diluted and then duplicate 25 μL aliquots were plated out onto selective mannitol salt agar (MSA; Oxoid). Plates were incubated as before and viable counts were performed.

5.2.5.4 Lethal photosensitisation following prolonged irradiation of coatings

The experimental protocol was identical to that described in chapter 2, section 2.5.3. However, prior to the experiment, all coatings were subjected to 7, 14, or 28 cycles of alternating light and dark periods (16 h of light and 8 h of darkness). The target organism was *S. aureus* NCTC 6571 and irradiation was for 6 hours.

5.2.6 Nebuliser experiments

5.2.6.1 Target organisms

The organisms and culture conditions used were as described in chapter 2, section 2.6.1.

5.2.6.2 Nebuliser experiments in PBS

This has been described in chapter 2, section 2.6.2.

5.2.6.3 Nebuliser experiments in saliva

The method used was the same as that described in chapter 2, section 2.6.2, with the following exceptions:

1.) The overnight culture was re-suspended and diluted 1:500 in PBS and then further diluted 1:1 in fresh unsterilised whole human saliva (the author's).

2.) The control agar plates were MSA.

3.) For the agar overlay, 3 mL of 75 % MSA was used.

5.2.6.4 Nebuliser experiments in horse serum

The method used was the same as that described in chapter 2, section 2.6.2, with the following exception:

The overnight culture was re-suspended and diluted 1:1000 in horse serum (Sigma).

5.2.7 Time-course experiments with *S. aureus*

5.2.7.1 Six hour time course experiment

The method used was the same as that described in chapter 2, section 2.5.3, with the following exceptions:

1.) 500 μL of the bacterial suspension was aliquoted on to each coating at the start of the experiment.

2.) For irradiated TBO/RB coatings (L+S+ samples), the following procedures were carried out at 5 time points during a 6 hour irradiation (30 min, 1h, 2h, 4h and 6 h):

(i) Duplicate 25 μL aliquots were removed from each coating and plated out on MSA.

(ii) One 10 μL aliquot was removed from each coating and serially diluted in sterile PBS in a microtitre plate (Sarstedt). Duplicate 25 μL aliquots of each dilution were then plated out on MSA.

3.) For the controls (L+S-, L-S+ and L-S-), samples were only processed after the end of the 6 hour irradiation. The coatings were vortexed to remove any adherent bacteria and then the bacterial suspensions were serially diluted in PBS and duplicate 25 μL aliquots of each dilution were plated out on MSA.

5.2.7.2 Time course experiment during the second hour of a 6 hour irradiation

The methods used were the same as those described in the previous section except that the time points measured were at 60, 75, 90, 105 and 120 minutes during the 6 hour irradiation.

5.2.8 Statistics

The Mann-Whitney *U* test was used as described in chapter 2, section 2.8.

5.2.9 Electron microscopy

Electron microscopy was carried out on two of the test bacteria, *S. aureus* and *E. coli*. Both organisms were grown as described in chapter 2, section 2.5.1, except that the microbial suspensions used were a 1:2 dilution (in PBS) of an overnight culture. These microbial suspensions were then aliquoted (550 μL) onto duplicate 25 μM TBO/RB or photosensitiser-free coatings that were either irradiated (L+S+, L+S-) or kept in the dark (L-S+ and L-S-) as described in chapter 2, section 2.5.3. For *S. aureus* irradiation was for 6 hours while *E. coli* was subjected to 16 hours of light exposure.

Following the photodynamic treatment, 500 μL was removed from each set of duplicate coatings and added to one eppendorf to give a final volume of 1 mL. All samples were centrifuged for 5 minutes at 16,110 x g in an Eppendorf 5415D microcentrifuge. The supernatant was removed and the pellet was resuspended in 3 % glutaraldehyde (Agar Scientific, Stansted, U.K.) in 0.1 M sodium cacodylate buffer and left for 24 hours at 4°C. In addition, for the *S. aureus* samples, one of each

of the different coatings (L+S+, L+S-, L-S+ and L-S-) was immersed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer and left for 24 hours at 4°C.

All further processing and image capture was carried out by Mrs. Nicola Mordan (Eastman Dental Institute, London, UK).

5.2.9.1 Scanning electron microscopy (SEM)

The coating specimens were dehydrated at room temperature in a series of graded ethyl alcohols of 20%, 50%, 70% and 90% for 10 min each and 3 changes in 100 % for 10 min each. Specimens were then critical point dried by immersion in hexamethyldisilazane (TAAB Ltd., Reading, U.K.) for 2 min with subsequent drying in a fume hood for at least an hour. Following this, the specimens were placed onto adhesive carbon tabs (Agar Scientific) attached to aluminium pin stubs. All specimens were sputter-coated with gold/palladium (Polaron E5000, Quorum Technology, Newhaven, U.K.). Specimens were examined in a Cambridge 90B Stereoscan electron microscope (Cambridge Scientific Instruments Ltd., Ely, U.K.) operating at 15 kV.

5.2.9.2 Transmission electron microscopy (TEM)

The fixed bacterial suspensions were dehydrated at room temperature in a series of graded ethyl alcohols of 20%, 50% and 70% for 15 min each and 3 changes in 90 % for 10 min each. Specimens were then infiltrated with, and embedded in, LR White resin (London Resin Co. Ltd., London, U.K.) and ultra-thin sections of 90 - 100 nm were cut. Specimens were stained with lead citrate and uranyl acetate and then

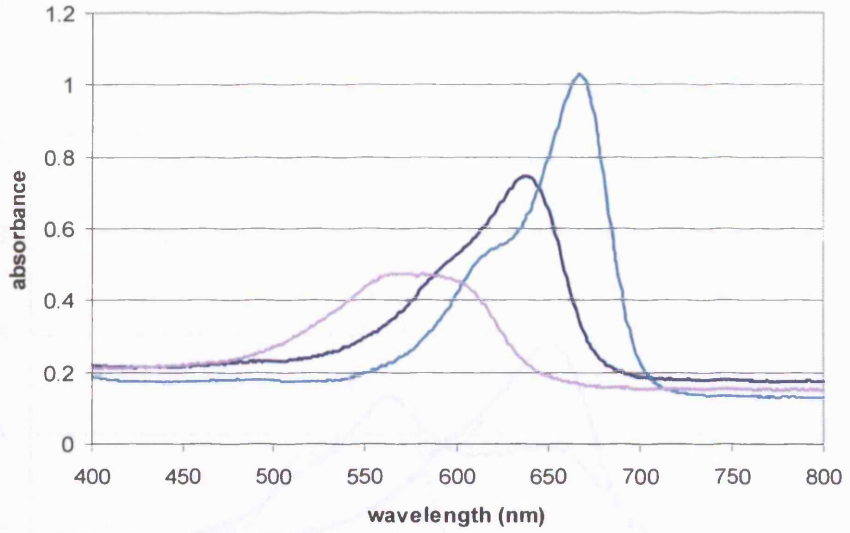
viewed with a Jeol 100 CX transmission electron microscope (Jeol Ltd., Welwyn Garden City, U.K.) operating at 80 kV.

5.3 Results

5.3.1 Absorption spectra of coatings

To ensure that the absorption spectra of the photosensitisers were not altered when they were dissolved in acetone and incorporated into the coatings, the absorption spectra of several different coatings were also measured. Individually, these spectra (Figure 5.1a) appeared almost identical to those obtained when these photosensitisers were dissolved in dH_2O (see figure 3.1b). Similarly, the coatings containing combinations of photosensitisers (Figure 5.1b) simply showed an additive effect: as expected, these coatings produced a broadened spectrum which encompassed the spectra of both the dyes present. Interestingly, the absorption maximum of each photosensitiser was lower when the agents were incorporated into coatings in combination as opposed to individually.

(a)



(b)

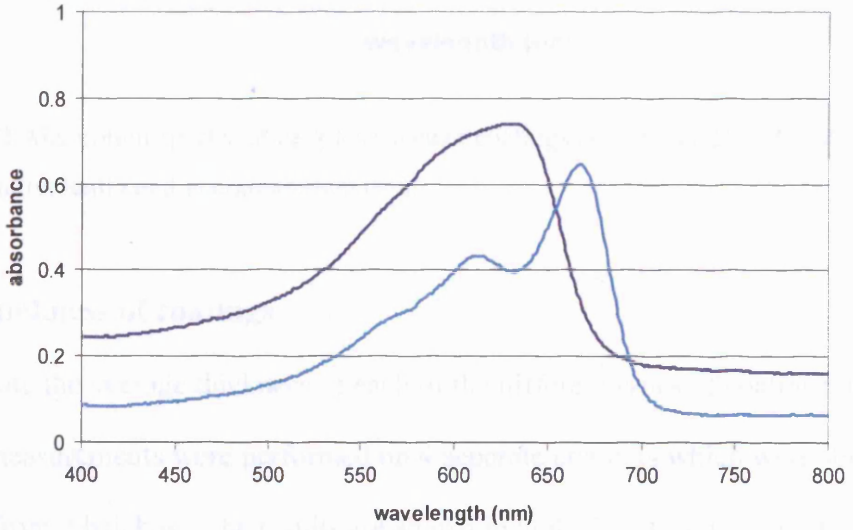


Figure 5.1 Absorption spectra of cellulose acetate coatings containing (a) the individual photosensitisers TBO (—), MB (—), and MV (—) and (b) two combinations of photosensitisers, TBO/MV (—) and MB/MV (—).

The absorption spectrum of the TBO/RB coating (which had a thickness of 43.2 ± 6 μm) is shown in figure 5.2 where it can be seen that strong absorbance occurs

between 500 nm and 675 nm which includes three of the main emission peaks of the light source.

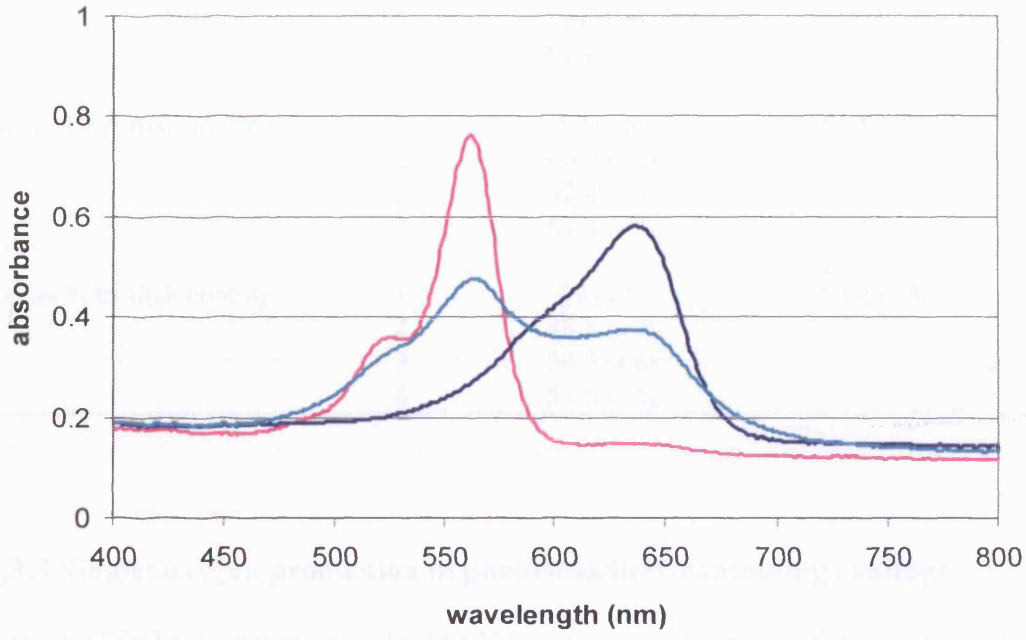


Figure 5.2 Absorption spectra of cellulose acetate coatings containing 25 μM TBO (—) and RB (—) individually and in combination (--).

5.3.2 Thickness of coatings

To estimate the average thickness of each of the different types of coatings used, several measurements were performed on 4 separate coatings which were selected at random from 2 batches. The results are shown in table 5.1. Coatings used for all laboratory kill experiments were 43.2 ± 6 μm thick while the coatings (in small glass Petri dishes) used for nebuliser and $^1O_2$ experiments were 51.5 ± 5 μm thick. Measurements for the coatings (in large glass Petri dishes) used in Chapter 7 are also shown in table 5.1; these coatings had an average thickness of 55.4 ± 5 μm.

Table 5.1. Thickness of all types of coatings used for singlet oxygen and kill experiments.

Type of coating	Replicate	Thickness (μm)	Average Thickness
Bijou bottle coating	1	41.7 (± 7)	43.2 (± 6)
	2	44.7 (± 8)	
	3	42 (± 7)	
	4	44.3 (± 10)	
Small Petri dish coating	1	50 (± 6)	51.5 (± 5)
	2	46.2 (± 6)	
	3	52.4 (± 6)	
	4	57.4 (± 4)	
Large Petri dish coating	1	59 (± 5)	55.4 (± 5)
	2	48.8 (± 3)	
	3	54.3 (± 6)	
	4	59.6 (± 2)	

5.3.3 Singlet oxygen production of photosensitiser-containing coatings

Both the TBO/MV coating and the MB/MV coatings produced singlet oxygen after four hours of irradiation (Figure 5.3). For the low concentration coatings (10 μM), the MB/MV coating generated nearly twice as much $^1O_2$ as the TBO/MV coating during both the initial ($p = 0.002$) and secondary ($p = 0.001$) irradiations. In contrast, no significant difference was seen between the 2 types of coatings when high concentrations (150 μM) of photosensitisers were used ($p = 0.529$ and $p = 1$ for the first and second irradiations respectively). For both the TBO/MV and the MB/MV coatings, the 150 μM coatings were much more effective than the 10 μM coatings, producing 3.2 ($p = 0.001$) and 1.9 ($p = 0.001$) times more $^1O_2$ respectively after the first illumination. In all cases, the $^1O_2$ yields were higher following the second irradiation when compared to the first but this difference was only significant for the 150 μM coatings ($p = 0.001$ for TBO/MV and $p = 0.021$ for MB/MV).

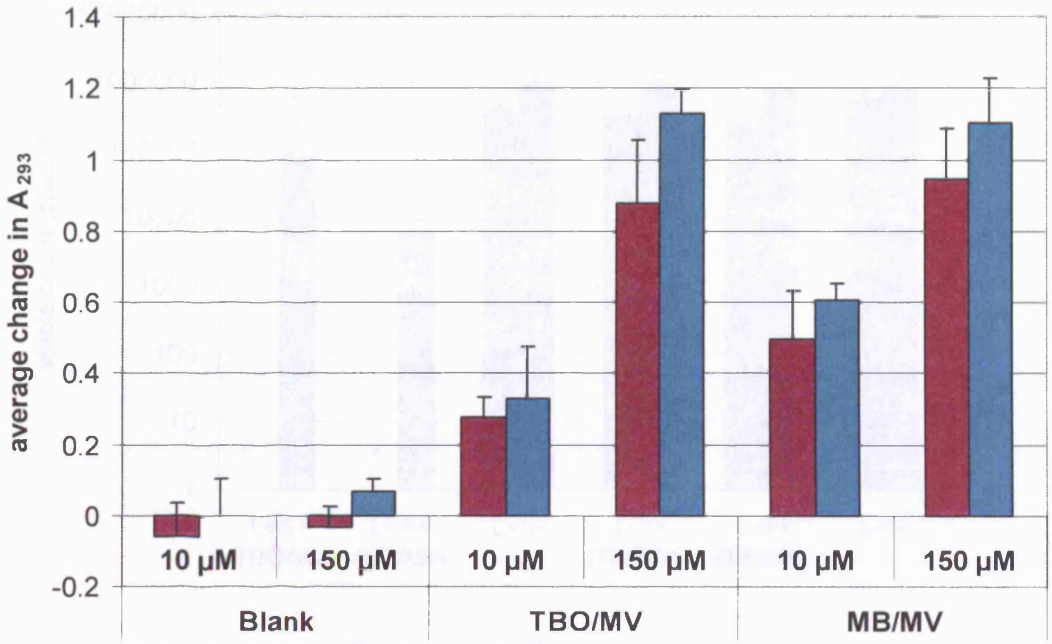


Figure 5.3 Singlet oxygen production by 10 µM and 150 µM TBO/MV and MB/MV coatings following an initial 4 hour irradiation (■) and again after being air-dried in the dark overnight and subjected to a second 4 hour illumination (■).

5.3.4 Lethal photosensitisation

5.3.4.1 Lethal Photosensitisation in PBS (TBO/MV and MB/MV coatings)

When using high concentration (150 µM) coatings containing either a combination of TBO and MV or MB and MV (Figure 5.4), a 100 % kill of *S. aureus* was obtained with either coating ($p = 0.013$ for both). For *E. coli*, kills were lower but still significant, with the TBO coating achieving a kill of 86.1 % and the MB coating a 99.1 % kill ($p = 0.020$ for both); the difference between the 2 types of coatings was not significant ($p = 0.386$).

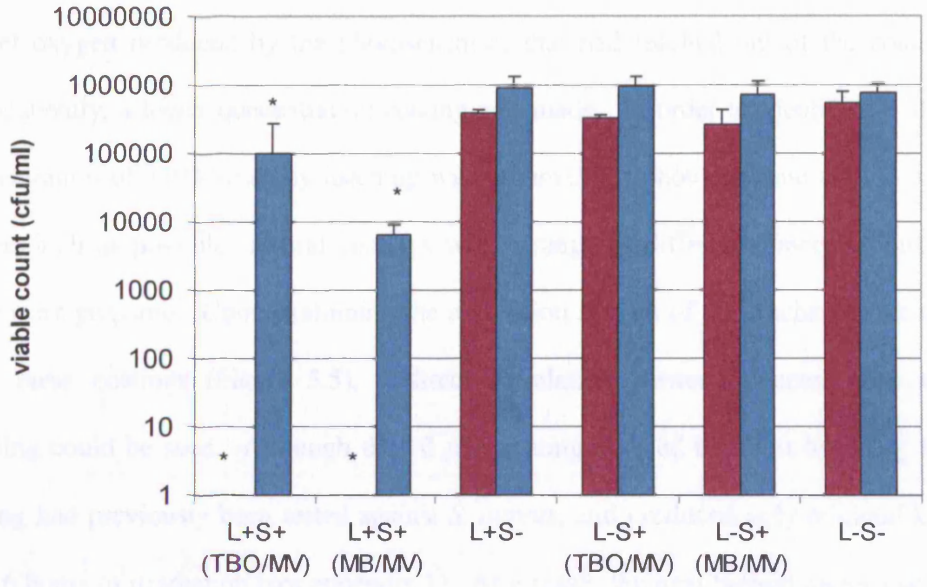


Figure 5.4 Viability of *S. aureus* (■) and *E. coli* (■) following exposure to light from a 28-W fluorescent lamp for 6 hours. Irradiated bacterial suspensions were in contact with cellulose acetate containing either 150μM TBO and 150μM MV (L+S+ (TBO/MV)) or 150μM MB and 150μM MV(L+S+ (MB/MV)). As a control, some were placed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing either TBO/MV or MB/MV (L-S+). Bars represent mean values of the viable counts and error bars represent standard deviations (n = 8). * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

5.3.4.2 Leaching experiments

Although both of the 150 μM coatings proved effective against *S. aureus* and *E. coli*, the bacterial suspensions placed on the TBO/MV coatings appeared slightly blue, indicating that leaching of the TBO out of the coating was occurring. This is not a problem from a practical point of view because as long as the coatings are active against a variety of microorganisms they will have an application within the hospital setting.

From a scientific standpoint, however, it was desirable to ascertain whether the antimicrobial effect was due to the singlet oxygen diffusing out of the coating or to singlet oxygen produced by the photosensitiser that had leached out of the coating. Consequently, a lower concentration coating was made. In order to identify the ideal concentration of TBO whereby leaching was minimal but photodynamic activity was still as high as possible, several coatings with a range of different concentrations of TBO were prepared. Upon examining the absorption spectra of the leachates obtained from these coatings (Figure 5.5), a direct correlation between concentration and leaching could be seen. Although the 10 μM coating showed the least leaching, this coating had previously been tested against *S. aureus*, and produced only minimal kills after 6 hours of irradiation (see appendix 1). As a result, the next highest concentration (25 μM) was chosen. With this 25 μM coating leaching was not observable with the naked eye, and appeared minimal when measured using the spectrophotometer.

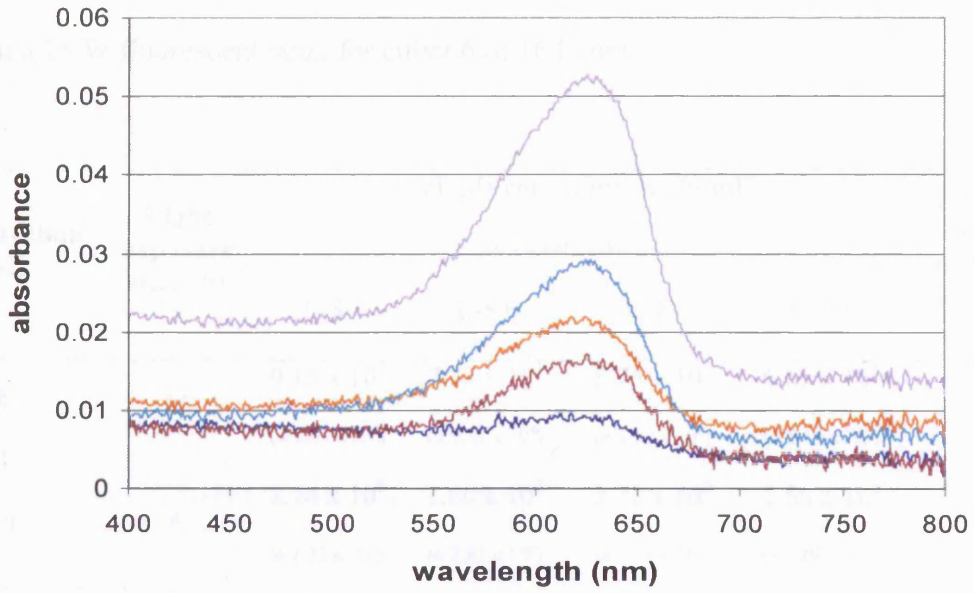


Figure 5.5 Spectra of TBO coatings with a range of different concentrations: 10 (—), 25 (—), 50 (—), 75 (—), and 100 (—) μM.

5.3.4.3 Activity of MV coatings

After selecting 25 μM as the ideal concentration for TBO, it was necessary to begin making and testing these coatings against a range of organisms. Before doing this, however, it was of interest to determine the antimicrobial effect of each photosensitiser individually so as to make sure that each compound was contributing to the activity of the coating. Interestingly, kill experiments with these coatings indicated that MV had only a low level of activity against *S. aureus* on its own within a coating (Table 5.2). The 25 μM MV coating achieved a 27.3 % reduction in the number of viable bacteria ($p = 0.11$). The 100 μM coating was similarly inactive after 6 hours of irradiation (26.2 %, $p = 0.069$) but was able to achieve a substantial kill of 72.9 % after 16 hours of illumination ($p = 0.021$).

Table 5.2. Effect on the viable count of suspensions of *S. aureus* when in contact with cellulose acetate containing either 25 or 100 μM MV when exposed to light from a 25 W fluorescent lamp for either 6 or 16 hours.

