Nano-scale systems for the detection and treatment of bacterial infections in burn wounds: Modes of action and efficacy

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The University of Bath
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Signed
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Abstract

Bacterial infections are and likely always will be a serious and costly complication to treatment in a healthcare environment. However consistent rises in the number of both healthcare associated and antibiotic resistant infections over the last of decades has the potential to turn a serious problem into a catastrophe. Control of infections in hospital wards has improved over the last five years but data from the European Centre for Disease Prevention and Control suggests a stale mate. While the numerical rise in drug resistant organisms has slowed, the severity of drug resistance appears to be on the increase with the prolific emergence of multiple drug resistant isolates.

On the front lines of the threat that these organisms represent are some of the most susceptible. In hospitals those who are already sick are more vulnerable, those with co-morbidities, those with surgical or other wounds, the very old and the very young. Children especially show high susceptibility as they are often incapable of communicating clinical complications in the way an adult might. This coupled with higher commonality of specific aetiologies in children such as scalds, open wounds that are prone to infection without proper treatment, creates population in need.

Antibiotics are often thought to be part of the problem in drug resistance, indeed to an extent they are. However their real downfall may be improper use. In order to improve treatment outcomes and simultaneously decrease antimicrobial resistance a combination of rapid diagnosis and prophylaxis can be utilised to decrease selection of resistance.

As such, this study focuses on the development of a novel vesicle based sensor system for the detection of bacterial infections in burn wounds. Additionally an organometallic antimicrobial system has been developed with the potential for surface attachment. Work with the vesicle based biosensor demonstrates high sensitivity to both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The toxins involved in activation of the sensor have been determined in both cases and an in-depth study into the activity of the staphylococcal agents of lysis (Phenol Soluble Modulins and delta haemolysin), shows a high degree of plasticity and tunability in the sensors function. Work with the zinc based antimicrobial reveals a highly complex system which demonstrates possible functions as a not only an antimicrobial but as a sensor system in its own right.
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P.aeruginosa

The activity of the Staphylococcal PSMs against DPPC53 vesicles

Structural differences between vesicle lytic species regulated by AGR

Screen of the lytic potential of burn pathogen supernatant towards phospholipid vesicles

Haemolytic activity of common burn pathogens.

Aims and Objectives

Retention of plasma deposited compound

Energy dispersive X-ray spectroscopy

Measurement of Relative Fluorescence

FTIR of Fabric swatches

The effect of varying lipid composition on toxin bilayer interactions

The lytic capacity of burn pathogen supernatant against phospholipid vesicles

Lytic potential and mediators of lysis of Staphylococcus aureus

Phenol soluble modulins as vesicle lytic agents of S.aureus lysogenic species regulated by AGR

The lytic potential and mediators of lysis of Pseudomonas aeruginosa

Staphylococcus aureus

Aims & Objectives

Anylytical techniques

FT-IR of Fabric swatches

Measurement of Relative Fluorescence

Scanning electron microscopy

Energy dispersive X-ray spectroscopy

Retention of plasma deposited compound

Data processing

Anylytical techniques

Introduction

Tuning phospholipid vesicles to bacterial toxins

Aims & Objectives

Activity of bacterial toxins against natural and synthetic bilayers

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The activity of the Staphylococcal PSMs against DPPC53 vesicles