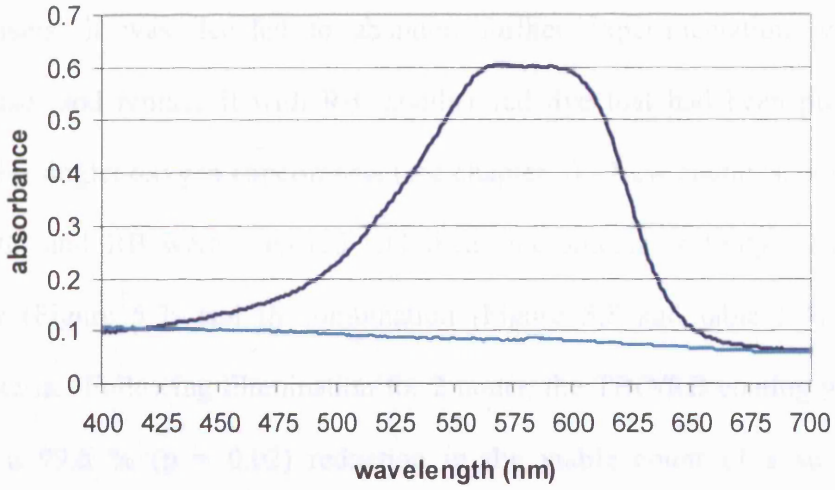
Concentration (μM)	Light exposure time (h)	Viable count: mean cfu/ml (± standard deviation)				% reduction in viable count (L+S+ versus L-S-)
		L-S-	L-S+	L+S-	L+S+	
25	16	9.15×10^5 ($\pm 2.54 \times 10^4$)	7.35×10^5 ($\pm 9.57 \times 10^4$)	8.75×10^5 ($\pm 1.34 \times 10^5$)	6.65×10^5 ($\pm 9.14 \times 10^4$)	27.3
100	6	2.14×10^6 ($\pm 2.23 \times 10^5$)	1.66×10^6 ($\pm 2.83 \times 10^5$)	1.78×10^6 ($\pm 1.18 \times 10^5$)	1.58×10^6 ($\pm 5.08 \times 10^5$)	26.2
100	16	8.4×10^5 ($\pm 1.66 \times 10^5$)	6.91×10^5 ($\pm 9.76 \times 10^4$)	7.16×10^5 ($\pm 4.21 \times 10^5$)	2.28×10^5 ($\pm 1.8 \times 10^4$)	72.9

To further investigate the activity of MV within the polymer, a $^1O_2$ experiment was performed on the 150 μM MV coatings. After 4 hours of irradiation, the amount of $^1O_2$ generated was similar to that produced by the 150 μM TBO/MV and MB/MV coatings, with an average change in A_{293} of 0.78 (Table 5.3). No leaching was observed but complete loss of colour from the coatings was noted during both the kill experiments and the $^1O_2$ assay. Subsequent spectroscopic analysis of MV coatings before and after irradiation confirmed this loss of colour (Figure 5.6a). Measurement of the absorption spectra of aqueous solutions of MV and polymer-embedded MV, demonstrated the effect that both the solvent and state can have on the light absorbing properties of MV (Figure 5.6b).

Table 5.3. Singlet oxygen production of 150 μM MV coatings following 4 hours of illumination.

Concentration (μM)	Light exposure time (h)	Average change in A_{293}	$^1O_2$ yield relative to 150 μM TBO/MV coatings	$^1O_2$ yield relative to 150 μM MB/MV coatings
150	4	0.78	0.89	0.82

(a)



(b)

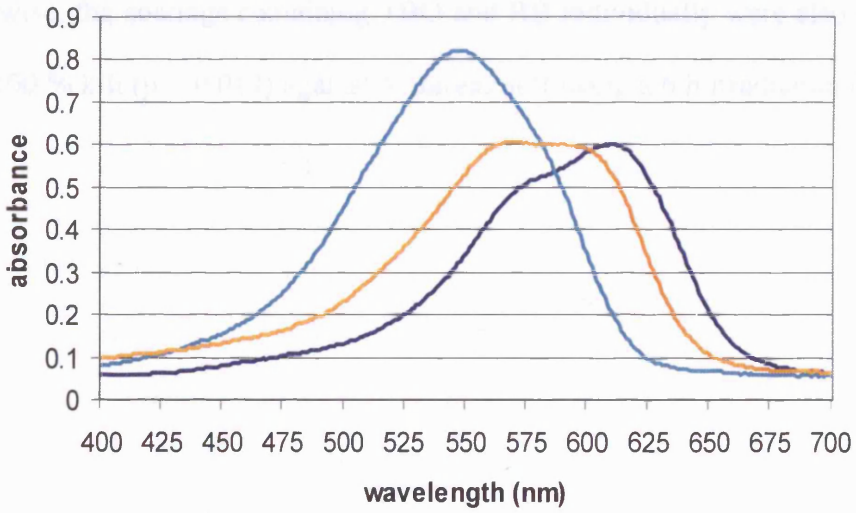


Figure 5.6 Absorption spectra of (a) 150 μM MV coatings before (—) and after (—) 16 hours of irradiation and (b) comparative absorption spectra of MV in acetone (— 20 μM), in dH2O (— 150 μM) and when incorporated into a cellulose acetate coating (— 150 μM).

5.3.4.4 Activity of coatings containing TBO, RB or both (TBO/RB)

Since MV appeared to be less active within the coating than the other two photosensitisers, it was decided to abandon further experimentation with this photosensitiser and replace it with RB, another red dye that had been previously studied in the singlet oxygen experiments (see chapter 3). New coatings, containing 25 μM TBO and RB were prepared and their microbicidal activity tested both individually (Figure 5.7) and in combination (Figure 5.8 and table 5.4) against various bacteria. Following illumination for 2 hours, the TBO/RB coating was able to achieve a 99.6 % ($p = 0.02$) reduction in the viable count of a suspension containing approximately 2×10^6 cfu/mL of *S. aureus* (Figure 5.8). Increasing the illumination time to 6 hours resulted in complete (100 %, $p = 0.014$) killing (Figure 5.8). Likewise, the coatings containing TBO and RB individually were also able to achieve a 100 % kill ($p = 0.014$) against *S. aureus* following a 6 h irradiation (Figure 5.7).

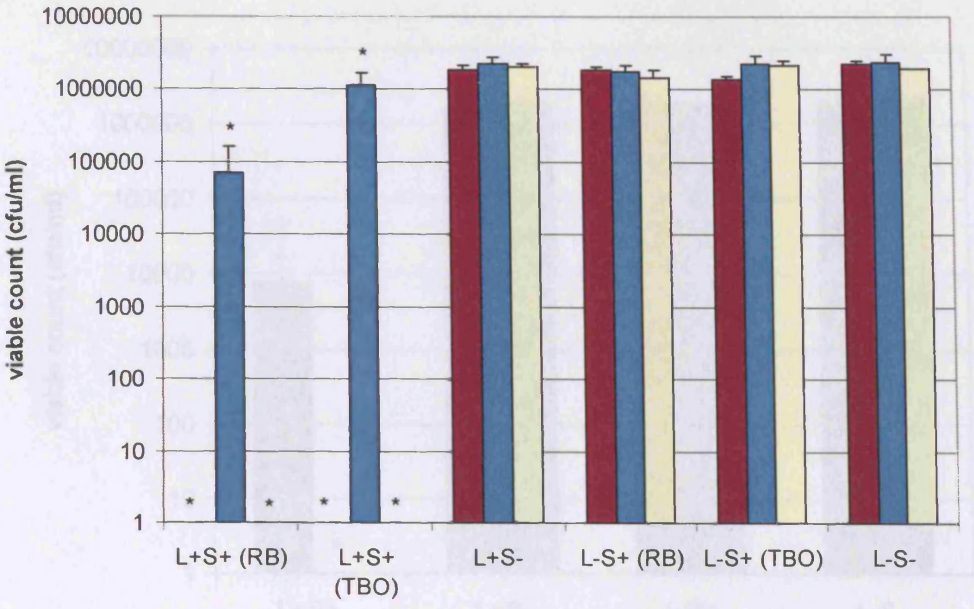


Figure 5.7 Viability of *S. aureus* (■) after 6 hours of irradiation and of *E. coli* after 6 (■) and 16 (□) hours of irradiation when in contact with cellulose acetate containing either 25μM TBO or 25μM RB (L+S+). As a control, some were placed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing either 25μM TBO or RB (L-S+). * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney U test).

A 100 % ($p = 0.014$) kill of a methicillin-resistant strain of the organism was also achieved after 6 h illumination (Table 5.4). Furthermore, *S. aureus* was susceptible to killing when suspended in human saliva – no viable cells remained in a suspension containing 4.1×10^6 cfu/mL ($p = 0.021$) following 16 h illumination (Table 5.4).

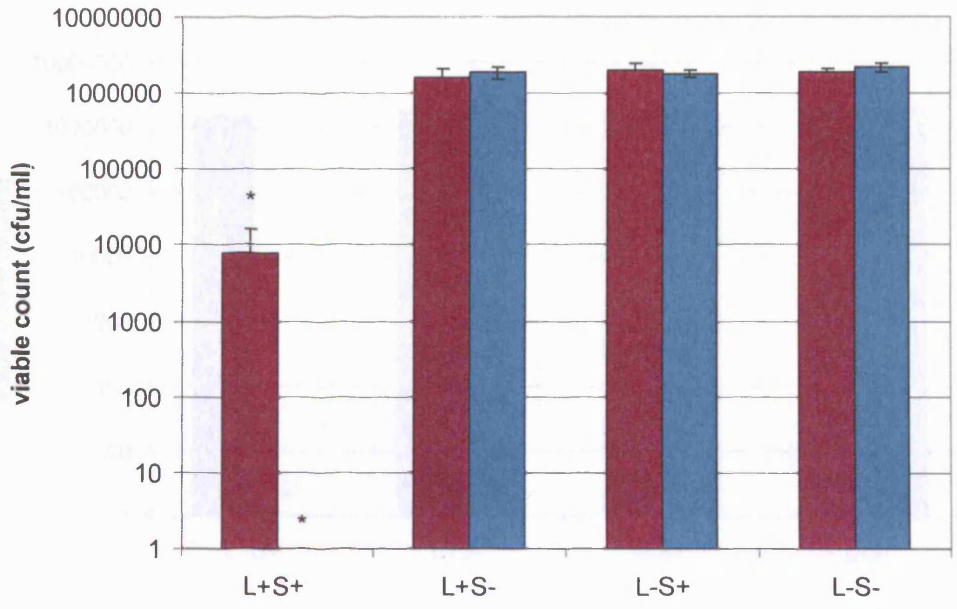


Figure 5.8 Viability of *S. aureus* NCTC 6571 after 2 (■) and 6 (■) hours of irradiation when in contact with cellulose acetate containing 25μM TBO and 25μM RB (L+S+). As a control, some were placed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing 25μM TBO and RB (L-S+). * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

For *E. coli*, all three coatings (TBO, RB and TBO/RB) achieved significant kills ($p = 0.02$) after 6 hours of irradiation with the RB coating being the most effective and obtaining a kill of 96.4 % (Figures 5.7 and 5.9). When the irradiation time was increased to 16 hours, the TBO/RB coating was able to kill 100 % of *E. coli* ($p = 0.014$).

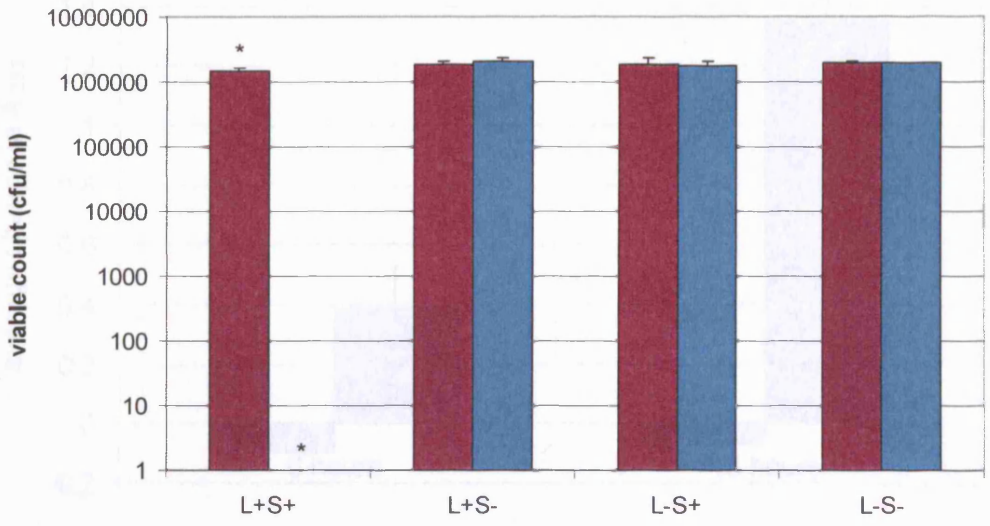


Figure 5.9 Viability of *E. coli* when in contact with cellulose acetate containing 25 μM TBO and 25 μM RB (L+S+) and irradiated for either 6 (■) or 16 (■) hours. As a control, some were placed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing 25μM TBO and RB (L-S+). * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney U test).

Singlet oxygen experiments performed on the 25 μM TBO/RB coatings revealed that a small amount of $^1O_2$ was generated after 6 hours of irradiation but that the yield after 16 hours of irradiation was 3.5 times higher (Figure 5.10).

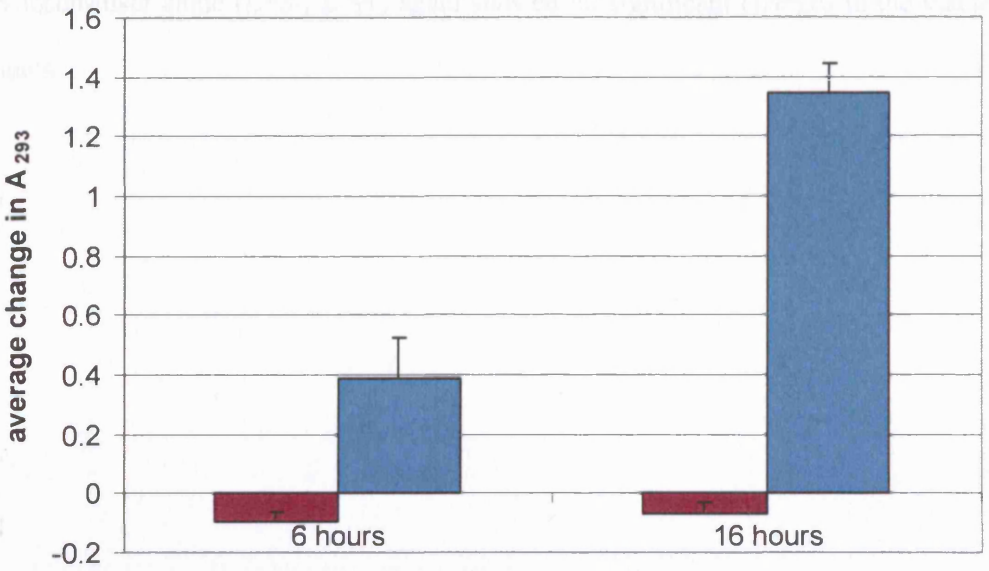


Figure 5.10 Singlet oxygen production by either control (photosensitiser-free) coatings (■) or 25 μM TBO/RB coatings (■) following irradiation with a compact fluorescent lamp for either 6 or 16 hours.

C. difficile also proved to be susceptible to killing by the illuminated coating – a 100 % kill ($p = 0.014$) of a suspension of the organism containing 5.19×10^6 cfu/mL being achieved after 4 h illumination (Table 5.4). In addition, the combination coating was also tested against the Gram-negative opportunistic pathogen *P. aeruginosa*, obtaining a small but statistically significant reduction (52.2 %, $p = 0.021$) in the number of viable bacteria (Table 5.4). Bacteriophage ΦX174 was susceptible to killing when the coatings were illuminated - a 91% reduction ($p = 0.043$) in the viable count of a suspension containing 1.34×10^6 pfu/mL being achieved after 16 h illumination (Table 5.4). Finally, the TBO/RB coating also exhibited activity against the yeast *C. albicans*, producing an appreciable kill of 88 % ($p = 0.021$). For all experiments, exposure of the suspensions to either light or

photosensitiser alone (L+S-, L-S+) again showed no significant changes in the viable counts.

Table 5.4. Viable count of suspensions of organisms when in contact with cellulose acetate containing 25 μM TBO and 25 μM RB and exposed to light from a 25 W fluorescent lamp for various periods of time. L-S-: microbial suspension in contact with photosensitiser-free coatings and not illuminated. L+S-: microbial suspension in contact with photosensitiser-free coatings and illuminated. L-S+; microbial suspension in contact with photosensitiser-containing coatings and not illuminated. L+S+; microbial suspension in contact with photosensitiser-containing coatings and illuminated. * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

Organism	**Light exposure time (h)**	**Viable count: mean cfu/ml** (± standard deviation)[1]				**% reduction in viable count (L+S+ versus L-S-)[2]**	**Log reductions in viable count (L+S+ versus L-S-)**
		L-S-	L-S+	L+S-	L+S+		
Methicillin-resistant *Staphylococcus aureus*	6	**2.69×10^6** ($\pm 4.58 \times 10^5$)	**2.81×10^6** ($\pm 3.19 \times 10^5$)	**3.02×10^6** ($\pm 5.55 \times 10^5$)	**< 5 *** ($\pm$ 0)	100	6.4
Clostridium difficile	4	**5.19×10^6** ($\pm 3.85 \times 10^5$)	**2.63×10^6** ($\pm 1.2 \times 10^6$)	**1.57×10^6** ($\pm 2.48 \times 10^5$)	**< 5 *** ($\pm$ 0)	100	6.7
Pseudomonas aeruginosa	16	**5.59×10^6** ($\pm 8.91 \times 10^5$)	**3.29×10^6** ($\pm 1.43 \times 10^6$)	**5.34×10^6** ($\pm 7.57 \times 10^5$)	**2.67×10^6 *** ($\pm 7.24 \times 10^5$)	52.2	0.3
Candida albicans	16	**1.99×10^5** ($\pm 1.51 \times 10^5$)	**2.18×10^5** ($\pm 1.78 \times 10^5$)	**2.33×10^5** ($\pm 1.48 \times 10^5$)	**2.39×10^4 *** ($\pm 1.01 \times 10^4$)	88	0.9
Bacteriophage ΦX174	16	**1.34×10^6** ($\pm 9.77 \times 10^4$)	**9.13×10^5** ($\pm 4.3 \times 10^5$)	**8.15×10^5** ($\pm 2.62 \times 10^5$)	**1.2×10^5 *** ($\pm 1.61 \times 10^5$)	91	1.1
S. aureus	6 (after 7 light/dark cycles)	**2.26×10^6** ($\pm 2.42 \times 10^6$)	**1.17×10^6** ($\pm 1.16 \times 10^6$)	**2.44×10^6** ($\pm 2.64 \times 10^6$)	**5 *** ($\pm$ 10)	100	5.6
S. aureus	6 (after 14 light/dark cycles)	**4.99×10^6** ($\pm 1.5 \times 10^6$)	**4.09×10^6** ($\pm 8.01 \times 10^5$)	**5.46×10^6** ($\pm 1.63 \times 10^6$)	**< 5 *** ($\pm$ 0)	100	6.7
S. aureus	6 (after 28 light/dark cycles)	**3.8×10^6** ($\pm 3.04 \times 10^5$)	**3.43×10^6** ($\pm 6.55 \times 10^5$)	**4.27×10^6** ($\pm 4.98 \times 10^5$)	**< 5 *** ($\pm$ 0)	100	6.6
S. aureus (in saliva)	16	**3.14×10^6** ($\pm 5.49 \times 10^5$)	**3.45×10^6** ($\pm 6.37 \times 10^5$)	**4.07×10^6** ($\pm 4.5 \times 10^5$)	**1.46×10^3 *** ($\pm 1.5 \times 10^3$)	100	3.3

[1] The minimum detection limit for each set of experiments was 5 cfu/mL.
[2] When the mean count of the L+S+ sample was below the detection limit, a 100% kill is recorded.

5.3.4.5 Lethal photosensitisation following prolonged irradiation of coatings

Exposure of the coatings to prolonged periods of irradiation (up to 28 days of alternating light and dark incubations) appeared to have no negative effect on either their singlet oxygen production (Figure 5.11) or their bactericidal activity (Table 5.4). In fact, for the coatings that underwent 14 and 28 cycles of light and dark incubation, singlet oxygen production was actually higher than for coatings that were simply subjected to one 6 hour irradiation. With respect to bactericidal activity, coatings that had been subjected to either 7, 14, or 28 light/dark cycles were still able to achieve 100 % reductions ($p = 0.018$, 0.014, and 0.014 for all 3 experiments respectively) in the viable counts of *S. aureus* (Table 5.4) following a further 6 hour illumination.

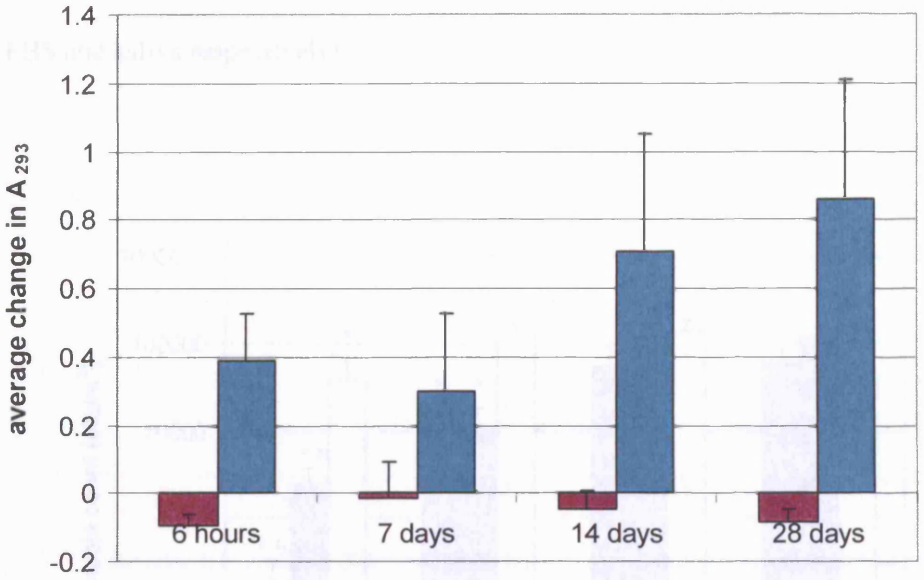


Figure 5.11 Singlet oxygen production by 25 μM TBO/RB coatings (■) and control coatings (■) following irradiation with a compact fluorescent lamp for either 6 hours without previous exposure or for 6 hours following 7, 14, or 28 days of prolonged light exposure (1 day = 1 cycle of 16 hours of light and 8 hours of darkness).

5.3.5 Nebuliser experiments

When a suspension of *S. aureus* in PBS was sprayed onto the 25 μM TBO/RB cellulose acetate coatings using a nebuliser, near complete kills (99.7 %, p = 0.001) were again achieved after 6 hours of illumination (Figure 5.12). *S. aureus* remained susceptible when suspended in whole human saliva and horse serum with significant kills of 98 % (p = 0.004) and 76.9 % (p = 0.004) being obtained respectively. For the experiments performed in PBS and saliva, significant kills (p = 0.002 and p = 0.004 for PBS and saliva respectively) were also observed upon exposure to light on photosensitiser-free coatings (L+S-). In each case, the kills achieved on the TBO/RB (L+S+) coatings were

significant when compared to those achieved with light alone ($p = 0.001$ and $p = 0.004$ for PBS and saliva respectively).

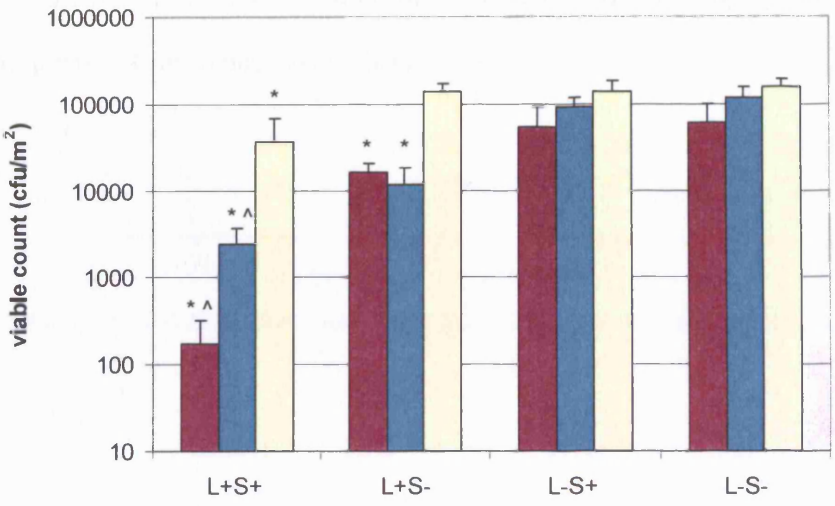


Figure 5.12 Comparative viability of *S. aureus* in PBS (■), saliva (■), or horse serum (□) when sprayed onto 25 μM TBO/RB coatings using a nebuliser and irradiated for 6 hours (L+S+). As a control, some were sprayed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing 25μM TBO and RB (L-S+). * denotes that the viable count was significantly different from that of the L-S- suspension and ^ indicates that the viable count was significantly different from that of the L+S- suspension (Mann-Whitney *U* test).

The nebuliser experiment was also carried out using a suspension of *P. aeruginosa* in PBS (Figure 5.13). In contrast to the *S. aureus* experiments however, the irradiation was only for 1 hour since *P. aeruginosa* did not appear to be viable when sprayed onto

control cellulose acetate coatings and left for longer periods (data not shown). The 1 hour irradiation proved sufficient to kill 97.5 % ($p = 0.004$) of the bacteria remaining on the coating during this period. Again, as for the experiments with *S. aureus*, significant kills ($p = 0.01$) were also observed upon exposure to light on photosensitiser-free coatings (L+S-) but the kills achieved on the TBO/RB (L+S+) coatings proved to be significant ($p = 0.004$) in comparison to these.

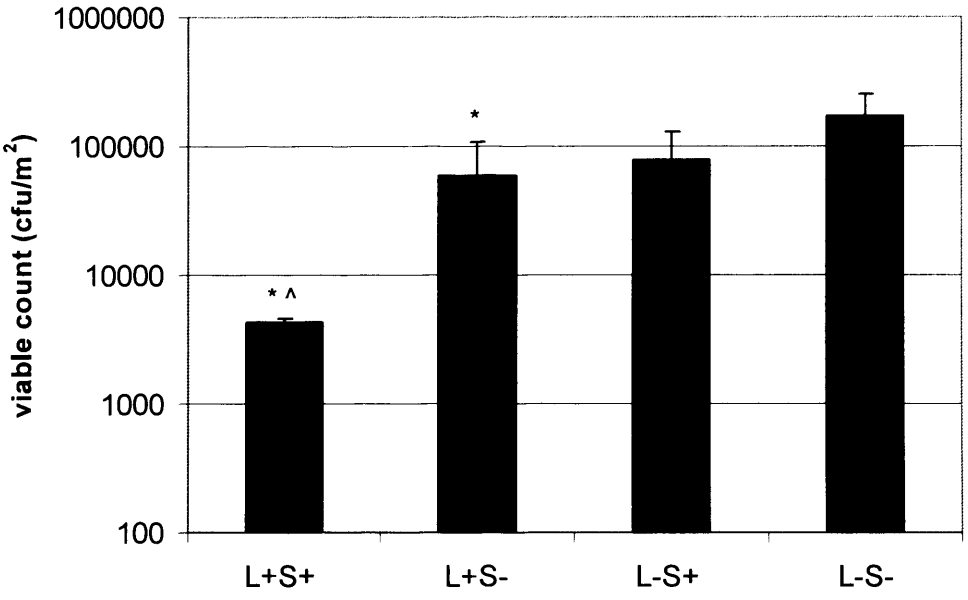


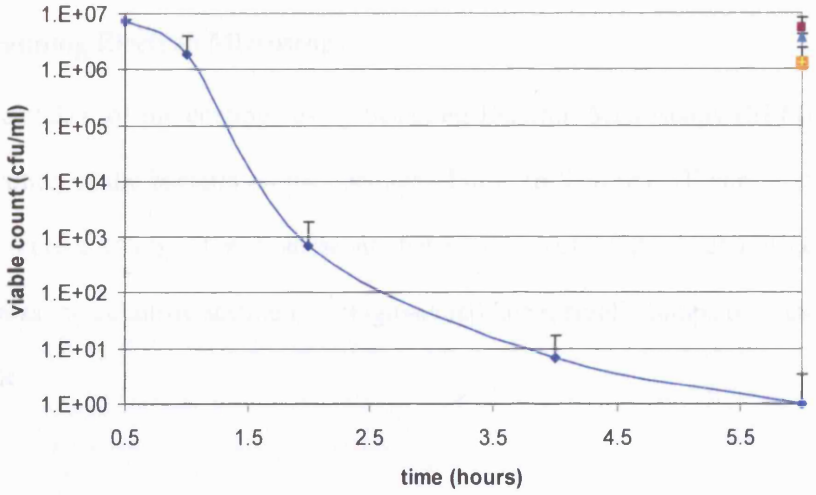
Figure 5.13 Viability of *P. aeruginosa* in PBS when sprayed onto 25 μM TBO/RB coatings using a nebuliser and irradiated for 6 hours. As a control, some were sprayed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing 25μM TBO and RB (L-S+). * denotes that the viable count was significantly different from that of the L-S- suspension and ^ indicates that the viable count was significantly different from that of the L+S- suspension (Mann-Whitney *U* test).

5.3.6 Time-course experiments with *S. aureus*

In order to investigate when most of the killing takes place, a time-course experiment was carried out using *S. aureus* (Figure 5.14a). Although no viable counts were performed at time 0 (due to limitations on the maximum volume - 500 μL - that could be aliquoted on to the coatings), it is clear from looking at the controls that little or no killing occurred within the first 30 minutes of irradiation. During the following 30 minutes of illumination however, a significant 73.5 % ($p = 0.004$) reduction in the viable

count was observed. Of the bacteria remaining viable at this second time point, 99.96 % ($p = 0.004$) were subsequently killed during the second hour of irradiation, thus giving a total kill of 99.99 % ($p = 0.004$). A second detailed time-course experiment was carried out to look at the killing during this second hour of light exposure (Figure 5.14b). The results showed that of the viable bacteria remaining after the initial hour of light exposure, 87.8 % ($p = 0.01$), 96.8 % ($p = 0.006$), and 99.8 % ($p = 0.004$) were killed after a further 15, 30, and 45 minutes of irradiation respectively. By the end of the second hour, near complete (99.9996 %, $p = 0.003$) kills were achieved.

(a)



(b)

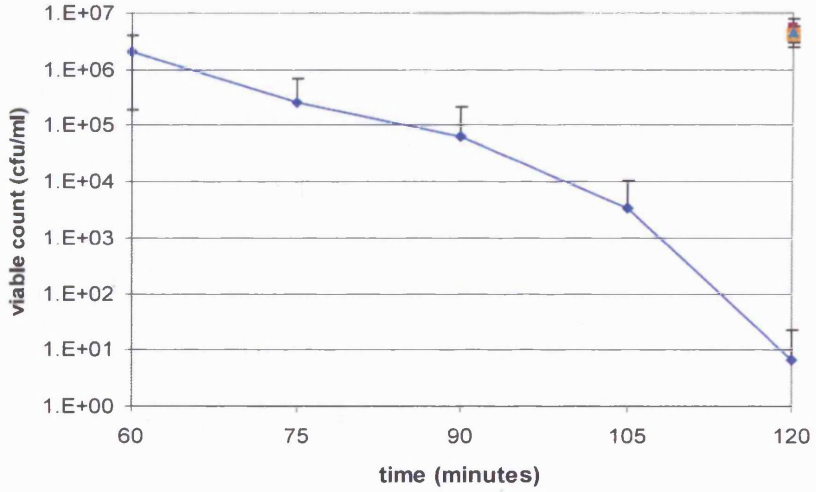


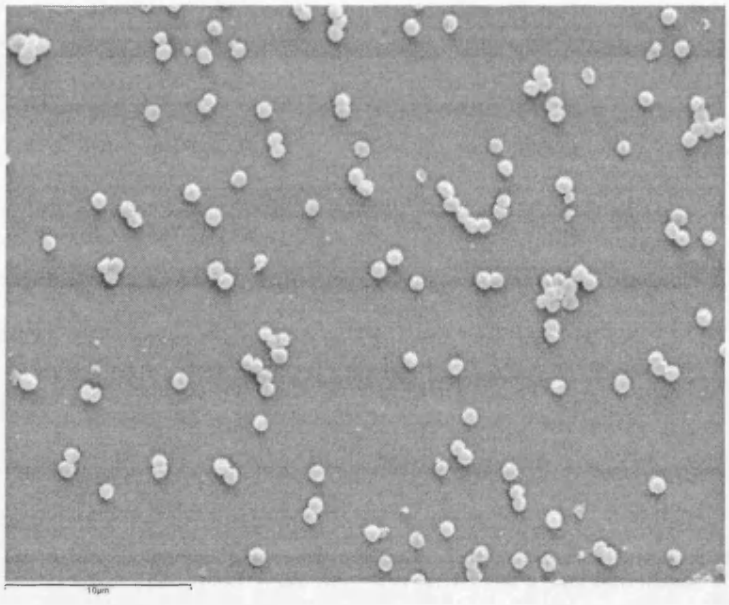
Figure 5.14 Time course experiments showing the rate at which *S. aureus* is killed (a) during the course of a 6 hour irradiation and (b) during the second hour of a 6 hour irradiation. Test suspensions (L+S+ ◆) were in contact with cellulose acetate containing 25 μM TBO and 25 μM RB. As a control, some were placed on photosensitiser-free cellulose acetate and kept in the dark (L-S- ▲). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S- ■) and bacteria kept in the dark that were in contact with cellulose acetate containing 25μM TBO and RB (L-S+ ✚).

5.3.7 Electron Microscopy of samples

5.3.7.1 Scanning Electron Microscopy

The images taken of the coatings using Scanning Electron Microscopy (SEM) showed the distribution of the bacteria on the coatings. For both *S. aureus* (Figure 5.15a,b) and *E. coli* (Figure 5.16a,b), it was apparent that while most of the bacterial cells were spread across the cellulose acetate in a single-celled layer, small clumps of bacteria were also visible.

(a)



(b)

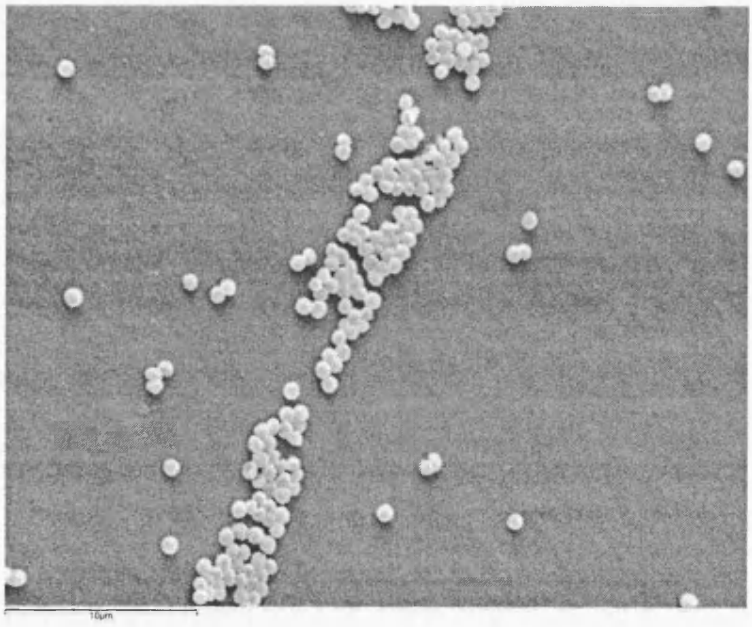
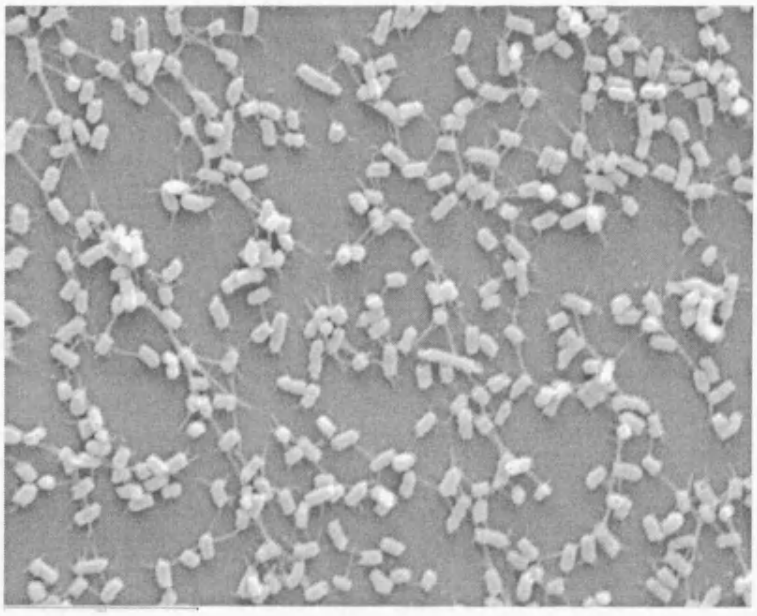


Figure 5.15 SEM images of *S. aureus* on TBO/RB coatings illustrate that the bacteria adhere to the coating both individually (a) and in clumps (b).

(a)



(b)

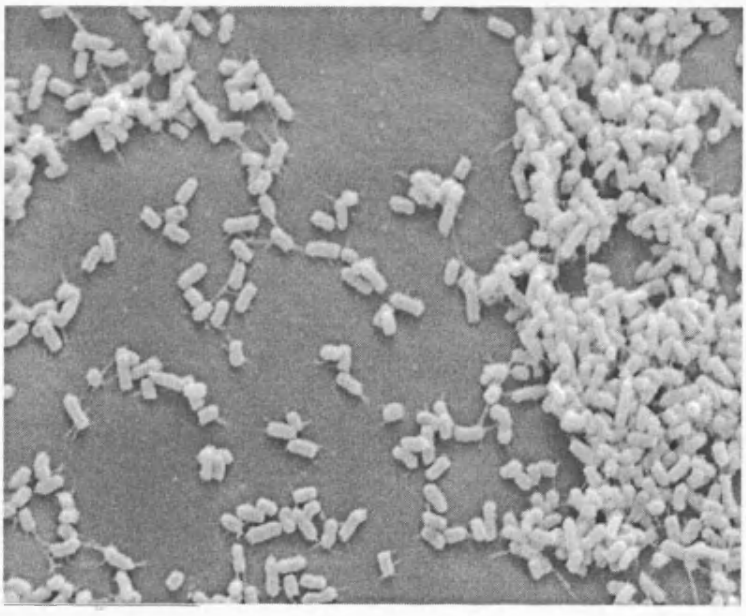


Figure 5.16 SEM images of *E. coli* on TBO/RB coatings illustrate that the bacteria adhere to the coating both individually (a) and in clumps (b).

5.3.7.2 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) revealed the extent of the damage done to bacterial cells following photodynamic treatment. For both *S. aureus* (Figure 5.17) and *E. coli* (Figure 5.18), the cells in the control samples (L+S-, L-S+ and L-S-) appeared robust and healthy. In contrast, bacterial cells that had been exposed to the TBO/RB coatings in the presence of light appeared to have undergone extensive vacuolation.

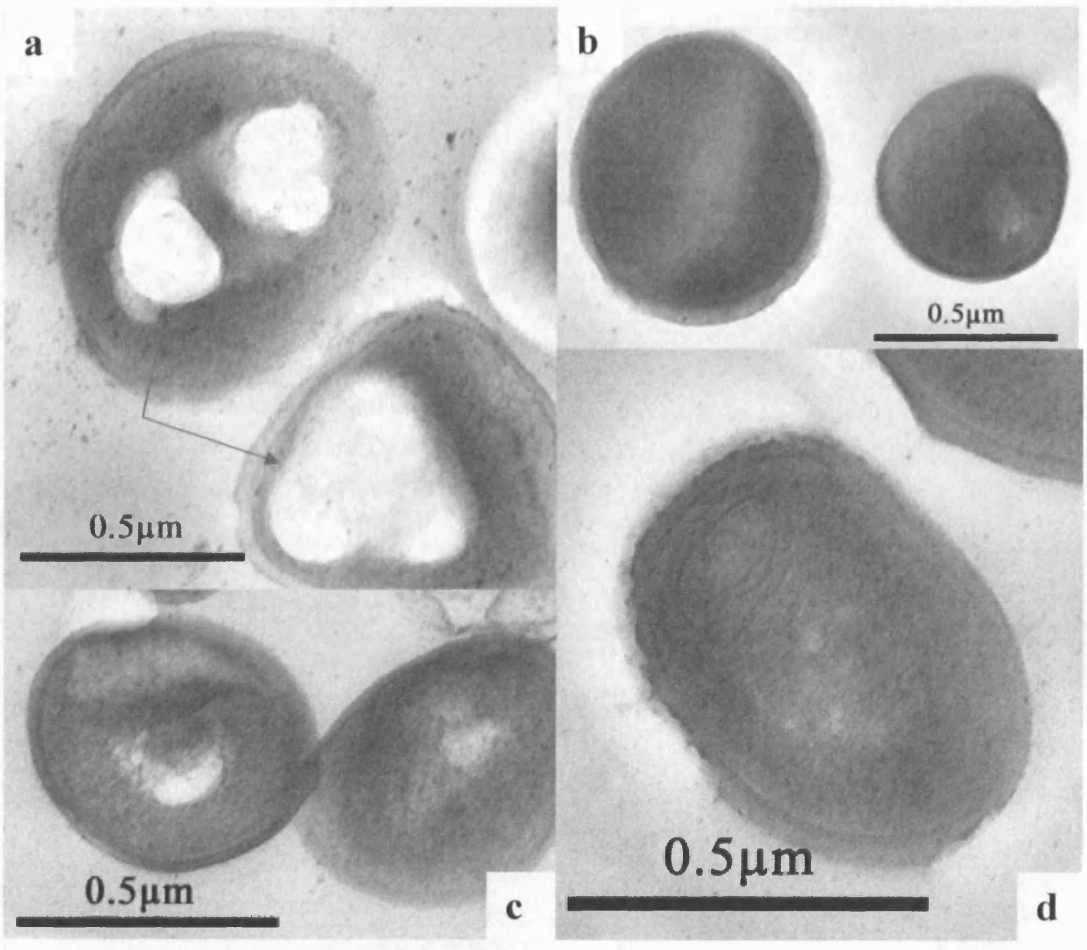


Figure 5.17 TEM images of *S. aureus* samples following different treatments. (a) L+S+: bacteria placed on TBO/RB coatings and irradiated. (b) L+S-: bacteria placed on control coatings and irradiated. (c) L-S+: bacteria placed on TBO/RB coatings and kept in the dark. (d) L-S-: bacteria placed on control coatings and kept in the dark. Red arrows indicate vacuolation.

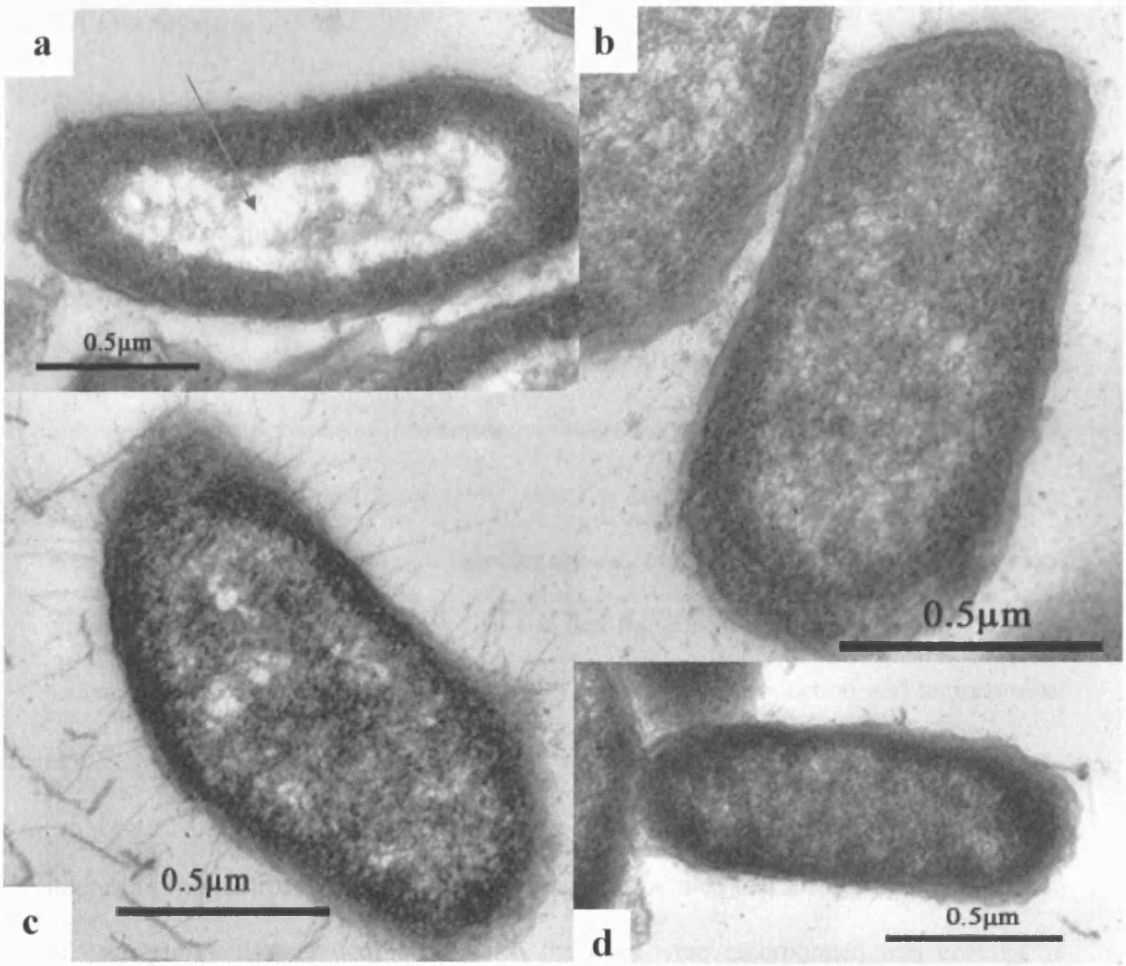


Figure 5.18 TEM images of *E. coli* samples following different treatments. (a) L+S+: bacteria placed on TBO/RB coatings and irradiated. (b) L+S-: bacteria placed on control coatings and irradiated. (c) L-S+: bacteria placed on TBO/RB coatings and kept in the dark. (d) L-S-: bacteria placed on control coatings and kept in the dark. Red arrow indicates vacuolation.

5.4 Discussion

The broad-spectrum antimicrobial activity of the phenothiazines in aqueous suspensions is well documented in the literature and the results described in the previous chapter supported these findings by demonstrating the successful killing of a range of different organisms by all three of the phenothiazine dyes tested (TBO, MB, and MV). The activity of the photosensitisers in solution, however, may not reflect their activity when immobilized in a polymer. Since research into the activity of light-activated antimicrobial materials is limited (Bonnett *et al.*, 1993; Bozja *et al.*, 2003; Wilson 2003, Wainwright *et al.*, 2006), little information exists regarding the behaviour and activity of these dyes when embedded in a polymer. It was to this end that the photosensitiser-containing cellulose acetate coatings were extensively tested both for their $^1O_2$ production and antimicrobial activity.

Interestingly, spectroscopic analysis of the coatings revealed that the absorption maximum of each photosensitiser was lower when the dyes were incorporated into coatings in combination as opposed to individually. Such alterations in absorption may result from interactions between the compounds. For example, each agent may affect the solubility of the other, thereby reducing the overall absorption.

The coatings were formed following evaporation of the solvent (acetone), and it is assumed that each photosensitiser molecule is embedded in the cellulose acetate layer, thereby trapping it but allowing the $^1O_2$ molecules generated on exposure to light to diffuse out and thus interact with, and kill, any microbes associated with the surface of the coating. The overall antimicrobial activity of a photosensitiser is dependent upon the quantum yield of

cytotoxic species produced (i.e. the yield per absorbed photon), as well as the type of cytotoxic species produced and their relative quantities, and this was not expected to be altered when the compound was incorporated into a coating. In reality, however, this proved not to be the case for MV, which was able to successfully kill microorganisms in solution (see chapter 4) but was markedly less effective than either TBO and MB when incorporated into a coating (see table 5.2). The initial hypothesis formulated to explain these findings was that the interaction of MV with the cellulose acetate or the solvent acetone somehow quenched or altered its $^1O_2$ -generating ability. However, when a uric acid assay was performed on the 150 μM MV coatings, the results revealed that the levels of $^1O_2$ were nearly as high as those generated by the TBO/MV and MB/MV coatings which achieved complete killing after 6 hours of irradiation. Consequently, reduced $^1O_2$ production can not explain the reduced antimicrobial activity of the MV coatings. Instead, the discrepancy between $^1O_2$ production and killing can probably be attributed to the previously described phenomenon of "photobleaching" (see chapter 3), whereby the photosensitiser is itself degraded by the $^1O_2$ generated. When it occurs under controlled conditions, photobleaching has been shown to have potential benefits in certain applications such as tumour photosensitisation. Researchers have demonstrated that by using a low dose of the photosensitiser Photofrin® in combination with a high dose of light, it is possible to achieve high levels of tumour destruction while at the same time limiting damage to normal tissues (Mang *et al.*, 1997). Likewise, it has been suggested that photobleaching could be exploited to help reduce skin photosensitivity - the major side-effect of PDT treatment using Photofrin® (Bonnett *et al.*, 2001). However, as is the case in this study, photobleaching can also be disadvantageous because it limits the activity of the photosensitiser. This is because not only are the active species being used up before they

can reach their target (i.e. microbial or tumour cells), but the source of active species is also rapidly destroyed, thus further reducing the activity and lifetime of the coating.

As described in chapter 3, photobleaching is manifested in the loss of absorption or emission intensity (Verhoeven, 1996) and can occur via two irreversible processes: photomodification and true photobleaching. Again, as with the xanthene dyes studied in chapter 3, the MV coatings appeared essentially colourless after being exposed to 4 hours of irradiation during the uric acid assay thus indicating that true photobleaching was taking place. Interestingly, as illustrated by the photobleaching experiment carried out in chapter 3, true photobleaching of MV does not seem to occur in solution. The reasons for this are not clear but are probably related to the different properties associated with the photosensitiser molecules being matrix-bound as opposed to free in solution. As discussed, the coatings are formed following the evaporation of acetone which leaves behind a thin photosensitiser-embedded film. The photosensitiser molecules will therefore be in a more dense configuration within the polymer. This close proximity of photosensitiser molecules increases the likelihood that the $^1O_2$ molecules generated will interact with a neighbouring photosensitiser molecule rather than being quenched by other molecules. This will therefore speed up the photobleaching process. The larger exposed surface area of the coatings, as well as the relative transparency of the overlaid uric acid/PBS solution, also means that more light is able to reach more photosensitiser molecules. This is in contrast to the situation in aqueous solution where reduced light penetration establishes a photosensitiser gradient. Thus, increasingly fewer molecules towards the bottom of a solution will be activated, with the end result being that there is less $^1O_2$ produced in the solution overall. Finally, the fact that MV is much more soluble in acetone than in water

may also have had an effect because solubility inevitably affects the absorption properties of the photosensitiser. When completely dissolved, not only does MV have a higher absorption coefficient but there is also a shift in its absorption spectrum so that it is absorbing more high-energy light towards the blue end of the spectrum (Figure 5.6b). All of these different factors may help to explain the differences in the photobleaching kinetics between the MV solution and the MV cellulose acetate coatings.

Following the discovery that MV was not contributing to the kills achieved using the TBO/MV and MB/MV coatings, another blue-light absorbing photosensitiser was needed in order to harvest light in the blue region that was being emitted by the lamp. In addition to the dyes screened in chapter 3, additional dyes were screened at this stage (see appendix 2) but while some did not produce any $^1O_2$ (ponceau S, phenol red, acid alizarin, acid fuchsin, acid red 1 and temoporfin), others could not sustain their $^1O_2$ production following cycles of light and dark incubation (Eosin Y and Safranin O). Consequently it was decided that, despite evidence of some photobleaching (see chapter 3), RB was the best choice for incorporation into the coating alongside TBO. Together these dyes are able to absorb strongly at many of the prominent wavelengths (545, 588 and 610 nm) emitted by fluorescent lamps of the type commonly used in hospitals in the United Kingdom.

As expected, the irradiation times necessary to achieve significant kills were much longer than those used in experiments performed in aqueous solutions. In suspension, significant kills were achieved for all photosensitisers following only 2 hours of irradiation for bacteria and after 6 hours for the yeast and virus. In contrast, much longer light exposure times of 6 hours for *S. aureus* and 16 hours for all of the other organisms were needed to obtain

appreciable kills. The same trend was apparent in the singlet oxygen data, where a 1 hour irradiation of photosensitisers in solution was able to generate between 1.6 and 3.5 times more singlet oxygen than a 4 hour irradiation of photosensitiser-containing coatings at the same concentration (10 μM). Wainwright *et al.*, (2006) reported a similar discrepancy between the $^1O_2$ production of a 10 μM ethanolic solution of new methylene blue (NMB) and that of matrix-bound NMB. In their study however, much larger differences were observed, with the NMB solution producing between 15 and 22.5 times more $^1O_2$ than the styrene and acrylate polymer-NMB matrices respectively. It is thought that such greatly reduced levels of $^1O_2$ generation may be explained by the heterogeneous nature of the singlet oxygen reaction when a photosensitiser is embedded in a polymer as compared with the rapid solution kinetics associated with dissolved photosensitiser molecules (Wainwright *et al.*, 2006). Furthermore, the increased potential for relaxation via interaction with the surrounding matrix may cause a reduced population of the excited singlet state of the immobilised photosensitiser (Wainwright *et al.*, 2006).

While considerable reductions in the viable counts of the Gram-positive bacterium *S. aureus* (including a methicillin-resistant strain of the organism) and *C. difficile* were achieved when the coatings were illuminated with light from the fluorescent lamp for periods of 6 h or less, much longer irradiation periods of 16 h were needed to obtain similar kills for Gram-negative bacteria, a yeast and a virus. These results directly mirror those described in chapter 4, where it was previously explained that increased size and/or structural complexity of these organisms is responsible for their decreased susceptibility. While there are no studies describing the activity of photosensitiser-embedded polymers against viruses and yeasts, there are several reports comparing the activity of

photobactericidal materials against both Gram-positive and Gram-negative bacteria. Bozja *et al.* (2003) reported that nylon fibres to which porphyrins had been attached were more effective at killing *S. aureus* than *E. coli* when illuminated with white light. Wainwright and co-workers (2006) also showed the same trend, reporting increased susceptibility of *S. epidermidis* when compared with *E. coli*. In contrast, Wilson (2003) reported that a cellulose acetate film containing TBO had greater efficacy against *P. aeruginosa* than against MRSA-16.

As seen with the MV coatings, a common problem associated with the use of light-activated compounds is photobleaching which can either occur immediately, as with the MV coatings, or over a longer time via photomodification. However, when the photosensitiser-containing coating was exposed to 7, 14 or even 28 cycles of alternating light and dark periods (16 hours of light and 8 hours in the dark), no reduction in its bactericidal activity was detectable. These findings suggest that photobleaching, at least in the short term, would not be a problem. If longer periods of light/dark, or continuous light exposure did result in photobleaching then it would be necessary to renew the coating on a regular basis – perhaps by spraying with a solution of the coating in a volatile solvent.

It was encouraging to find that substantial kills of *S. aureus* were also achieved when the organism was suspended in saliva rather than phosphate buffered saline as aerosols and droplets derived from oral and respiratory secretions are an important mode of transmission of infectious agents in a clinical setting (Sehulster & Chinn, 2003). Furthermore, other authors have repeatedly reported that the presence of organic matter such as blood, serum

or saliva can interfere with lethal photosensitisation and lead to reduced kills as compared with experiments carried out in PBS or saline (Nitzan *et al.*, 1989; Wilson *et al.*, 1993; Wilson & Pratten, 1995; Bhatti *et al.*, 1997; Kömerik & Wilson, 2002). This diminished killing efficacy can be best explained by the high protein content of these fluids since proteins can affect light-mediated killing in a number of ways. Firstly, proteins can absorb light which reduces the number of photons available to activate the photosensitiser molecules and thereby leads to a reduction in the yield of cytotoxic species (Wilson & Pratten, 1995). Secondly, proteins can directly compete with microorganisms for the photosensitiser molecules, thereby decreasing the number available for binding to the target microbes. Furthermore, the extremely short lifespan and short diffusion distance of $^1O_2$ necessitates close proximity to cells to produce a cytotoxic effect, the proteins may simply act as physical barrier shielding the microbial cells from the $^1O_2$. Lastly, proteins can also act as $^1O_2$ quenchers, scavenging the $^1O_2$ from the solution and thus conferring protection for the microorganisms.

As revealed by the nebuliser experiments, horse serum has a greater inhibitory effect than saliva in terms of the kills achieved. In a study investigating the effects of serum and saliva on the lethal photosensitisation of *E. coli*, *P. aeruginosa*, and *Klebsiella pneumoniae* using TBO, Kömerik and Wilson (2002) reported similar findings with serum having a greater protective effect than saliva for all three bacteria. The reason for this is most probably the higher protein content of horse serum (5 – 9.5 g 100 mL^{-1}) compared with that of saliva (0.15 – 0.25 g 100 mL^{-1}) (Cole & Eastoe, 1988). In fact, Nitzan *et al.* (1998) demonstrated that an inverse relationship existed between protein content and light-mediated killing by

studying the effect that increased protein content in the suspension medium had on the lethal photosensitisation of *Acinetobacter baumannii* using a cationic porphyrin.

The nebuliser experiments were particularly significant because they showed that the coatings were effective even when bacteria were sprayed onto the surface; a situation which more closely mimics the settling out of bacteria from aerosols. While this has not been tested previously for light-activated antimicrobial materials, several studies have examined the effectiveness of other antimicrobial surfaces using aerosol challenge. For example, investigators studying the activity of glass surfaces modified with poly-(4-vinyl-N-hexylpyridinium bromide) (hexyl-PVP) used a commercial chromatography sprayer to deposit bacteria on the surface of the glass (Tiller *et al.*, 2001). These authors found that the hexyl-PVP modified glass could achieve > 99 % kills against *S. epidermidis*, *P. aeruginosa*, and *E. coli* but was less successful at killing *S. aureus* (94 % kill) following a 30 minute contact time. Likewise, polyurethane surfaces modified with alkylammonium side chains achieved 3.6 and 4.3 log reductions in the numbers of *S. aureus* and *P. aeruginosa* respectively following a 30 minute contact time (Kurt *et al.*, 2007). Neither of these studies evaluated the effect that the presence of organic matter might have on the antimicrobial activity of such coatings. Moreover, the activity of these polymers against viruses and yeasts has not been not described using either a sprayed suspension or an aqueous suspension.

In addition to the polymers described above, various other types of antimicrobial films have been developed and tested using aqueous suspensions of bacteria deposited on the surface. One substance which has been extensively studied as an antimicrobial coating

is titanium dioxide (TiO_2). Like the TBO/RB coatings, TiO_2 is also light-activated, although ultraviolet light is needed rather than the white light used in the case of the TBO/RB coatings. Killing by TiO_2 coatings results from the production of reactive oxygen species - predominantly hydroxyl radicals. Kühn *et al.* (2003) reported the antimicrobial activity of TiO_2-coated Plexiglas® against a range of organisms following 1 hour of UVA irradiation, with 3.9 and 6.3 $\log_{10}$ kills obtained for *S. aureus* and *E. coli* respectively. Page *et al.* (2007) demonstrated that silver-doped TiO_2 coatings could achieve 99.997 % kills for *S. aureus* but only managed a 69 % reduction in the number of *E. coli* following 6 hours of UV irradiation. However, the need to use UV light to activate such coatings would limit their use in hospitals.

Finally there are those coatings which rely on the release of biocides from the material in order to mediate microbial killing. One such coating uses the organic disinfectant dodecyl-di(aminoethyl)-glycine (TEGO 51®) immobilised in a silicon oxide matrix (Copella *et al.*, 2006). When the concentration of TEGO 51® is 1 %, this material is able to kill > 99 % of *E. coli* and *P. aeruginosa* of an initial inoculum of 10^5 cfu/mL after 24 hours but interestingly a higher concentration of 1.5 % is needed to achieve comparable kills of *S. aureus*. When considering the use of this type of coating it should be remembered that release of biocides may rapidly foster resistance and also (in the case of the disinfectant) may have adverse health implications (skin irritation etc.). In addition to the conventional disinfectants and antimicrobial agents, metal ions such as copper, zinc or silver ions are also known to be biocidal and consequently surfaces made of, or plated with, these metals can be self-disinfecting. For example, Noyce *et al.* (2006) showed that pure copper surfaces were able to kill 10^7 MRSA, EMRSA-1, and EMRSA-

16 after 45, 60, and 90 minutes respectively. Unfortunately, the effect of the presence of proteins on the antimicrobial activity of these surfaces was not investigated. Many proteins are able to interact with metal atoms and ions and neutralise their antimicrobial effects.

The kinetics of photodynamic killing using white light have not been well studied and may help to explain the variable results achieved in different investigations. In their study of protoporphyrin-based antimicrobial nylon fibers, Bozja *et al.* (2003) showed a direct correlation between light intensity and kill rate. While they found that a 30 minute exposure to an incandescent lamp emitting light at an intensity of 10,000 lux only achieved a 45 % kill, a 95 % kill reduction in the number of viable *S. aureus* was observed after a 30 minute irradiation at 60,000 lux. In contrast, the time-course study carried out in the present study using the TBO/RB coatings revealed that 99.99 % of viable *S. aureus* were killed after 2 hours of illumination with a fluorescent lamp emitting light at an intensity of only 3,700 lux. Despite differences in the irradiation times and light intensities used, a 30 minute irradiation at 60,000 lux should be comparable to a 2 hour irradiation at 3,700 lux. Thus, at first glance, it appears that the TBO/RB coatings are more effective against *S. aureus* than the porphyrin-grafted fibers. However, as the time-course experiment shows, there is no significant reduction in the viable counts during the first 30 minutes of irradiation on the TBO/RB coatings so it is possible that a similar effect is seen with other photosensitisers and that if the irradiation time was increased, more appreciable kills could be achieved.

Transmission electron microscopy (TEM) allows analysis of the bacterial cell structure following photodynamic treatment, thus giving some insight into the potential targets of $^1O_2$. As discussed in chapter 1, there is still considerable debate concerning the primary target of lethal photosensitisation. While this project has not dealt with this question in any detail, the implication from the TEM images obtained is that the majority of damage in bacteria is taking place inside the cell. For both *S. aureus* and *E. coli*, differences in the cytoplasm structure between treated and non-treated bacteria were apparent, with treated bacteria displaying large vacuoles in their cytoplasms. It is likely that the initial $^1O_2$-mediated disruption of the membrane leads to an influx and efflux of ions and subsequent disorganisation of the cytoplasm. However, owing to the magnification used, specific alterations to the cell membrane could not be identified. Other authors have reported photosensitiser-mediated structural changes for *S. aureus* (Nitzan *et al.*, 1983), *Porphyromonas gingivalis* (Bhatti *et al.*, 2002), *E. coli* (Nitzan *et al.*, 1992; Nitzan & Ashkenzi, 2001) and *P. aeruginosa* (Nitzan *et al.*, 1992) using electron microscopy. Since the photosensitisers are embedded in the cellulose acetate and can therefore neither bind to, nor enter, the bacterial cells, any visible damage must be caused by extracellularly produced $^1O_2$ and other free radicals. Bearing in mind the short diffusion distance of $^1O_2$, this lends further support to the hypothesis that $^1O_2$ largely targets the cell membrane in the first instance and that this damage leads to subsequent intracellular structural changes.

The data presented here demonstrate the feasibility of using a coating containing photosensitisers to photoinactivate microbes. The TBO/RB coating was not only an efficient producer of $^1O_2$ but was also successful at killing a range of different microorganisms when activated using light from a fluorescent lamp. Moreover, the levels

of killing achieved (up to a 6 $\log_{10}$ reduction) should be more than sufficient for surface disinfection as microbial densities encountered on hospital surfaces are much lower. One study for example, showed that between 4 and 7 cfu/cm^2 of *S. aureus* were present on the surfaces of rooms occupied by patients infected with the organism (Rutala *et al.*, 1983).

As discussed in chapter 3, the regulations governing lighting in healthcare buildings in the UK specify minimum light levels for all areas within hospitals. In operating theatres, for example, the recommended light intensity is 50,000 lux, while for Accident and Emergency resuscitation and major treatment rooms a light intensity of 15,000 lux is advised (CIBSE Lighting Guide LG2, 1989). In GP examination rooms and general wards lower light intensities of 1,000 and 200 lux respectively are recommended. The CIBSE guidelines also specify lighting levels for dental clinics and stipulate that the light intensity at work surfaces should be 300 – 500 lux. As the light intensity used in this study was 3,700 ± 20 lux, the light-activated coating described and tested here would be of particular use in examination rooms and operating theatres where light intensities are highest. However, it is also possible that these coatings could achieve appreciable kills under lower light intensities if the illumination time was increased to produce higher light energy doses e.g. by leaving lights on for 24 h per day. Alternatively, lamps emitting a higher light intensity could be used. Furthermore, it should be remembered that in areas such as general wards and GP examination rooms, natural sunlight can augment the light intensity to as high as 30,000 lux (CIBSE Lighting Guide LG2, 1989). Overall, these coatings show potential as self-disinfecting surfaces which would be useful in reducing the microbial load on surfaces in hospitals, clinics and in other buildings requiring high levels

of environmental hygiene. The next step will be to perform a detailed evaluation of the activity of the coatings in the hospital environment.

CHAPTER 6

Assessment of microbial contamination of surfaces in a dental clinic

6.1 Introduction

Given that the oral cavity of humans is densely populated with a wide variety of microbes, there is tremendous potential for aerosol transmission of these microbes to the environment and other individuals (Wilson, 2005). The expulsion of microbes from the oral cavity is increased by coughing and sneezing and during periods of dental treatment. With regard to the latter, many of the procedures carried out in the dental clinic result in the production of aerosols (particles < 50 μm in diameter) and splatter (particles > 50 μm in diameter) e.g. tooth preparation, ultrasonic scaling and tooth polishing (Harrel & Molinari, 2004). Particles comprising aerosols are small enough to remain suspended for considerable periods of time before they settle on surfaces or enter the respiratory tract of individuals in the vicinity (Micik *et al.*, 1969). In contrast, particles present in splatter do not remain airborne for long and quickly settle on neighbouring surfaces (Miller *et al.*, 1971). Microbes present in the oral cavity (originating from the respiratory tract as well as from the oral cavity itself) may be associated with either type of particle and so can be transmitted directly to other individuals or can settle on surfaces from which they can subsequently be transferred to patients and clinical personnel (Kohn *et al.*, 2003). The avoidance of both of these means of transmission is a key feature of measures designed to prevent cross-infection in dental clinics (Miller & Palenik, 2001). While a number of studies have evaluated the generation of microbe-containing aerosols produced by particular procedures or instrument use (Logothetis *et al.*, 1988; Bentley *et al.*, 1994), little is known concerning environmental contamination in dental clinics. In particular, although there have been several reports monitoring the numbers of microbes present in the air (Grenier, 1995; Monarca *et al.*, 2000) or on surfaces (Monarca *et al.*, 2000; Cellini *et al.*, 2001; Motta *et al.*, 2005; Rautmaa *et al.*, 2006), there have been very

few detailed studies documenting the variety of bacterial species most commonly found on surfaces in dental clinics.

Before trialling the TBO/RB cellulose coatings *in situ* in a busy dental clinic, it was of interest to investigate the types and numbers of bacteria that are present on the surfaces in such clinics. The aim of this part of the study, therefore, was to ascertain the numbers as well as the types of organisms arriving on a surface in a dental clinic by the air-borne route.

6.2 Materials and Methods

6.2.1 Sample collection and microbial enumeration

Settle plates were used to assess the degree and nature of environmental contamination in a dental clinic. One Columbia blood agar (CBA) plate and one Anaerobe basal agar (ABA) plate (Oxoid Ltd., Basingstoke, UK), both supplemented with 5 % (v/v) defibrinated horse blood, were placed side by side on a partitioning wall in the centre of Dental Clinic #2 in the School of Hygiene at the Eastman Dental Hospital. The clinic is open plan with 10 separate treatment bays, each housing one dental chair (see figure 6.1). The height of the wall was 129 cm and the plates were 244 cm from the nearest dental chair. Samples were taken on 10 separate days, and on each occasion there was a clinic running from 9am to 5pm. Clinics were either paediatric, periodontal, or adult restorative (i.e. periods of high activity). Samples were also taken on 10 separate control days during which no clinics were taking place (i.e. periods of low activity). Following exposure of the plates for 8 hours, the CBA plate was incubated aerobically with 5 % CO_2 at 37°C for 48 hours, while the

ABA plate was incubated anaerobically at 37°C for 7 days. Colonies were enumerated by viable counting and the counts expressed as cfu/m^2/h, calculated by dividing the cfu by the area of the agar plate (56.7 cm^2) dividing by the exposure time (8h).

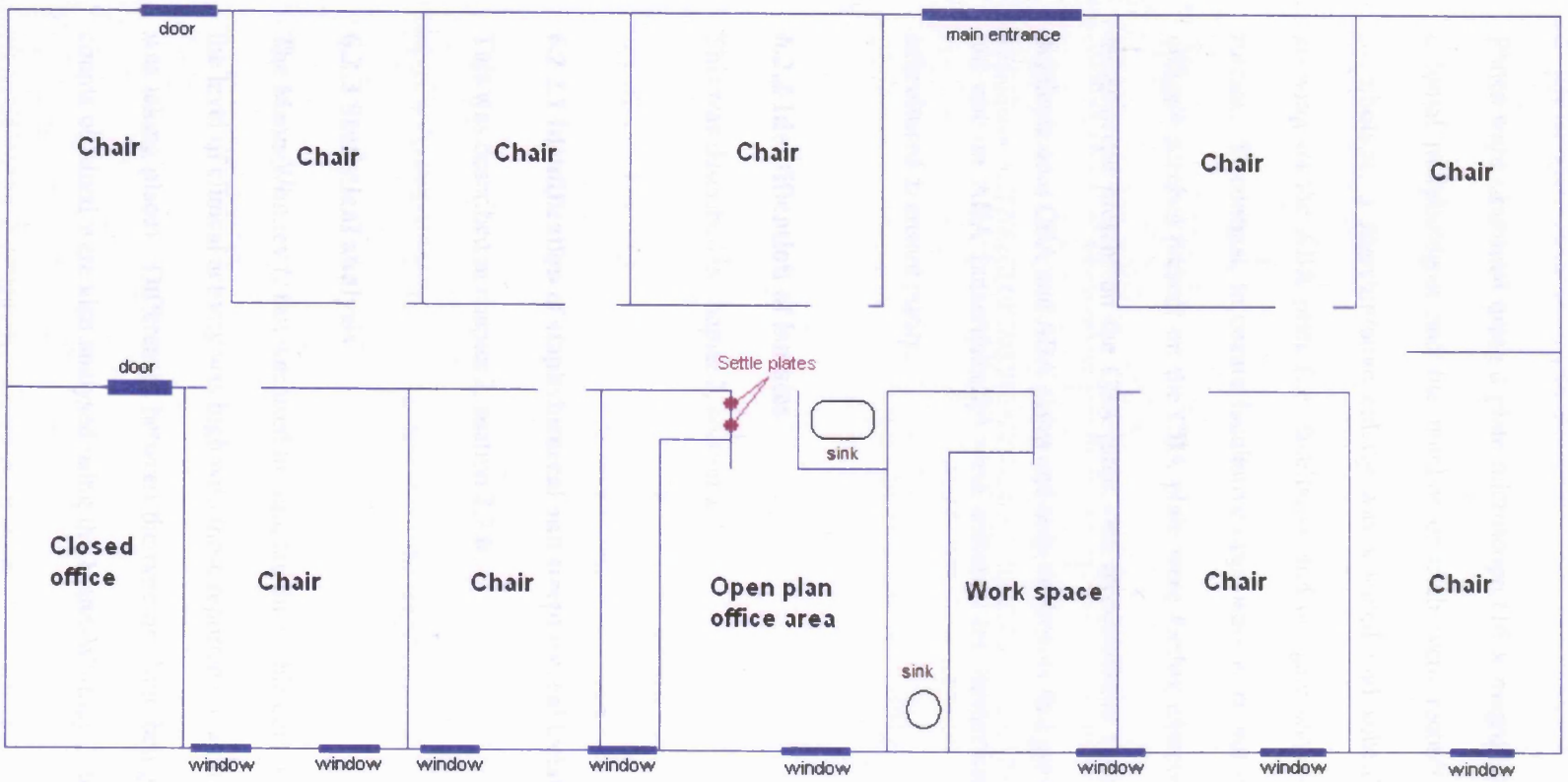


Figure 6.1 Plan of the dental clinic and location of settle plates.

Plates were examined using a plate microscope (16 x magnification) and the different colonial morphologies and the number of each were recorded. For each colonial morphotype, a representative colony was selected and subcultured. All organisms growing on the ABA plate (i.e. facultative and obligate anaerobes) were investigated further. In contrast, to ensure facultative organisms were not counted twice, only the obligate aerobes present on the CBA plate were further examined. For each colonial morphotype present on the CBA plate, one representative colony was subcultured in duplicate onto CBA and ABA plates and only organisms that grew on CBA (aerobically) but not on ABA (anaerobically) were selected for further study. All isolates were subcultured to ensure purity.

6.2.2 Identification of isolates

This was described in chapter 2, section 2.7.

6.2.2.1 Identification of staphylococcal and streptococcal isolates

This was described in chapter 2, section 2.7.6.

6.2.3 Statistical analysis

The Mann-Whitney *U* test was used to compare the viable counts obtained on days when the level of clinical activity was high with those reported on control days (when no clinic was taking place). Differences between the type of clinic being held and the bacterial counts obtained were also analysed using the Mann-Whitney *U* test. Since the morning

and afternoon clinics were often different, days were divided into groups based on whether each type of clinic took place at all (am, pm, or all day). For the days when clinics were run, the number of patients treated was also examined as a potential factor influencing the environmental microbial load. The samples were divided into two groups and compared using the Mann-Whitney *U* test: one group contained all samples collected on days when ≤ 23 patients were treated and the other comprised all samples taken on days when ≥ 24 patients were treated. Finally, for the control samples, temperature was taken to be another potential factor since 4 out of the 10 samples were collected during the summer. The viable counts were therefore divided into those taken between September and April and those taken between May and August and the Mann-Whitney *U* test was again used to compare these groups. $P < 0.05$ was considered statistically significant in all cases. All statistical analyses were carried out using the SPSS statistical package (version 12.0, SPSS Inc.).

6.3 Results

6.3.1 Number and variety of bacteria isolated

The number of viable bacteria detected on each sampling occasion during periods of high clinical activity ranged from 12.3 x 10^2 to 34 x 10^2 cfu/m^2/h (Table 6.1) with a mean of 21.9 x 10^2 cfu/m^2/h (median: 22.4 x 10^2 cfu/m^2/h). In contrast, during periods of low clinical activity the counts were significantly lower (p = 0.000), ranging from 0 to 10.1 x 10^2 cfu/m^2/h with a mean of 2.3 x 10^2 cfu/m^2/h (median: 0.7 x 10^2 cfu/m^2/h). There was no correlation between the bacterial counts and the type(s) of clinic carried out on each day (p = 0.138, 0.602, and 0.569 for periodontal, paediatric and adult

restorative clinics respectively). In addition, neither the number of patients treated ($p = 0.347$) nor seasonal temperature fluctuations ($p = 0.054$) had any significant effect on the numbers of bacteria detected.

A total of 193 colony morphotypes, comprising 73 species, were isolated during the study. When the clinic was being used, as many as 16 different colony morphotypes could be isolated during an 8 h period (Table 6.1). When the clinic was not in use this variation was generally much lower, with a mean of only 5 colony morphotypes per sampling occasion (Table 6.1).

Table 6.1. Number of microbes detected on settle plates in the dental clinic during periods of clinical activity and when no clinical activities were taking place.

		Clinical activity			No clinical activity	
Sampling occasion	**Type of clinic**	**No. of patients treated**	**Viable count ($x10^2$ cfu/m^2/h)**	**Number of distinct colony morphotypes**	**Viable count ($x10^2$ cfu/m^2/h)**	**Number of distinct colony morphotypes**
1	(AD Perio)*	24	26.4	14	4.8	9
2	(Perio/AR)	16	23.3	13	0.4	2
3	(Perio/Paed)	23	22.4	14	0.6	3
4	(Paed/AR)	19	34	16	2.5	10
5	(AD Paed)	30	29.1	16	10.1	10
6	(Perio/AR)	29	14.1	11	3.4	10
7	(AD Paed)	21	20.5	15	0.8	3
8	(Paed/Perio)	26	13.9	16	0.3	1
9	(AD Perio)	18	23.4	15	0	0
10	(AD Perio)	29	12.3	13	0.4	2
Mean		23.5	21.9	14.3	2.4	5

* AD = All day; Perio = periodontal; AR = adult restorative; Paed = paediatric.

6.3.2 Types of organisms isolated

Overall, many members of the cutaneous and oral microbiota as well as a wide range of environmental organisms were detected (Table 6.2). The organisms most frequently isolated during periods of high activity were *Propionibacterium acnes*, *Micrococcus luteus* and *Staphylococcus epidermidis* – these were detected on all 10 sampling occasions (Table 6.2). Other frequently-isolated species included *Acinetobacter lwoffii* and *Streptococcus mitis* which were detected on 70 % and 50 % of sampling occasions respectively. On most sampling occasions, *P. acnes* was the numerically-dominant organism and comprised 29 % (range: 23-44 %) of the organisms cultivated on each sampling occasion. *M. luteus* and *S. epidermidis* were generally the next most dominant organisms, comprising 22 % (range: 13-32 %) and 15 % (range: 1-28 %) of the organisms cultivated.

The organisms most frequently isolated during periods of low activity included, again, *P. acnes* and *M. luteus* which were detected on 40 % of sampling occasions (Table 6.2). Other frequently-isolated species were *S. epidermidis* and *Staphylococcus hominis* which were each detected on 30 % of sampling occasions. On most of these occasions, only small numbers of microbes were recovered so that analysis of the dominant organisms present would not have been meaningful. On those occasions when larger numbers of microbes were detected (#1, #4, #5 and #6 – Table 6.1), *M. luteus* comprised the highest proportion of the organisms cultivated, with a mean of 22 % (range: 7–35 %) while *S. epidermidis* (mean: 13 %, range: 0-36 %) and *P. acnes* (mean: 14 %; range: 0–29 %) comprised lower proportions.

Other members of the cutaneous microbiota detected in the study included *Corynebacterium* spp. (four species), *Brevibacterium* spp. (two species), coagulase-negative staphylococci other than *S. epidermidis* (ten species), *Propionibacterium* spp. other than *P. acnes* (two species), *Micrococcus lylae, Kytococcus* spp. (two species), *Acinetobacter* spp. (two species) and *Kocuria* spp. (two species). Organisms detected that are indigenous to the oral cavity and/or the respiratory tract included streptococci (five species), *Actinomyces* spp. (two species), *S. aureus, Lactobacillus casei, Haemophilus parainfluenzae, Neisseria* spp. (two species) and *Veillonella dispar.* As well as members of the indigenous microbiota, a wide range of environmental organisms were detected including *Bacillus* spp. (seven species), *Pseudomonas* spp. (two species), *Clostridium* spp. (two species), *Empedobacter brevis, Dietzia maris* and *Gordonia terrae.*

Table 6.2. Identities of microbes detected on settle plates in the dental clinic during periods of clinical activity and when no clinical activities were taking place.

organism	Periods of clinical activity		Clinically-inactive periods	
	frequency of isolation (%)[a]	(average no. of cfu isolated per sampling day)	frequency of isolation (%)[a]	(average no. of cfu isolated per sampling day)
Acinetobacter johnsonii	0	0	10	1
Acinetobacter lwoffii	70	9	10	2
Actinomyces naeslundii	10	2	0	0
Actinomyces odontolyticus	30	3.7	0	0
Anaerococcus octavius	10	10	0	0
Arsenicicoccus bolidensis	10	1	10	1
Bacillus cereus	10	2	0	0
Bacillus circulans	10	5	0	0
Bacillus ehimensis	0	0	10	2
Bacillus licheniformis	20	1	10	1
Bacillus megaterium	10	1	0	0
Bacillus niabensis	0	0	10	1
Bacillus pumilus	20	16.5	10	1
Brevibacillus borstelensis	10	1	0	0
Brevibacterium casei	0	0	10	3
Brevibacterium paucivorans	10	2	0	0
Clostridium perfringens	30	1.3	0	0
Clostridium sordellii	10	1	0	0
Corynebacterium afermentans	10	9	0	0
Corynebacterium amycolatum	20	1	0	0
Corynebacterium freneyi	0	0	10	1

Corynebacterium tuberculostearicum	10	6	0	0
Curtobacterium flaccumfaciens	0	0	10	1
Curtobacterium herbarum	0	0	10	2
Dietzia maris	20	17.5	0	0
Empedobacter brevis	10	1	0	0
Enterococcus faecalis	10	8	0	0
Exiguobacterium lactigenes	10	5	0	0
Exiguobacterium sp.	0	0	10	1
Facklamia hominis	10	9	0	0
Gordonia terrae	10	5	0	0
Haemophilus parainfluenzae	10	1	0	0
Kocuria rhizophila	20	4.5	0	0
Kocuria rosea	0	0	10	
Kytococcus schroeteri	10	1	0	0
Kytococcus sedentarius	20	1	0	0
Lactobacillus casei	10	2	0	0
Lactococcus lactis	10	1	0	0
Leuconostoc mesenteroides	0	0	10	1
Luteococcus sanguinis	0	0	10	1
Micrococcus luteus	100	26	40	5.5
Micrococcus lylae	10	1	10	2
Neisseria cinerea	10	1	0	0
Neisseria sp.	10	1	0	0
Paenibacillus amylolyticus	10	2	0	0
Pantoea agglomerans	0	0	10	1
Propionibacterium acnes	100	33.8	40	4
Propionibacterium granulosum	10	1	0	0
Propionibacterium propionicus	10	1	0	0

Pseudomonas oleovorans	30	1.3	0	0
Pseudomonas stutzeri	20	1	0	0
Rhodobacter massiliensis	0	0	10	1
Staphylococcus aureus	20	2	0	0
Staphylococcus capitis	40	4.3	0	0
Staphylococcus chromogenes	20	1	0	0
Staphylococcus cohnii	20	4	0	0
Staphylococcus epidermidis	100	18.8	30	3
Staphylococcus haemolyticus	20	5	0	0
Staphylococcus hominis	40	5.8	30	2.3
Staphylococcus lugdunensis	0	0	10	1
Staphylococcus saprophyticus	10	4	10	1
Staphylococcus simulans	10	9	0	0
Staphylococcus warneri	10	2	10	1
Staphylococcus xylosus	0	0	20	2
Streptococcus mitis	50	4.5	10	1
Streptococcus mutans	30	1.3	0	0
Streptococcus salivarius	10	1	20	1
Streptococcus sanguinis	20	1	0	0
Streptococcus thoraltensis	0	0	10	1
Streptomyces sp.	10	1	10	1
Unidentified Gram-negative rod	10	1	0	0
Veillonella dispar	10	1	0	0
Zimmermannella faecalis	20	14	10	2

[a] the frequency of isolation is given as the percentage of sampling occasions on which the organism was isolated.

6.4 Discussion

As discussed in chapter 1, the ability of organisms to survive on a particular surface depends on a number of factors including the type of organism, the nature of the surface, the temperature and the presence of organic matter. The types and numbers of organisms present in turn depend on the nature of the activities being carried out in the vicinity. As a result, it is difficult to predict which organisms will be present on the different hospital surfaces where the TBO/RB coatings might be applied. Since the last stage of the project was going to involve a trial of the coatings within a dental clinic, it was logical to first evaluate the level of microbial contamination present on surfaces within this clinic. The purpose of this part of the project, therefore, was to determine the type, and number, of air-borne microbes that arrive at a surface during, and in the absence of, routine activities in a dental clinic.

The first step in designing the experimental protocol for this contamination study was deciding which sampling technique to employ. While the use of settle plates is a passive air sampling technique, active air samplers can also be used to determine the level of microbial air contamination. Active air samplers measure the number of colony-forming units present in 1 m^3 of air collected over a 15 minute sampling period (Pasquarella *et al.*, 2000). This method forms the basis for official standards in air control (Pasquarella *et al.*, 2000) and is often thought to be the more appropriate sampling strategy. However, there are several problems surrounding the use of active samplers. Firstly, they are expensive, heavy, difficult to sterilise and need to be continuously calibrated (Pasquarella *et al.*, 2000). Secondly, many different types of active sampler exist and

the results of several studies (Nakhla & Cummings, 1981; Casewell *et al.*, 1984; Jensen *et al.*, 1992; Cage *et al.*, 1996) have shown that there is tremendous variation between each of these devices, thus making comparisons difficult. Thirdly, because air is being sucked in or pushed out by these volumetric samplers, this can cause artificial turbulence in the surrounding area and thereby alter the counts obtained (Ljungqkvist & Reinmuller, 2004). Furthermore, the situation in a dental clinic requires special consideration due to the evident fluctuation in contamination resulting from frequent aerosol production (Rautemaa *et al.*, 2006). Consequently, the use of settle plates is more appropriate as it allows for the cumulative measurement of contamination following a period of clinical activity. Moreover, since this thesis is focused on designing coatings for surface disinfection, only the organisms actually landing on hospital surfaces are of interest.

Another possibility would have been to use contact plates to sample the surface. However, as with air samplers, this method only provides a snap-shot of the numbers and types of bacteria present on surfaces; any bacteria that transiently landed on the surface during the course of the day would not have been recovered using just one set of contact plates. As previously discussed, the purpose of this investigation was to evaluate the level of environmental contamination that accumulates during an entire day of clinical activity and in order to achieve this, several pairs of contact plates (used at regular time intervals) would have been needed. The increased workload that would have resulted from using several sets of contact plates on each sampling day was not feasible in the time-frame of the current project.

One problem associated with the use of settle plates in this study was that time and resource constraints limited the number of agar plates that could be processed. Thus only 2 plates were used, both of which were placed in the centre of the clinic; in order to form a more complete picture of the environmental contamination throughout the clinic, it would have been better to use several pairs of plates, perhaps one positioned in each treatment bay.

During an eight hour period of high activity, the mean number of air-borne microbes arriving at a surface was found to be 21.9 x 10^2 cfu/m^2/h. This result is similar to that reported in an assessment (using settle plates) of air-borne contamination in four dental clinics in Italy, in which the mean number of viable microbes was found to range from 5 x 10^2 - 23 x 10^2 cfu/m^2/h (Cellini *et al.*, 2001). Encouragingly, all of these counts are below the standard for surface contamination of < 350 cfu/m^2/h as suggested by Friberg *et al.* (1999). Not surprisingly, high levels of activity increased the number of air-borne microbes, with the number of microbes on the settle plates increasing almost ten-fold during these periods. Interestingly, Fischer *et al.* (1972) found that the number of air-borne microbes in hospital operating theatres increased by the same factor during use. Both Cellini *et al.* (2001) and Osorio *et al.* (1995) have reported that after clinical activity, the number of air-borne bacteria in dental clinics was approximately two-fold greater than that present before such activity. However, it is also possible that the observed increase in the number of microbes may not be a direct result of the clinical activities but rather a consequence of there simply being more people moving around near the sampling site.

Overall, no statistically significant differences in the viable counts were observed when comparing viable counts obtained on days when at least one of the clinics (a.m. or p.m.) was AR. This is in contrast to a recent study which investigated microbial surface contamination in a dental surgery using settle plates and found that samples collected from rooms where restorative dentistry (using high-speed rotating instruments) was carried out yielded viable counts that were nearly twice as high as those taken from rooms where orthodontic or periodontal treatment was taking place (Rautemaa *et al.*, 2006). Of course, the viable counts presented here can be less easily compared between sample days based on the type of treatment since one clinic is used for all three modes of treatment and because on several occasions two different types of clinics took place. The very small sample size (n = 10; AR clinics took place on only 3 days versus 7 for other treatment modes) may also conceal the existence of any real differences and limits the usefulness of statistical analysis. A much larger study would need to be conducted in order to evaluate the potential effects that the types of treatment may have on the level of environmental contamination.

Surprisingly, on the days when clinics took place, the number of patients treated did not have a significant effect on the bacterial counts obtained. It would be expected that the more patients are treated, the more dental procedures are carried out and therefore more aerosols would be produced. However, the location of the settle plates in this study and the relatively large size of the clinic mean that any such effects were more widely dissipated. If the plates had been positioned near just one dental chair rather than in the

centre of the clinic, the number of patients treated in that specific chair would likely have more of an effect on the levels of bacterial contamination detected. Again, as before, the small sample size will also affect the significance of the findings.

Another factor which is often thought to influence the levels of microbial contamination is the ventilation. Good ventilation (air-conditioning) can indirectly reduce the air-borne microbial load simply by its diluting effect and also directly by filtration and/or purification (Legnani *et al.*, 1994). At the same time, air-conditioning systems have been implicated in the dissemination of microbes throughout a dental surgery (Osorio *et al.*, 1995). In this study, however, the clinic investigated did not have any air-conditioning system and instead airflow was achieved using numerous portable fans placed around the clinic. Airflow near the sampling site was also caused directly by the movement of patients and staff throughout the clinic and the opening and shutting of the door near the sample collection site. With the absence of air-conditioning, another factor that should be considered is the ambient temperature of the clinic since this plays a role in determining the survival and growth of bacteria landing on surfaces. Unfortunately, ambient air temperatures in the clinic were not measured and therefore it is only possible to hypothesise that seasonal differences in the sampling dates may have influenced microbial survival and/or growth. While the samples taken during periods of clinical activity were collected in winter, between the months of October and March, the control samples were collected between February and November, with 4 of these control samples (# 4-7) being taken during the summer months. It is possible that elevated temperatures within the clinics during these warmer months may have enhanced

bacterial survival and growth on the agar plates. Statistical analysis of these samples indicated that in fact any observed differences between the two groups of samples were not significant, although again a larger sample size might reveal more appreciable differences.

While some data are available with respect to the number of air-borne bacteria in dental clinics, no detailed analysis (i.e. to the species level) of the types of air-borne microbes present in dental clinics appears to have been published. In this investigation, during periods of high activity, *P. acnes, M. luteus* and *S. epidermidis* were the most frequently-detected organisms, being recovered on all sampling occasions. Furthermore, these organisms also generally comprised the largest proportions of the organisms cultivated on each occasion. Even when the clinic was not being used, these three organisms were among the four most-frequently detected. The normal habitat of *P. acnes, M. luteus* and *S. epidermidis* is the skin (Wilson, 2005). However, all three organisms are also frequently found in the oral cavity and so may be present in the aerosols created during dental treatment (Moore *et al.*, 1982; Funke *et al.*, 1997; Smith *et al.*, 2001; Anesti *et al.*, 2005; Wilson, 2005). Surprisingly, species considered to be members of the indigenous oral microbiota (e.g. viridans streptococci, *Actinomyces* spp., *Haemophilus* spp., *Neisseria* spp. and *Lactobacillus* spp.) were detected at much lower frequencies, although at least one of these was detected on every sampling occasion. These results are in keeping with those reported in the limited number of studies that have identified the air-borne organisms present in dental clinics. Osorio *et al.* (1995) found that staphylococci were the dominant air-borne microbes in dental clinics both before and

after clinical activity and accounted for between 73 % and 82 % of the organisms detected. Similarly, in a study in Japan, coagulase-negative staphylococci accounted for 22 % of the air-borne microbes detected in a dental clinic, while micrococci, coryneforms and streptococci comprised 23 %, 21 % and 12 % respectively of the organisms isolated (Noro *et al.*, 1998). One explanation for the infrequent detection of members of the indigenous oral microbiota in the current study is likely to be the location of the settle plates which were positioned approximately 2.5 metres from the nearest dental chair. Osorio *et al.* (1995) showed that, during clinical activity, the detection rate of streptococci decreased with increasing distance from the dental chair. It has been shown that particles present in splatter (i.e. > 50 μm in diameter) originating from the oral cavity do not remain airborne for long and quickly settle on neighbouring surfaces (Miller *et al.*, 1971). These are likely to contain large numbers of oral bacteria and so may not have reached the settle plates in this study. As indicated previously, the principal habitat of the three most-frequently identified air-borne bacterial species is human skin (Wilson, 2005) and so they are likely to have originated from skin squames shed by staff and patients or indeed by the investigator during sample set-up and/or collection. In contrast to all other reports found in the literature, a study by Rautemaa *et al.* (2006) observed that bacterial counts were actually higher in the more remote sampling points. The authors attributed this to the increased rotating speeds of the instruments used which gives a higher angular velocity and longer trajectory of the bacteria. Once again, however, it seems likely that staff movement around the more distant sampling sites could have been responsible for the increased counts, especially

since the most commonly isolated organisms were Gram-positive cocci, in particular staphylococci.

This study also illustrated that a large variety of environmental microbes can be found in dental clinics. Although the majority of these species belonged to well known genera such as *Bacillus* and *Clostridium*, several less common species were also detected. These included the plant pathogen *Curtobacterium flaccumfaciens* as well as *Empedobacter brevis, Dietzia maris* and *Arsenicicoccus bolidensis*, which inhabit soil. Of particular interest was the isolation of *Exiguobacterium* spp., which has recently been implicated in periodontitis (Zijnge *et al.*, 2003).

The results presented here have shown that a wide variety of cutaneous, oral, respiratory and environmental bacteria can be detected on settle plates in a dental clinic and that, of those microbes that can be cultivated on routine media in the laboratory, *P. acnes, M. luteus* and *S. epidermidis* are the most likely to contaminate surfaces in a dental clinic by the air-borne route. In the case of this particular dental clinic, it is encouraging to note that the majority of isolated bacteria are members of the skin microbiota that rarely cause infection under normal circumstances. However, it should be remembered that in the dental setting, as in the hospital environment, patients may be immunocompromised and, as many of the bacteria recovered in this study are opportunistic pathogens, the infection risk posed should not be ignored. Overall, these findings highlight the need for effective surface disinfection practices in order to minimise the survival of microbes on surfaces and their subsequent spread in the clinical environment. Fortunately, because

the majority of bacteria detected were found to be Gram-positive bacteria, which have previously been shown (see chapter 5) to be easily killed using the TBO/RB coatings, this suggests that the coatings should be effective at reducing the numbers of bacteria *in situ* on surfaces within the dental clinic. The final stage of the project will investigate whether this hypothesis holds true.

CHAPTER 7

Assessment of the antimicrobial activity of TBO/RB coatings in a dental clinic

7.1 Introduction

So far, this study has established that 25 μM TBO/RB cellulose acetate coatings can generate $^1O_2$ and successfully kill a variety of organisms both when applied as a suspension and when sprayed on to the coating surface using a nebuliser. The coatings were also effective in the presence of organic matter such as saliva or serum, thus further emphasising the potential that such coatings might have in a real hospital environment where dust, skin squames and body fluids will be present. Having determined the levels of microbial contamination and the types of organisms present on surfaces within a dental clinic in the previous chapter, the next logical progression was to test the coatings *in situ* against these organisms in the dental clinic.

Of the few studies that have investigated photosensitiser-based antimicrobial films and fibres, none have yet evaluated the efficacy of their materials in a clinical setting. The purpose of this final stage of the project was to evaluate the ability of the TBO/RB coatings in reducing the microbial load found on environmental surfaces in the dental clinic.

7.2 Materials and Methods

7.2.1 Experimental set-up

A shelving unit (Argos Direct, Stafford, UK) was constructed and placed in the centre of Clinic 2 in the School of Hygiene at the Eastman Dental Hospital, adjacent to one of the partitioning walls. The clinic was open plan with 10 separate treatment bays, each housing one dental chair (see figure 7.1).

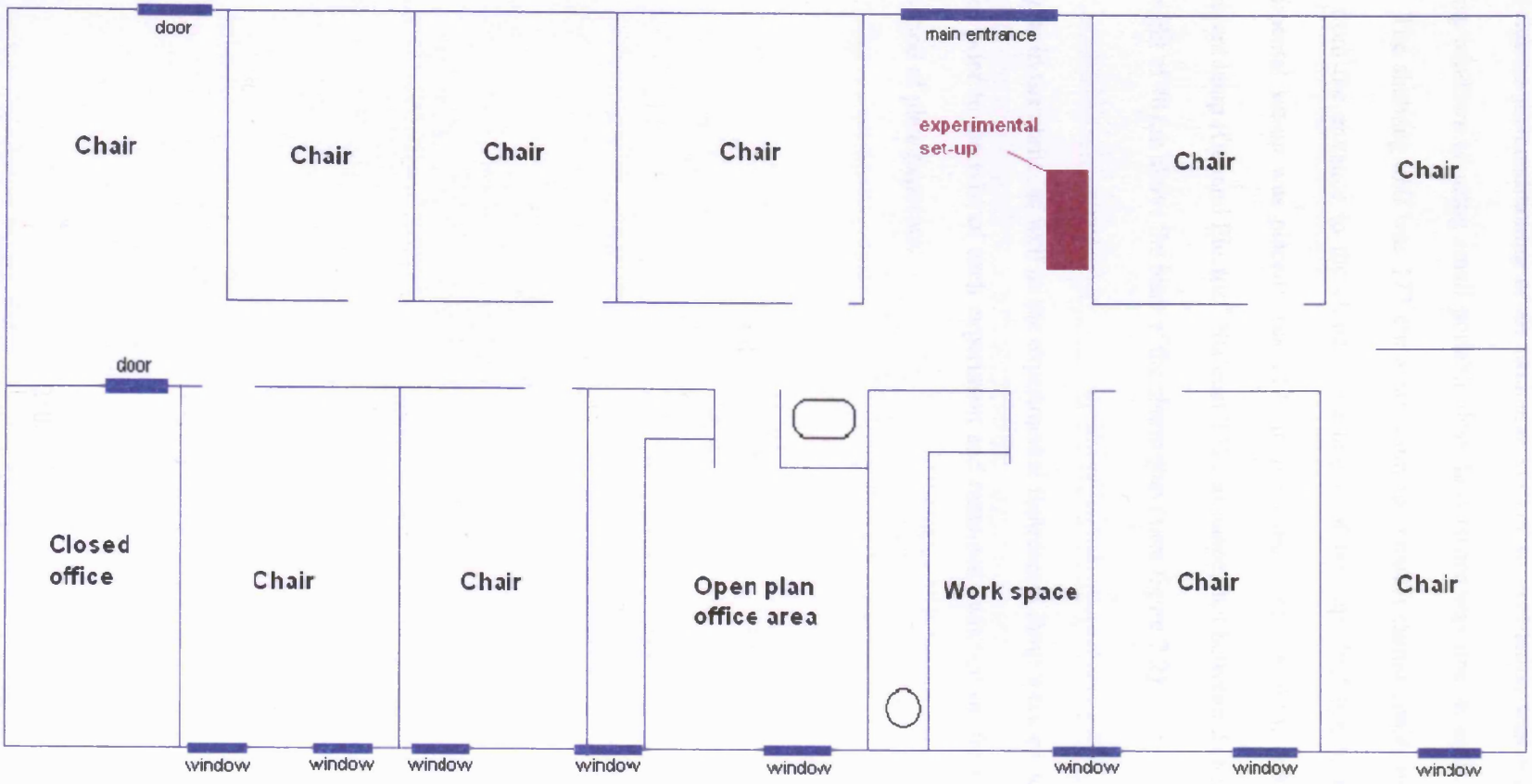
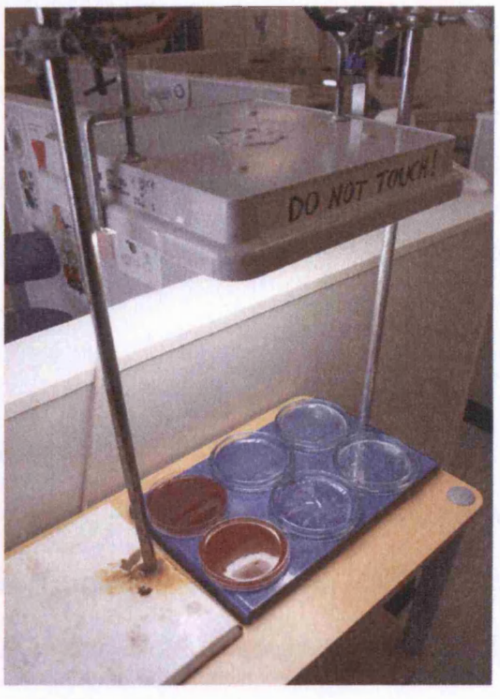


Figure 7.1 Plan of the dental clinic where experimental set-up was placed.

There was no air-conditioning or air exchange system so ventilation was achieved by opening windows or using small portable desk fans (there was one in each treatment bay). The shelving unit was 177 cm away from the nearest dental chair and 175 cm away from the entrance to the clinic. The height of the top shelf (on to which the experimental set-up was placed) was 112 cm from the floor. A 4-pin 2D compact fluorescent lamp (General Electric® Starcoat T5) was suspended between 2 clamp stands at a height of 40 cm above the base of the clamp stand (see figure 7.2).

All lights in the clinic, as well as the experimental fluorescent lamp were switched on 5 minutes prior to the start of each experiment and remained switched on for the full 24 hour period of plate exposure.

(a)



(b)

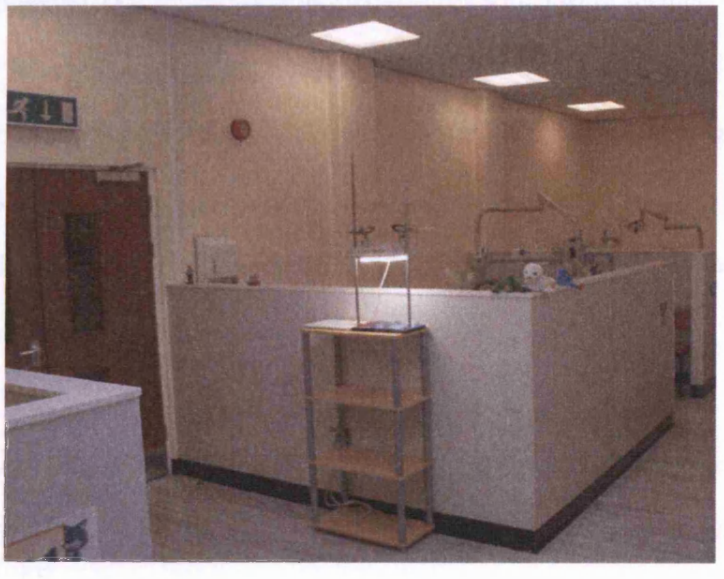


Figure 7.2 Experimental set-up of coatings (a) and their placement within the dental clinic (b).

7.2.2 Preparation of coatings

This was described in chapter 2, section 2.2.2.2.

7.2.3 Measurement of thickness of coatings

This was described in chapter 2, section 2.2.3. Measurements obtained are shown in Chapter 5, table 5.1.

7.2.3 Sampling procedure

Two large (diameter = 86 mm) control (photosensitiser-free) cellulose acetate coatings (see section 2.2.2.2), two 25 μM TBO/RB coatings, one thick (volume ~45 mLs) CBA plate and one thick ABA plate were placed alongside each other in 2 rows on the base of the clamp stand, underneath the suspended lamp. The lids were removed from all of the Petri dishes, inverted, and placed underneath the plates which were secured using double sided tape. Plates and coatings were placed in the clinic at 9 a.m. and collected at the same time the following day. On all days, treatment of patients did not begin until 9:15 a.m. but staff members were sometimes present in the clinic from 8:30 a.m. onwards. After the 24 hour exposure period, the lids were placed back onto all of the dishes and the plates were taken back to the laboratory for processing.

During each sampling period, the light intensity was measured three times using a digital luxmeter; once at the start of the experiment, once after 8 hours of exposure, and once at the end of the sampling period.

All staff and patients were advised of the study and instructed not to touch the plates or tamper with the experimental set-up.

7.2.4 Sample processing

Using sterile forceps, the two photosensitiser-free coatings were removed from their Petri dishes and placed in a sterile Seward Stomacher 80 standard bag (Seward Ltd., Thetford, UK). Five mL of sterile brain-heart infusion (BHI, Oxoid) which had been pre-reduced for 18 hours under anaerobic conditions were added to the bag. The bag was then stomached for 5 minutes at medium speed in a Seward Stomacher 80 biomaster lab system (Seward Ltd.). Triplicate 100 μL aliquots were plated out on to CBA and ABA plates. This procedure was then repeated with the TBO/RB coatings. All CBA plates (including the agar settle plate) were incubated aerobically with 5 % CO_2 at 37°C for 48 hours, while all ABA plates were incubated anaerobically at 37°C for 7 days.

7.2.5 Bacterial enumeration and isolation

Bacterial colonies were enumerated by viable counting. For the samples plated out from the photosensitiser-free coatings, the agar plate with the highest number and greatest diversity of colony morphologies was selected for further study while for the TBO/RB coatings, all three agar plates were studied. Each of these plates was examined using a plate microscope (16 x magnification). The different colony morphologies and the number of each were recorded. For each colonial morphotype, a representative colony was selected and subcultured. Only bacteria surviving on the TBO/RB coatings were

subjected to 16S rDNA sequencing (see section 2.8.3). All bacteria were stored on Microbank^{TM} beads (Pro-lab diagnostics, Neston, UK) and stored at -80°C.

7.2.6 Identification of isolates

This was described in chapter 2, section 2.7.

7.2.6.1 Identification of staphylococcal and streptococcal isolates

This was described in chapter 2, section 2.7.6.

7.2.7 Statistical analyses

The Mann-Whitney U test was used to compare the number of aerobic and anaerobic bacteria recovered from the control (S-) coatings with the number recovered from the TBO/RB coatings. $P < 0.05$ was considered statistically significant.

7.3 Results

7.3.1 Number of bacteria detected on control versus TBO/RB coatings

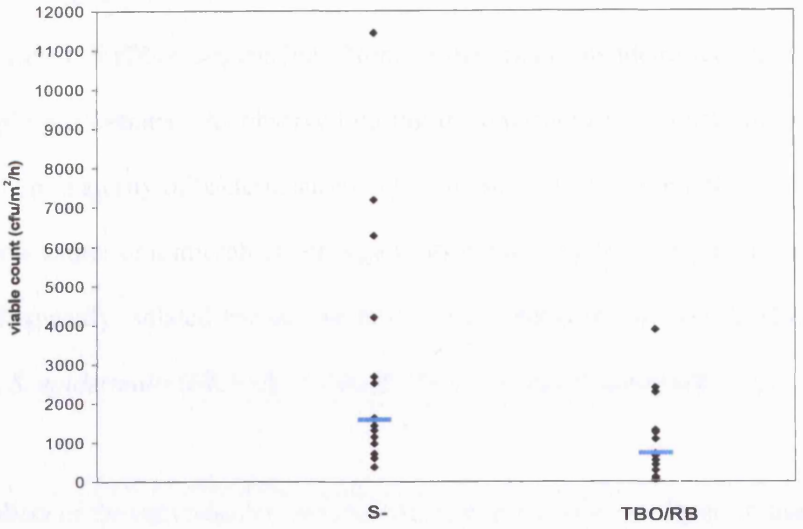
The first thing to note about the results obtained in this study is that the viable counts from both the S- and TBO/RB coatings showed a large degree of variability (see figure 7.3). As a result, the median rather than the mean will be referred to throughout this chapter. Furthermore, when examining the percentage kills, the negative values achieved on days 4, 10, and 13 (see table 7.1) inevitably affect the arithmetic means and consequently it is again more representative to compare the medians. For the aerobic

bacteria, the viable counts detected on the photosensitiser-free (S-) coatings during each 24 hour sampling period ranged from 6 x 10^2 to 114.2 x 10^2 cfu/m^2/h (Table 7.1) with a median of 15.5 x 10^2 cfu/m^2/h. For the anaerobic bacteria, counts were lower, ranging from 0 to 93.2 x 10^2 cfu/m^2/h, with a median of 6.6 x 10^2 cfu/m^2/h. Overall, the numbers of bacteria recovered on the 25 μM TBO/RB coatings were significantly lower than the numbers detected on the S- coatings ($p = 0.014$ for aerobes and $p = 0.02$ for anaerobes). Reduced counts were achieved on 13 out of the 15 days (86.7 % of the sampling occasions) for the aerobes and 12 out of the 15 days (80 %) for the anaerobes (Table 7.1). The median reduction was 63.8 % (range: 0 - 95.8 %) and 81.8 % (range: 0 - 100 %) for aerobic and anaerobic bacteria respectively (Table 7.1). For aerobic bacteria, viable counts on the TBO/RB coatings ranged from 0.6 to 38.9 x 10^2 cfu/m^2/h with a median of 7.2 x 10^2 cfu/m^2/h. As observed for the S- coatings, the numbers of anaerobic bacteria were lower than the numbers of aerobic bacteria, ranging from 0 to 14.9 x 10^2 cfu/m^2/h, with a median of 1.8 x 10^2 cfu/m^2/h . For the agar plates, median counts of 9.2 x 10^2 cfu/m^2/h (range: 3.7 x 10^2 to 19.9 x 10^2 cfu/m^2/h) and 5.7 x 10^2 cfu/m^2/h (range: 1.9 x 10^2 to 8.9 x 10^2 cfu/m^2/h) were observed for aerobic and anaerobic bacteria respectively.

Table 7.1 Number of bacteria recovered on control and TBO/RB coatings

	Aerobes				Anaerobes			
	Viable count (x10^2 cfu/m^2/h)			% kill	Viable count (x10^2 cfu/m^2/h)			% kill
Sampling occasion	Agar (CBA) plate	Control (S-) coatings	25 µM TBO/RB coatings		Agar (FAA) plate	Control (S-) coatings	25 µM TBO/RB coatings	
1	10.1	62.8	22.7	63.8	6.1	47.8	2.4	95
2	16.1	114.2	38.9	66	8.9	93.2	7.8	91.7
3	9.2	41.8	10.8	74.3	5.7	12.6	1.8	85.7
4	10.3	7.2	12.6	0	6.8	9	11.4	0
5	7.6	11.4	7.2	36.8	7	8.4	4.2	50
6	5.8	16.1	5.4	66.7	4.7	1.8	1.2	33.3
7	6.3	9.6	0.6	93.8	4.3	4.2	0	100
8	9.3	26.9	4.2	84.4	6	3.6	0.6	83.3
9	7.2	13.2	7.8	45.5	3.2	3.6	1.8	50
10	7.9	3.6	13.2	0	4.8	0	0.6	0
11	9	6	4.2	30	5.7	4.2	2.4	42.9
12	14.4	71.7	3	95.8	5.4	6.6	1.2	81.8
13	19.9	25.1	23.9	4.8	8.7	6.6	14.9	0
14	3.7	15.5	1.2	92.3	1.9	1.8	0	100
15	10.6	14.3	6.6	54.1	4.7	11.4	1.8	84.2
Median	**9.2**	**15.5**	**7.2**	**63.8**	**5.7**	**6.6**	**1.8**	**81.8**

(a)



(b)

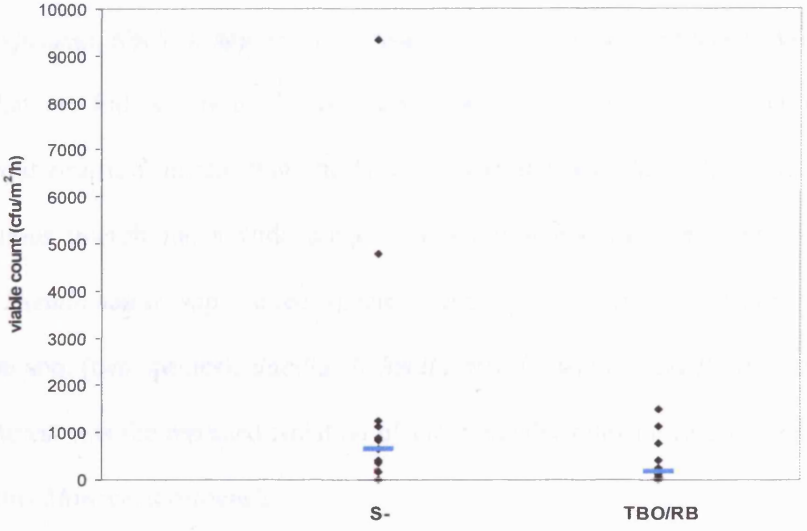


Figure 7.3 Scatter diagram comparing the viable counts recovered from photosensitiser-free (S-) and TBO/RB-containing coatings for: (a) aerobes and (b) anaerobes. The horizontal lines represent median values of the viable counts.

7.3.2 Types of organisms isolated

Due to time constraints, only the bacteria recovered from the TBO/RB coatings were identified using 16S rDNA sequencing. None of the organisms identified were detected on all sampling occasions. As observed during the environmental contamination study (chapter 6), the majority of bacteria surviving on these coatings were either members of the cutaneous and/or oral microbiota or organisms commonly found in the environment. The most frequently isolated species were *M. luteus* (detected on 93.3 % of sampling occasions), *S. epidermidis* (67.7 %), *A. lwoffii* (53.3 %), and *P. acnes* (46.7 %).

Other members of the cutaneous microbiota detected in the study included *Acinetobacter johnsonii*, a *Brevibacterium* species, coagulase-negative staphylococci other than *S. epidermidis* (eight species – these are also found in the oral cavity), *Corynebacterium* spp. (four species), *Kocuria* spp. (two species), and *Kytococcus schroeteri*. Organisms detected that are indigenous to the oral cavity and/or the respiratory tract included *Streptococcus oralis*, *S. aureus* and one *Streptomyces* species. As well as members of the indigenous microbiota, a wide range of environmental organisms were detected including *Pseudomonas* spp. (three species), *Brachybacterium* spp. (three species) *Clostridium* spp. (two species), *Bacillus licheniformis*, *G. terrae,* and *Paracoccus yeeii*. Also of interest was the repeated isolation of the clinically relevant rare Gram-negative coccobacillus *Moraxella osloensis*.

Table 7.2 Identities of microbes surviving on TBO/RB coatings in the dental clinic during a 24 hour period.

Organism	**frequency of isolation (%)[a]**	**(average number of cfu isolated per sampling day)**
Acinetobacter junii	6.7	1
Acinetobacter lwoffii	53.3	4.3
Arsenicoccus bolidensis	6.7	1
Arthrobacter sp.	6.7	2
Bacillus licheniformis	6.7	1
Brachybacterium fresconi	13.3	6
Brachybacterium rhamnosum	6.7	3
Brachybacterium sp.	6.7	5
Brevibacterium sp.	6.7	1
Brevundimonas vesicularis	6.7	1
Clostridium sp.	6.7	1
Corynebacterium coyleae	6.7	2
Corynebacterium imitans	13.3	1.5
Coynebacterium pseudogenitalium	6.7	1
Corynebacterium tuberculostearicum	20	1.3
Gordonia terrae	6.7	1
Kocuria rhizophila	6.7	2
Kocuria sp.	20	2.3
Kocuria varians	6.7	1
Kytococcus schroeteri	6.7	1
Luteococcus sanguinis	6.7	1
Micrococcus luteus	93.3	5.1
Moraxella osloensis	13.3	2
Paracoccus yeeii	6.7	19

Propionibacterium acnes	46.7	3
Pseudomonas luteola	6.7	1
Pseudomonas oleovorans	6.7	18
Pseudomonas stutzeri	26.7	3.5
Roseomonas sp.	6.7	1
Staphylococcus aureus	6.7	1
Staphylococcus auricularis	6.7	1
Staphylococcus capitis	26.7	2.3
Staphylococcus epidermidis	66.7	4.3
Staphylococcus haemolyticus	20	9
Staphylococcus hominis	26.7	2
Staphylococcus lugdunensis	13.3	1
Staphylococcus saprophyticus	13.3	3
Staphylococcus sciuri	6.7	1
Staphylococcus warneri	13.3	2
Streptococcus oralis	6.7	2
Streptomyces sp.	6.7	1
Unidentified Gram-positive cocci	13.3	4

[a] the frequency of isolation is given as the percentage of sampling occasions on which the organism was isolated

7.4 Discussion

The encouraging results demonstrating the effectiveness of the coatings against various organisms in laboratory-based experiments (chapter 5) implied that they had potential as antimicrobial coatings in a clinical setting. The next stage of the project, therefore, was to test the efficacy of the coatings in a clinical environment. The first challenge to be

overcome was the experimental design, both in terms of the set-up and the sampling technique used.

The experimental set-up was designed to be similar to that used in the environmental study described in chapter 6. However, some modifications were necessary. Firstly, due to the low level of light intensity (250 ± 10 lux) in the clinic, it was decided that a secondary light source (Starcoat 2D T5 compact fluorescent lamp) should be used to supplement the existing ambient lighting conditions. In this way it was possible to achieve the same light intensity (3,700 lux) as that used in laboratory experiments. This allowed testing of the coatings using uncontrolled conditions and inocula while maintaining a comparable light intensity to that used in the laboratory experiments which previously demonstrated the efficacy of coatings using controlled inocula. Ideally, the light intensity would have been augmented using a light source commonly found in dental clinics such as the dental chair lamps already present in the clinics (light intensity ranges from 8,000 - 23,000 lux at a distance of 30 cm) but this was not possible since the experimental set-up would have impeded the work of the dental hygienists.

To suspend this lamp above the coatings, two clamp stands were needed and this in turn necessitated the use of a separate shelving unit to support the experimental set-up (as shown in figure 7.2a). Space constraints within the clinic meant that the only place that the shelving unit could be placed without causing any disruption to the running of the clinic would be near the main entrance door (Figure 7.2b). Logically, the placement of the experimental set-up will affect the numbers and types of organisms detected during

the study. While the proximity to the entrance door would be expected to increase the overall numbers of bacteria due to the frequent movements of both patients and staff around the sampling site, the plates were partially sheltered from nearby dental chairs by the partitioning walls which may have prevented bacteria aerosolised during treatments from reaching the plates. In fact, the overall viable counts recovered from the agar plates were lower than those detected during the previous environmental study thus indicating that proximity to the main entrance door did not lead to increased levels of contamination. Rather, this may be explained by the position of the settle plates during the environmental study which were placed on top of one of the partitioning walls and were therefore completely exposed to all circulating air currents. Likewise, the results showed that nearly all of the bacteria recovered were members of the cutaneous microbiota or environmental organisms rather than members of the oral microbiota. This is in keeping with the hypothesis that the partitioning wall may prevent oral bacteria from settling on the coatings but it may also reflect the reduced susceptibility of these organisms to the antimicrobial action of the coatings.

Although both the general public and the media place a great deal of emphasis on hospital cleanliness, the only real assessment of cleaning efficacy is visual; microbiological examination of surfaces is not mandatory in the UK and is rarely carried out in hospitals in this country. Several different culture methods can be used for environmental sampling but a large amount of variation exists with respect to the recovery rates and reproducibility of these protocols. When carried out, sampling of environmental surfaces is most often performed by swabbing (Das *et al.*, 2002; French *et*

al., 2004; Lemmen *et al.*, 2004; Hayden *et al.*, 2006) but this method has been repeatedly reported to give reduced recovery rates, particularly with respect to dry sampling areas (Davidson *et al.*, 1999; Moore & Griffith, 2002; Obee *et al.*, 2007). Since the coatings were in glass Petri dishes which could be covered with lids and transported back to the laboratory, it was decided that a more direct sampling technique should be employed to maximise recovery rates. Initially, the direct agar contact plate method was trialled but the size of the agar plates and irregular shape (near the edges) of the coatings proved to be incompatible with this approach. Stomaching the coatings overcame the problem of reduced recovery by ensuring that all organisms were washed off the coatings but, because of the dilution factor involved in placing the coatings in 5 mLs of BHI, some bacteria may have been missed.

It was encouraging to observe that the TBO/RB coatings consistently had a reduced microbial load when compared to the control (S-) coatings, with a decrease in the number of bacteria recovered achieved on 86.7 % and 80 % of the sampling occasions for the aerobes and anaerobes respectively. While the reductions obtained were not as high as might have been expected (median: 63.8 % for aerobes and 81.8 % for anaerobes) on the basis of the results presented in Chapter 5, they were nevertheless statistically significant. The reasons for the discrepancy between the results obtained in laboratory experiments and those obtained when the coatings are tested *in situ* remain unclear. It may be due to the organic matter present in the dirt and dust particles which will inevitably settle out on to the coatings. As discussed in chapters 1 and 5, organic matter such as serum, blood, and saliva decreases the efficacy of lethal photosensitisation by

interfering with light absorption, obstructing access to $^1O_2$, and competing with $^1O_2$. While the coatings did not achieve complete killing of organisms deposited on their surface, any reduction in the number of organisms will be beneficial since it will help to diminish the chance of cross-contamination and subsequent spread of microorganisms.

The sampling conditions and experimental set-up also inevitably had an effect on the results. On days when no reductions were apparent (sampling days 4, 10, and 13), it is possible that there was a higher level of staff activity on the morning of sample collection; staff were known to arrive from 8 a.m. onwards and the samples were not collected until 9 a.m. Such increased levels of activity will inevitably affect the movement of particles in the air and may have increased the numbers of bacteria landing on the coatings just before sample collection. If this was the case then it is entirely possible that the coatings did not have enough time to kill these microbes. With respect to days 10 and 13, an alternative explanation of the lack of successful killing may have been an increase in the temperature of the clinic. While the temperature was not routinely measured, during the late spring and early summer months the clinic felt extremely warm and on several occasions colonies were already visible on the agar settle plates after the 24 hour sampling period. Such an increase in temperature would not only affect the growth and therefore the susceptibility and survival of the organisms, but it may also have necessitated the use of the portable fans positioned around the clinic and these would increase the air flow around the clinic, thus facilitating the movement of bacteria through the air.

One of the biggest inconsistencies encountered during the course of the study was the environmental conditions in the clinic; it was impossible to control or monitor variables such as ambient temperature and the behaviour of staff and patients in the vicinity of the samples. The changeability of environmental conditions is evidenced by the large degree of variability that exists between the inoculum size (viable counts) recorded on each of the 15 sampling days (figure 7.3). Ideally, in order to get more representative and consistent results, a much larger study with samples being taken at regular intervals over the course of an entire year should be carried out and environmental factors such as ambient temperature should be routinely measured.

Unfortunately, time constraints meant that it was impossible to sequence all of the bacteria isolated from both the control and the TBO/RB coatings and consequently only those bacteria surviving on the TBO/RB coatings were identified. As a result, performing any meaningful analysis of the types of species that were being killed was impossible. However, comparing these data with the background environmental contamination observed in chapter 6 indicates that no large differences are apparent. As in the environmental study, the most frequently isolated organisms were the Gram-positive bacteria *S. epidermidis*, *M. luteus* and *P. acnes* which, based on the previous lab-based kill experiments (chapter 5), would be expected to be easily killed by the TBO/RB coatings. However, while these bacteria were detected on every sampling occasion during the environmental study, on the TBO/RB coatings *S. epidermidis*, *M. luteus* and *P. acnes* were only detected on 66.7 %, 93.3 %, and 46.7 % of the sampling occasions respectively thus reflecting the overall reduction in microbial load.

It should also be highlighted that while the dental clinic provided a convenient setting for the evaluation of the TBO/RB coatings *in situ*, there are several important differences between dental clinic and hospital wards which should be borne in mind if these coatings are to be used in the hospital environment. Firstly, the levels of dirt and dust present in hospital wards are likely to be substantially higher than those present in the dental clinic, thus potentially reducing the efficacy of the coatings. Secondly, the presence of patients who may shed high levels of organisms will inevitably cause higher levels of environmental contamination which will comprise a larger proportion of pathogenic and infectious organisms. This again will have implications for the efficacy of the coatings since shorter killing times would be needed to interrupt transmission of infectious agents such as norovirus. The greater number of patient and staff movements in busy hospitals will not only increase the microbial load present on surfaces but will also enhance the chance for cross-contamination between surfaces. Perhaps one of the biggest differences is the consistently high workload; hospital staff are often over-stretched and consequently infection control practices such has hand washing will be overlooked, thus adding to environmental contamination. Finally, the presence of inpatients provides a constant source of environmental contamination.

Overall, the results from this part of study have demonstrated the difficulties that arise when attempting to carry out experiments in the clinical environment and have highlighted the differences in the results obtained between laboratory and *in situ* experiments. The results have also shown that while the coatings are effective at reducing the environmental microbial load in a clinical environment, they appear to be

less active than previously shown in laboratory experiments. It is clear that TBO/RB coatings can not provide a complete solution to the problem of environmental contamination and contact transmission but they do have the potential to considerably reduce the microbial load on surfaces.

CHAPTER 8

Final Discussion and Conclusions

8.1 Discussion

The fight against hospital-acquired infections is ongoing and unfortunately no 'magic bullet' solution exists to combat the problem. The rapid emergence of new multi-antibiotic resistant organisms further exacerbates the situation, making treatment of patients more difficult. It is becoming increasingly clear that the most successful strategy is one that focuses on all areas of hospital hygiene including both hand and environmental hygiene. The results of the research project described here imply that light-activated antimicrobial coatings may be able to contribute to reducing surface contamination and therefore transmission of microorganisms in clinical environments. The ideal light-activated coating would be one that is safe to touch, activated by the ambient light available, easy to apply, and effective at killing a range of hospital-associated pathogens and each of these parameters had to be addressed in this project.

Correspondence with several NHS trusts indicated that the most common form of lighting used in the wards and ward corridors are fluorescent luminaires and, accordingly, a compact fluorescent lamp with similar specifications was chosen for use in all experiments. This light selection step was critical not only because of the importance of choosing a light source that mimicked hospital conditions but also because the emission spectrum of this lamp determined the choice of photosensitiser(s). Only photosensitisers which absorbed a large proportion of the light emitted by the lamp were selected for further study. Of the photosensitisers initially screened using the singlet oxygen ($^1O_2$) assay, only three phenothiazine (TBO, MB, MV) and two xanthene dyes (RB and Er B) were found to produce appreciable amounts of $^1O_2$. These

photosensitisers were then subjected to more in-depth $^1O_2$ assays and spectroscopic analyses which revealed that Er B could not maintain its $^1O_2$ production for prolonged periods of irradiation and that both Er B and RB became rapidly photobleached. In contrast, all three phenothiazine dyes were efficient $^1O_2$ producers and stable compounds thereby making them desirable candidates for further study.

TBO, MB, and MV are all agents frequently used in the photosensitisation of a wide variety of microorganisms but it was essential to determine their activity using the specific parameters employed in this study (e.g. room temperature, fluorescent light). Moreover, since research using white light for photosensitisation is limited, microbial kill experiments in suspension would also provide information about the irradiation times and photosensitiser concentrations necessary for killing. These kill experiments revealed that, at a concentration of 10 μM, all 3 photosensitisers could successfully kill *S. aureus* and *E. coli*, bacteriophage φX174 and *C. albicans* after either 2 or 6 hours of irradiation. However, variations existed between the photosensitisers in terms of their activity against each of these organisms. These experiments also demonstrated that bacteria are more susceptible to lethal photosensitisation than both viruses and fungi, and that Gram-negative bacteria are less susceptible to photosensitisation than Gram-positive species.

Once the microbicidal activity of TBO, MB, and MV solutions had been established, the next step was to evaluate the activity of these dyes when incorporated into a polymer subjected to the same experimental conditions. Cellulose acetate was the polymer

selected for use in the study due to its transparency, ease of preparation, and previously documented success as an antimicrobial coating (Wilson, 2003). However, it should be noted that the solvent used to dissolve cellulose acetate is acetone, a notoriously problematic solvent. Acetone necessitates the use of glassware in experiments due to its plastic-dissolving properties. Applying such a coating to environmental surfaces would require careful consideration of the compatibility between acetone and the surface to be coated. Nevertheless, cellulose acetate could be used to coat a variety of hospital surfaces including those made from stainless steel or glass. It must be borne in mind, of course, that the research described here is 'proof of principle' and was not intended to provide the optimum polymer/photosensitiser combination. Further development of this concept would have to involve research by polymer chemists and photochemists in order to produce a coating that could be mass-produced for use in hospitals etc..

Based on the light absorption properties of the dyes and the emission spectrum of the lamp, it was decided that the best way to capture the majority of light emitted would be to make one coating incorporating MV in combination with TBO and another which used MV together with MB. Both of these combinations proved effective at killing suspensions of *S. aureus* and *E. coli* placed on their surface although the irradiation time (6 hours) and the photosensitiser concentration (150 μM) were substantially higher than those used in the kill experiments in solution. Since there appeared to be little difference in the activity of the two coatings, the TBO coating was selected for further study. Unfortunately, excessive leaching of TBO out of the coating as well as the inactivity of MV when embedded in cellulose acetate necessitated further investigation and selection

of an alternative blue-light absorbing photosensitiser to use in combination with TBO. Leaching experiments indicated that the ideal concentration to prevent leaching but maintain activity was 25 μM, while RB was re-considered for incorporation into the coating. Once the antimicrobial activity of coatings containing TBO and RB individually had been ascertained, the combination (TBO/RB) coating was tested against a large variety of organisms, all of which proved susceptible to light-activated killing by the coating. The longer irradiation time (16 hours) necessary to achieve significant kills of *E. coli*, *P. aeruginosa*, bacteriophage φX174, and *C. albicans* confirmed the decreased susceptibility of these organisms to lethal photosensitisation in comparison to *S. aureus*. Importantly, the kill experiments carried out using the coatings were designed to determine the numbers of each organism that can be killed. This is in contrast to other reports documenting the activity of light-activated antimicrobial materials which have either qualitatively measured the presence or absence of bacterial growth following irradiation (Wainwright *et al.*, 2006) or reported the kills as percentages only (Bozja *et al.*, 2003).

Of particular importance was the finding that bacteria sprayed onto the coatings using a nebuliser were killed by the irradiated TBO/RB coatings. This situation more closely mimics the settling out of bacteria from aerosols and has not been tested previously for light-activated antimicrobial materials. The few studies that exist on the activity of photosensitiser-based materials have all been performed using liquid suspensions that are either spread (Wainwright *et al.*, 2006) or pipetted (Wilson, 2003) on to the surface of the film or, as in the study by Bozja *et al.* (2003), the materials themselves were

immersed in bacterial suspensions prior to irradiation. Because bacteria behave differently when dried on a surface as opposed to being free in a suspension, the nebuliser experiments were seen as the logical progression from the aqueous suspension experiments, to be performed prior to testing the coatings *in situ* in clinics. The fact that the nebulised bacteria could be killed even when suspended in saliva or horse serum was also critical since bacteria in aerosols are invariably suspended in fluids containing organic material (e.g. saliva, blood etc) and organic matter is often found on hospital surfaces.

Once the activity of the TBO/RB coatings had been extensively tested in the laboratory, the final stage of the project was to assess their efficacy in a clinical environment. Prior to this, however, a settle plate study was carried out to evaluate the numbers and types of bacteria that are deposited on surfaces in the dental clinic where the coatings would subsequently be tested. This investigation revealed that the numbers of bacteria recovered were much higher on days when clinics were being held than on days when no such clinical activity was taking place. Furthermore, the study showed that the most frequently isolated organisms were members of the cutaneous microbiota. In particular, *P. acnes*, *S. epidermidis* and *M. luteus* were isolated on each of the sampling days when clinics were taking place. Although these organisms are most often harmless commensals, they can also be opportunistic pathogens particularly in the hospital environment where many patients are immunocompromised. The previously described process of contact transmission emphasises the importance of reducing such microbial loads and it was encouraging that the majority of organisms isolated were Gram-positive

bacteria which were shown to be the most susceptible to the TBO/RB coatings of all the microbes tested.

In fact, when the TBO/RB coatings were tested *in situ* in the dental clinic, median reductions of 63.8 % and 81.8 % were achieved for aerobic and anaerobic bacteria respectively. These kills were lower than expected considering the high, and in many cases, complete (100 %) killing of microbes achieved in laboratory experiments. The most likely explanations for the diminished kills are elevated ambient temperature, the presence of organic material and the possible settling out of bacteria just before sampling.

As discussed in Chapter 5, light-activated antimicrobial polymers are by no means the only type of antimicrobial materials with potential applications as self-disinfecting surfaces within the hospital environment; a large variety of coatings are being developed and tested. Some of these, such as the hexyl-PVP-modified glass surfaces, have been shown to be more effective than the TBO/RB coatings, with comparable bacterial kills achieved using much shorter contact time of 30 minutes (Tiller *et al.*, 2001). These materials, however, have yet to be tested against viruses or yeasts. Furthermore, they are much more complex (and therefore presumably more expensive) to produce and have not been shown to achieve complete (100 %) killing. One of the advantages of using light-activated materials is that it is possible to achieve higher kills with shorter irradiation times simply by applying a higher light intensity. Hence the coatings produced in this study could very likely achieve substantial kills more rapidly. However,

the time restraints of the project prevented the testing of higher light intensities. Titanium dioxide photocatalysts have also been demonstrated to have superior (Ohko *et al.*, 2000; Kühn *et al.*, 2003) or comparable (Page *et al.*, 2007) antimicrobial efficacy. The disadvantage of these coatings is that they require activation using UVA (365 nm) light which not only has adverse health effects but this wavelength of light is not emitted by the light sources commonly employed in the hospital environment. The final class of antimicrobial materials are those which work by releasing either a disinfectant (Tiller *et al.*, 2005; El-Hayek *et al.*, 2006) or copper (Noyce *et al.*, 2006) or silver (Ewald *et al.*, 2006) ions. Regular use of such coatings would need careful consideration however since both intrinsic and acquired resistance to these compounds is well documented.

While current disinfection practices using detergents and disinfectants can also be successful at diminishing environmental contamination, these cleaning protocols can suffer from problems of staff compliance, microbial seeding of mops and cleaning solutions, resistance development and health concerns. Such issues have helped to drive the increased interest in developing self-disinfecting surfaces.

Although the alternative antimicrobial materials discussed above show potential, none have yet been investigated in as much detail as the coatings described in this study. The TBO/RB coatings have been shown to possess long-term efficacy, broad-spectrum activity, activity against aerosol challenge, efficacy in the presence of organic material and most importantly, activity when tested *in situ*.

In a recent review, Kenawy *et al.* (2007) stated that antimicrobial polymers should ideally possess the following characteristics:

- simple and inexpensive synthesis
- stability in long-term usage and storage at the temperature of its intended application
- no toxic bi-products generated or emitted
- safe to touch
- possibility of regeneration upon loss of activity
- biocidal to a broad-spectrum of pathogenic microorganisms in brief contact times

The photosensitiser-containing cellulose acetate coatings described here possess nearly all of these traits, providing a simple, inexpensive and stable means of reducing the microbial load on surfaces. In terms of safety, concerns surrounding photosensitser-containing coatings are 2-fold: toxic effects of $^1O_2$ and toxicity of the photosensitisers themselves. Since $^1O_2$ decays to normal ground state oxygen within fractions of a microsecond, a build-up is not possible. The negligible leaching of TBO and RB from the coatings also means that there should be no health concerns associated with photosensitiser phototoxicity. Moreover, even if minimal leaching were to occur, both TBO (Kömerik *et al.*, 2003) and RB (Wachter *et al.*, 2003) have been shown to be non-toxic in animal models at concentrations as high as 1 mg/mL. A 1 % solution of TBO was also topically administered for the early diagnosis of precancerous and cancerous cells, with no reported reactions or side-effects occurring after application (Warnakulasuriya & Johnson, 1996). Nevertheless, the safety of the coatings would

need to be assessed in detail in a clinical trial before being widely applied to hospital surfaces.

The last point listed by Kenawy *et al.* still needs to be addressed when considering the use of TBO/RB coatings since the contact time of > 6 hours is not ideal. This can however, be overcome by using a higher light intensity or a higher concentration of one or both photosensitiser(s) (although this may require selection of an alternative polymer due to the leaching problems described). Clearly, the shorter the contact time the better, since this shortens the survival time and therefore the transmission potential of the contaminating organism. Nevertheless, when using the coatings for continuous surface disinfection in hospitals, any reduction in the microbial load that can be achieved over the course of the day without the need for additional cleaning procedures will be beneficial.

8.2 Conclusions

Extensive experimentation in the laboratory demonstrated the broad-spectrum antimicrobial activity of cellulose acetate coatings containing TBO and RB while initial investigations in the clinical environment showed that these coatings could consistently reduce the microbial load present. Additional investigations might include carrying out tests in the hospital ward environment or performing contact testing whereby organisms are transferred to and from the coatings directly by touch.

With the ever-increasing levels of antimicrobial resistance, high rates of hospital-acquired infections and heightened public concern, all promising strategies for reducing the transmission of microorganisms should be investigated. While good compliance with hand hygiene remains of paramount importance, any reduction in the microbial load present on surfaces will help to minimise the cross-transmission that occurs between staff, patients and the environment.

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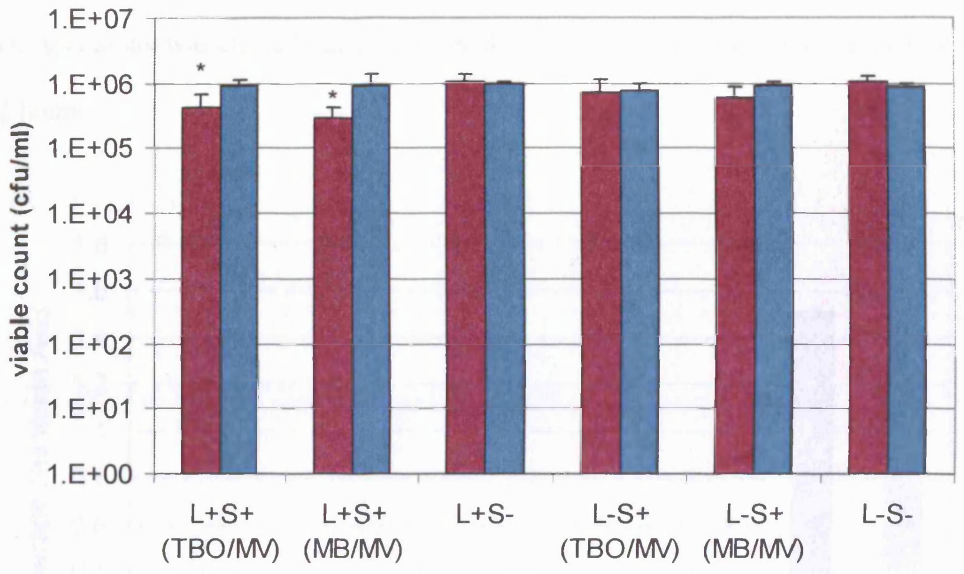
Appendices

Appendix 1: Lethal photosensitisation of S. aureus using 10 μM coatings

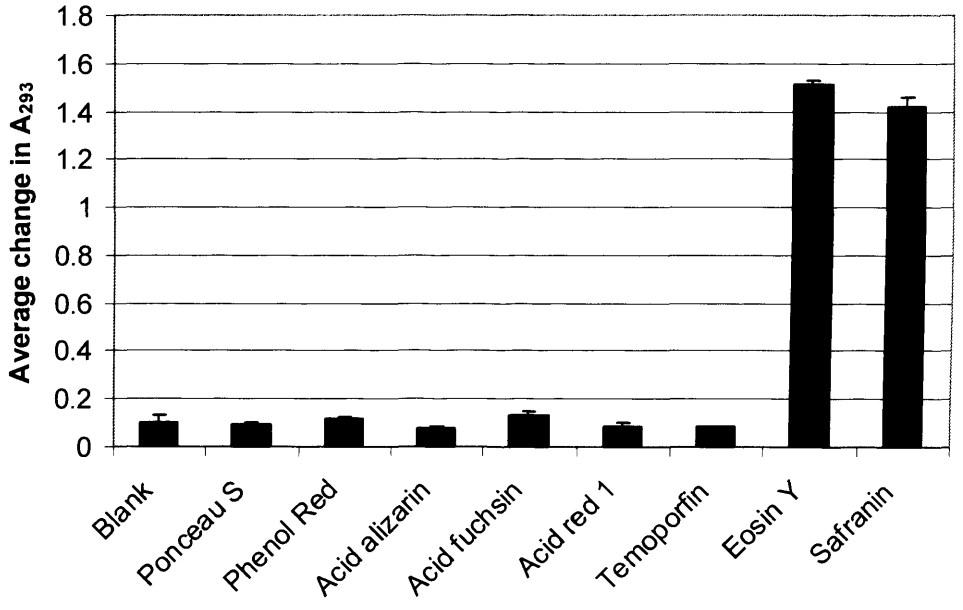


Viability of *S. aureus* (■) and *E. coli* (■) after 6 hours of irradiation when in contact with cellulose acetate containing either 10μM TBO and 10μM MV (L+S+ (TBO/MV))or 10μM MB and 10μM MV(L+S+ (MB/MV)). As a control, some were placed on photosensitiser-free cellulose acetate and kept in the dark (L-S-). Additional controls consisted of bacteria irradiated with light on photosensitiser-free cellulose acetate (L+S-) and bacteria kept in the dark that were in contact with cellulose acetate containing either 25μM TBO or RB (L-S+). * denotes that the viable count was significantly different from that of the control (L-S-) suspension (Mann-Whitney *U* test).

Appendix 2: Alternative dyes screened for singlet oxygen production

Two hour uric acid assay

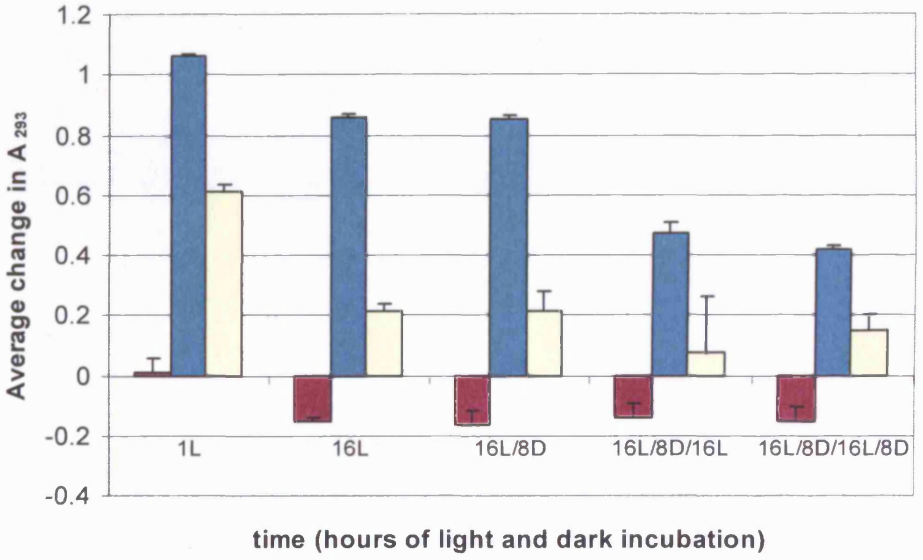
A uric acid assay was carried out as described in chapter 2, section 2.3.1. Irradiation was for 2 hours.



Singlet oxygen production of a selection of dyes following irradiation with a compact fluorescent lamp for 2 hours. Control = PBS. Bars represent mean values and error bars represent standard deviations (n = 3).

Light/dark experiment

To evaluate singlet oxygen production by Eosin Y and Safranin O following alternating cycles of irradiation and incubation in the dark was performed, a light/dark experiment was performed as described in chapter 3, section 3.2.3.4.



The average change in absorbance at 293 nm of Eosin Y (■) and safranin O (□) following cycles of light and dark incubation. ■ = PBS. 1L = 1 hour light, 16L = 16 hours light, 8D = 8 hours dark. Bars represent mean values and error bars represent standard deviations (n = 6).

Appendices 3 and 4: these can be found on the CD-ROM submitted together with this thesis.

Publication resulting from this research

SPECIAL NOTE

ITEM SCANNED AS SUPPLIED
PAGINATION IS AS SEEN

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2006, p. 4436–4439 Vol. 72, No. 6
0099-2240/06/$08.00+0 doi:10.1128/AEM.02945-05

Cellulose Acetate Containing Toluidine Blue and Rose Bengal Is an Effective Antimicrobial Coating when Exposed to White Light

Valérie Decraene, Jonathan Pratten, and Michael Wilson*

Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, London, United Kingdom

Received 14 December 2005/Accepted 29 March 2006

Simple methods of reducing the microbial load on surfaces in hospitals are needed to reduce the risk of hospital-associated infections. Here we report on the ability of a cellulose acetate coating containing the photosensitizers toluidine blue and rose bengal to kill microbes (*Staphylococcus aureus*, *Escherichia coli*, *Clostridium difficile*, a bacteriophage, and *Candida albicans*) on its surface when illuminated with white light.

In the United Kingdom, approximately 1 in 11 inpatients at any given time has an infection acquired in a hospital (9). Internationally, figures suggest a similar trend, with prevalence rates ranging from 4 to 10% (10). Hospital-acquired infections can often lead to complications with existing illnesses, may cause anxiety and discomfort, and can also lead to the death of the patient. Furthermore, infected patients remain in hospital on average 2.5 times longer than uninfected patients (11), resulting in increased costs. Although it was previously believed that the inanimate environment played little or no role in the transmission of infectious disease, this concept is now being reconsidered (3). The Centers for Disease Control and Prevention has now listed contact transmission—direct, from body to surface, or indirect, via contaminated inanimate objects—as one of the main routes of microbe transmission (6). The Department of Health (4) has also stated that "good hospital hygiene is an integral and important component of a strategy for preventing hospital-acquired infections." Contaminated environmental surfaces can contribute to the spread of microbes by acting as reservoirs from which personnel and patients can contaminate their hands (14). The problem is not only that such reservoirs exist but also that they can persist for long periods due to the ability of some microorganisms to survive on surfaces for days, weeks, and even months (8). It is widely recognized that the usual means of attempting to reduce the microbial load on environmental surfaces are ineffective. Detergent solutions, for example, often become contaminated during use, thus allowing the spread of microbes throughout the hospital via mopheads and cleaning cloths (14). There is a need, therefore, to develop new approaches to reducing microbial contamination of the hospital environment. One possible approach is to use light-activated antimicrobial agents incorporated into polymers which could be applied as coatings (either permanently or on a renewable basis) to hospital surfaces, provided that these could be activated by the ambient light conditions found in hospitals. A number of studies have shown that photosensitizers can retain their antimicrobial properties when attached to polymers (1, 17). In this study, the ability of such coatings to kill a range of microbes under lighting conditions likely to be present in hospitals has been investigated.

The light source used in this study was a General Electric 28-W Biax 2D compact fluorescent lamp that emits light across the visible spectrum. This lamp has the same color-rendering properties and spectral power distribution as the fluorescent luminaires used in hospitals in the United Kingdom. Prominent peaks were present at 435, 495, 545, 588, and 610 nm (Fig. 1A). The lamp was fitted into a refrigerated incubator (LTE Scientific Ltd., Oldham, United Kingdom) that maintained the temperature at a constant 22°C. The light intensity was measured using a digital luxmeter (Hagner Photometric Instruments Ltd., Bosham, United Kingdom). The photosensitizers toluidine blue O (TBO) and rose bengal (RB) were purchased from Sigma (Poole, United Kingdom). The coatings were prepared as follows. Cellulose acetate (Sigma) was dissolved in acetone (50 mg/ml), and stock solutions of the photosensitizers in acetone (100 μg/ml) were added to give a final concentration of 25 μM for each photosensitizer. Aliquots (450 μl) of each mixture were transferred to flat-bottom glass containers (diameter, 18 mm), and the acetone was left to evaporate overnight. The thickness of the coatings was measured using a Starrett (Athol, Mass.) micrometer and was found to be 43.2 ± 6 μm. The absorption spectrum of the coatings was determined using a UNICAM UV 500 UV/visible spectrophotometer (ThermoSpectronic) over the range 250 to 800 nm and is shown in Fig. 1B, where it can be seen that strong absorbance occurs between 500 nm and 675 nm, which includes three of the main emission peaks of the light source.

The organisms used were *Staphylococcus aureus* NCTC 6571, a methicillin-resistant strain of *S. aureus* (NCTC 13143), *Escherichia coli* NCTC 10418, *Candida albicans* (clinical isolate), *Clostridium difficile* 630 (clinical isolate), and bacteriophage ϕX174 (host organism, *E. coli* ATCC 13706). This particular bacteriophage was used because it has previously been used as a model virus in transmission studies and it has a stability comparable to the most resistant human-pathogenic viruses, such as the poliovirus (12). All bacteria (except for *C. difficile*) were maintained by weekly subculture on nutrient agar (Oxoid, Basingstoke, United Kingdom), while *Candida albicans* was subcultured weekly on Sabouraud dextrose agar (Oxoid). *C. difficile* was subcultured every 5 days onto brain

* Corresponding author. Mailing address: Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, 256 Gray's Inn Road, London WC1X 8LD, United Kingdom. Phone: 020-7915-1231. Fax: 020-7915-1127. E-mail: m.wilson@eastman.ucl.ac.uk.

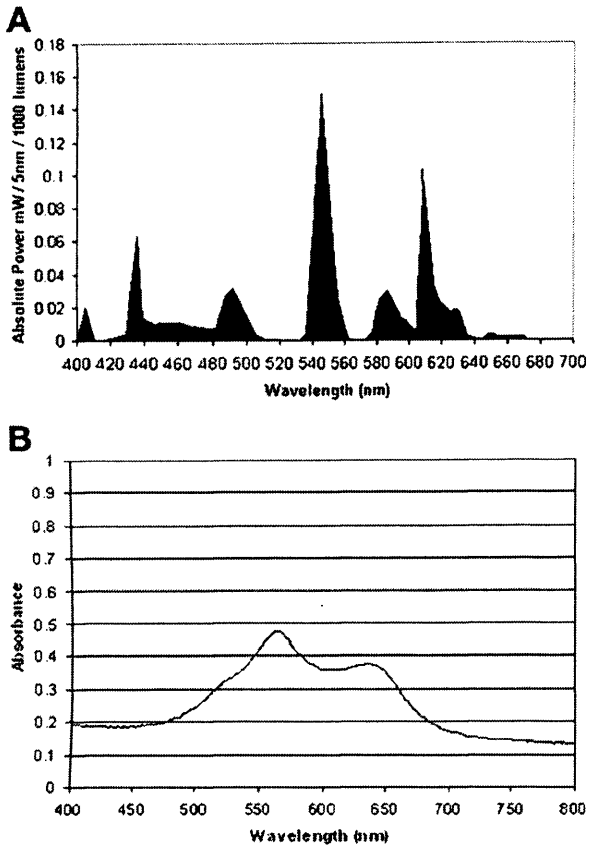


FIG. 1. (A) Emission spectrum of the 28-W fluorescent lamp used in the study. (B) Absorption spectrum of cellulose acetate coatings containing toluidine blue (25 μM) and rose bengal (25 μM).

heart infusion agar (Oxoid) supplemented with 5% horse blood, Clostridium supplement (Oxoid), and 10 μg/ml tetracycline. The phage was propagated and titered according to ATCC guidelines, and the resulting stock suspension was stored at 4°C. For experimental purposes, bacteria (except for *C. difficile*) were grown aerobically in nutrient broth, while *Candida albicans* was grown in Sabouraud dextrose liquid medium (Oxoid); all were incubated at 37°C for 16 h. *C. difficile* was grown overnight in brain heart infusion (Oxoid) at 37°C in an anaerobic cabinet (Don Whitley Scientific Ltd., United Kingdom). All organisms (except for the phage) were centrifuged, resuspended in an equal volume of phosphate-buffered saline (PBS), and then further diluted 1:1,000 in PBS. The resulting bacterial suspensions contained approximately 10^6 CFU/ml, while the count for *Candida albicans* was approximately 10^5 CFU/ml. In some experiments, *S. aureus* was resuspended in human saliva instead of PBS. The saliva was used undiluted without any further processing. For the bacteriophage, a 1:1,000 dilution of the stock suspension in PBS was used to give a PFU-per-milliliter count of around 10^6.

To determine the effectiveness of the photosensitizer-containing coatings, aliquots (250 μl) of each microbial suspension were placed on each of four photosensitizer-containing coatings in the glass containers, the mouths of which were then covered with transparent cling film. Two of these were exposed to light ($L^+ S^+$), while the other two were kept in the dark ($L^- S^+$). In addition, microbial suspensions were also inoculated onto four control coatings (containing no photosensitizer), two of which were exposed to light ($L^+ S^-$) and two of which were kept in the dark ($L^- S^-$). After incubation of the coatings in the refrigerated incubator at 22°C for 2, 4, 6, or 16 h, survivors were enumerated by viable counting of serial dilutions of the microbial suspension. Serial dilutions were prepared in sterile PBS, and 25-μl aliquots were plated on nutrient agar (for *S. aureus* and *E. coli*), Sabouraud dextrose agar (for *Candida albicans*), brain heart infusion agar supplemented with 5% horse blood, Clostridium supplement, and tetracycline (for *C. difficile*), or mannitol salt agar (for *S. aureus* when suspended in human saliva). Following incubation at 37°C under aerobic conditions (except for *C. difficile*, which was incubated anaerobically), the resulting colonies were enumerated. In the case of the bacteriophage, the number of PFU was determined as follows. Aliquots (30 μl)of each dilution were added to 300 μl of the host organism, *E. coli* ATCC 13706 (mid-exponential phase: 0.5 ml of overnight culture inoculated in 10 ml of nutrient broth and grown to an optical density of around 0.7 at 600 nm) in polypropylene phage tubes. Following incubation of the tubes at room temperature for 30 min, 3 ml of 0.5% nutrient agar (kept at 42 to 45°C) was added to each. Tubes were inverted and then poured evenly onto prewarmed nutrient agar plates. Plaques were counted after overnight incubation of the plates at 37°C.

In order to assess whether there had been any leaching of the photosensitizers out of the coating, in each experiment 1.0 ml of PBS was placed on a photosensitizer-containing coating, and following illumination, the absorbance of this solution at the peak absorbance of the photosensitizers (632 nm and 545 nm for TBO and RB, respectively) was measured. In all of the experiments, the absorbance of the solutions was found to be extremely low (A_{632}, <0.009; A_{545}, <0.003).

The Mann-Whitney U test was used to compare the number of survivors in the various suspensions ($L^+ S^+$, $L^+ S^-$, $L^- S^+$) with the number of survivors from the control samples ($L^- S^-$). A P value of <0.05 was considered statistically significant. In none of the experiments did exposure of the microbial suspensions to the coatings in the dark result in a significant reduction in the viable count of the suspension. Similarly, illumination of the microbial suspensions on the surfaces of the photosensitizer-free cellulose acetate coatings did not exert a microbicidal effect.

Following illumination for 2 h, the TBO-RB coating was able to achieve a 99.6% reduction in the viable count of a suspension containing approximately 2×10^6 CFU/ml of *S. aureus* (Table 1). After 6 h of illumination, a 100% kill was obtained (Table 1). A 100% kill of a methicillin-resistant strain of the organism was also achieved after 6 h of illumination (Table 1). *S. aureus* was also susceptible to killing when suspended in human saliva; no viable cells remained in a suspension containing 4.1×10^6 CFU/ml following 16 h of illumination. This is encouraging, because aerosols and droplets derived from oral and respiratory secretions are an important mode of transmission of infectious agents in a clinical setting (15).

C. difficile also proved to be susceptible to killing by the

TABLE 1. Effects on viable counts of contact with a cellulose acetate coating containing toluidine blue and rose bengal[a] and exposed to light from a 25-W fluorescent lamp

Organism	Light exposure time (h)	Viable count (CFU/ml)[b] under the following condition[c]:				% Reduction in viable count ($L^+ S^+$ vs $L^- S^-$)	Log_{10} reduction in viable count ($L^+ S^+$ vs $L^- S^-$)
		$L^- S^-$	$L^- S^+$	$L^+ S^-$	$L^+ S^+$		
Staphylococcus aureus	2	1.84×10^6	1.99×10^6	1.58×10^6	7.71×10^3*	99.6	2.4
Staphylococcus aureus	6	2.21×10^6	1.8×10^6	1.86×10^6	0*	100	6.3
Methicillin-resistant *Staphylococcus aureus*	6	2.69×10^6	2.81×10^6	3.02×10^6	0*	100	6.4
Clostridium difficile	4	5.19×10^6	2.63×10^6	1.57×10^6	0*	100	6.7
Candida albicans	16	1.99×10^5	2.18×10^5	2.33×10^5	2.39×10^4*	88	0.9
Bacteriophage ϕX174	16	1.34×10^6	9.13×10^5	8.15×10^5	1.2×10^5*	91	1.1
Escherichia coli	6	1.96×10^6	1.85×10^6	1.9×10^6	1.48×10^6*	24	0.1
Escherichia coli	16	1.92×10^6	1.79×10^6	2.09×10^6	0*	100	6.3

[a] Each at 25 μM.

[b] Asterisks indicate that the viable count was significantly different ($P < 0.05$) from that for the $L^- S^-$ condition by the Mann-Whitney U test.

[c] $L^- S^-$, microbial suspension in contact with photosensitizer-free coatings and not illuminated; $L^- S^+$, microbial suspension in contact with photosensitizer-containing coatings and not illuminated; $L^+ S^-$, microbial suspension in contact with photosensitizer-free coatings and illuminated; $L^+ S^+$, microbial suspension in contact with photosensitizer-containing coatings and illuminated.

illuminated coating: a 100% kill of a suspension of the organism (consisting mainly of vegetative cells) containing 5.19×10^6 CFU/ml was achieved after 4 h of illumination (Table 1). In contrast, the gram-negative organism *E. coli* appeared less susceptible, and little killing was observed after 6 h of illumination. However, illumination for 16 h resulted in 100% kills of a suspension containing approximately 2×10^6 CFU/ml of the organism (Table 1). It has been reported repeatedly that gram-positive bacteria are more susceptible to photodynamic inactivation than gram-negative bacteria, irrespective of which photosensitizer is used (16). The results obtained using photosensitizer-containing coatings in the current study support these findings. It is thought that the lower susceptibility of gram-negative bacteria is attributable to the presence of the outer membrane, which intercepts photogenerated reactive oxygen species (7). *Candida albicans* also appeared to be less susceptible than *S. aureus* to killing: an 88% reduction in the viable count of a suspension containing 1.99×10^5 CFU/ml was achieved after 16 h of illumination (Table 1).

Bacteriophage ϕX174 was susceptible to killing when the coatings were illuminated; a 91% reduction in the viable count of a suspension containing 1.34×10^6 PFU/ml was achieved after 16 h of illumination (Table 1). In terms of its ability to persist in the environment in an infectious state, bacteriophage ϕX174 has been shown to resemble the most resilient human-pathogenic viruses i.e., parvoviruses and polioviruses (12).

One possible problem associated with the use of such coatings is "photobleaching" of the photosensitizers, which can result from degradation of the photosensitizer by the singlet oxygen generated (5). However, when the photosensitizer-containing coating was exposed to seven cycles of alternating light and dark periods (16 h of light and 8 h of darkness), no reduction in its bactericidal activity was detectable; a 5.7 log_{10} reduction in the viable count of a suspension of *S. aureus* (2.3×10^6 CFU/ml) was obtained after 6 h of illumination. These findings suggest that photobleaching, at least in the short term, would not be a problem. If longer periods of light/dark or continuous light exposure did result in photobleaching, then it would be necessary to renew the coating on a regular basis, perhaps by spraying with a solution of the coating in a volatile solvent.

In this study we investigated the ability of a fluorescent lamp, similar to those used in United Kingdom hospitals, to activate photosensitizers embedded in a coating and thereby kill microbes in its vicinity. The results obtained demonstrate that it is possible to use light emitted by a fluorescent lamp to render cellulose acetate coatings containing TBO and RB effective at killing a range of microbes. TBO and RB were chosen for incorporation into the coating because together they are able to absorb strongly at many of the prominent wavelengths emitted by fluorescent lamps of the type commonly used in hospitals in the United Kingdom. The coatings were formed following evaporation of the solvent (acetone), and it is assumed that each photosensitizer molecule is embedded in the cellulose acetate layer, thereby trapping it but allowing the $^1O_2$ molecules generated upon exposure to light to diffuse out and thereby interact with, and kill, any microbes associated with the surface of the coating. The leakage experiments carried out in the study demonstrated that the amounts of TBO and RB released from the coatings were extremely small. It is unlikely, therefore, that the microbicidal effect observed could be attributed to the photosensitizers leaching out of the coatings and being activated while in the bacterial suspension.

The data presented here demonstrate the feasibility of using a coating containing photosensitizers to photoinactivate microbes. Moreover, the levels of killing achieved (up to a 6.7 log_{10} reduction) should be more than sufficient for surface disinfection, since microbial densities encountered on hospital surfaces are generally much lower. One study, for example, showed that between 4 and 7 CFU/cm^2 of *S. aureus* were present on the surfaces of rooms occupied by patients infected with the organism (13). Regulations governing lighting in hospitals in the United Kingdom specify minimum light levels for various locations within hospitals (2). For example, ward corridors need to have a minimum light intensity of 200 lx, while in Accident and Emergency examination rooms and operating theaters, light intensities of 1,000 and 50,000 lx, respectively, are necessary. Since the light intensity used in this study was 3,700 ± 20 lx, the light-activated coating described and tested here would be of particular use in examination rooms and operating theaters, where light intensities are highest. However, it is also possible that these coatings could achieve ap-

preciable kills under lower light intensities if the illumination time were increased to produce higher light energy doses, e.g., by leaving lights on for 24 h per day. Alternatively, lamps emitting a higher light intensity could be used. Overall, these coatings show potential as self-disinfecting surfaces. The next step will be to perform a detailed evaluation of the activity of the coatings in the hospital environment.

This work was supported by a grant from the Charles Wolfson Charitable Trust.

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