Acitivity of PSMs against eukaryotic cells

The lytic capacity of Staphylococcal supernatant

P.aeruginosa rhamnolipids

The effect of varying lipid composition on toxin bilayer interactions

Altering bilayer fluidity

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<th>Definition</th>
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<tr>
<td>a</td>
<td>Cross sectional area of lipid polar headgroup</td>
</tr>
<tr>
<td>aA</td>
<td>Attenuation coefficient of absorption</td>
</tr>
<tr>
<td>AGR</td>
<td><em>Staphylococcus aureus</em> accessory gene regulator</td>
</tr>
<tr>
<td>AIP</td>
<td>Auto inducing peptide</td>
</tr>
<tr>
<td>aS</td>
<td>Attenuation coefficient of scattering</td>
</tr>
<tr>
<td>b</td>
<td>Length scale between plates in membrane plate model</td>
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<tr>
<td>BMJ</td>
<td>British Medical Journal</td>
</tr>
<tr>
<td>C</td>
<td>Curvature</td>
</tr>
<tr>
<td>CA</td>
<td>Community acquired</td>
</tr>
<tr>
<td>Can</td>
<td>Staphylococcus aureus Collagen attachment protein</td>
</tr>
<tr>
<td>CF</td>
<td>5(6)-carboxyfluorescein</td>
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<tr>
<td>CF50</td>
<td>5(6)-carboxyfluorescein at a concentration of 50mmol dm(^{-3})</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>cho</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Clf</td>
<td>Staphylococcus aureus clumping factor</td>
</tr>
<tr>
<td>CN</td>
<td>Number of membrane configurations in membrane plate model</td>
</tr>
<tr>
<td>CR</td>
<td>Congo red</td>
</tr>
<tr>
<td>CRAC</td>
<td>Cholesterol recognition amino acid consensus</td>
</tr>
<tr>
<td>d</td>
<td>Thickness of an infinitesimal slice of an optically active region</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>DMPA</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimiristoyl-sn-glycero-phosphatidylcholine</td>
</tr>
<tr>
<td>DMPE</td>
<td>1,2-dimiristoyl-sn-glycero-phosphatidylethanolamine</td>
</tr>
<tr>
<td>DOGM</td>
<td>1,2-distearoyl-3-octaethyleneglycol ether methacrylate</td>
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<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
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<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
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<tr>
<td>DOPS</td>
<td>1,2-dioleoyl-sn-glycero-3-phospho-L-serine</td>
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<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-phosphatidylcholine</td>
</tr>
<tr>
<td>DPPE</td>
<td>1,2-dipalmitoyl-sn-glycero-phosphatidylethanolamine</td>
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<td>DSPC</td>
<td>1,2-distearoyl-sn-glycero-phosphatidylcholine</td>
</tr>
<tr>
<td>DSPE</td>
<td>1,2-distearoyl-sn-glycero-phosphatidylethanolamine</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
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<tr>
<td>EDANS</td>
<td>5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid</td>
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<tr>
<td>EDX</td>
<td>Energy-dispersive X-ray spectroscopy</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Esphere</td>
<td>Theoretical bending energy of a sphere</td>
</tr>
<tr>
<td>f</td>
<td>Equivalent packing factor of lipids</td>
</tr>
<tr>
<td>F</td>
<td>Free energy density</td>
</tr>
<tr>
<td>F</td>
<td>Ground state fluorophore</td>
</tr>
<tr>
<td>F*</td>
<td>Excited state fluorophore</td>
</tr>
<tr>
<td>FAb</td>
<td>Fragment of antibody binding (antibody)</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment of crystallisation (antibody)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Fnbp</td>
<td>Staphylococcus aureus fibronectin binding protein</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photo bleaching</td>
</tr>
<tr>
<td>FRSK</td>
<td>Foetal rat skin keratinocyte</td>
</tr>
<tr>
<td>g</td>
<td>Geometric packing factor of lipids</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GUVs</td>
<td>Giant unilamellar vesicles</td>
</tr>
<tr>
<td>h</td>
<td>Concentration of scattering particles within a sample</td>
</tr>
<tr>
<td>HA</td>
<td>Hospital acquired</td>
</tr>
<tr>
<td>HCAI</td>
<td>Health care associated infection</td>
</tr>
<tr>
<td>HELICS-ICU</td>
<td>Hospitals in Europe Link for Infection Control through Surveillance intensive care unit protocol</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>hla</td>
<td><em>Staphylococcus aureus</em> alpha haemolysin</td>
</tr>
<tr>
<td>hlb</td>
<td><em>Staphylococcus aureus</em> beta haemolysin</td>
</tr>
<tr>
<td>hld</td>
<td><em>Staphylococcus aureus</em> delta haemolysin</td>
</tr>
<tr>
<td>I</td>
<td>Light intensity</td>
</tr>
<tr>
<td>iBID</td>
<td>International Burn Injuries Database</td>
</tr>
<tr>
<td>If</td>
<td>Fluorescence intensity</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISDA</td>
<td>Infectious diseases society of America</td>
</tr>
<tr>
<td>K</td>
<td>Stern-Volmer quenching constant</td>
</tr>
<tr>
<td>kb</td>
<td>Bending rigidity</td>
</tr>
<tr>
<td>kg</td>
<td>Gaussian bending rigidity</td>
</tr>
<tr>
<td>knr</td>
<td>The rate of non-radiative decay</td>
</tr>
<tr>
<td>kq</td>
<td>Bimolecular quenching constant</td>
</tr>
<tr>
<td>l</td>
<td>Acyl chain length</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani Broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>LUVs</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>Lα</td>
<td>Liquid crystalline bilayer phase</td>
</tr>
<tr>
<td>Lβ</td>
<td>Liquid gel bilayer phase</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial surface components and adhesive matrix molecules</td>
</tr>
<tr>
<td>MSK</td>
<td>Membrane skeleton</td>
</tr>
<tr>
<td>MSSA</td>
<td>methicillin sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>N</td>
<td>Number of plates in the membrane plate model</td>
</tr>
<tr>
<td>n0</td>
<td>Number density of ions within a closed system</td>
</tr>
<tr>
<td>NICE</td>
<td>National institute for health and care excellence</td>
</tr>
<tr>
<td>NRK</td>
<td>Normal rat kidney fibroblast</td>
</tr>
</tbody>
</table>
P Averaged packing factor of a lipid mixture
PAMPS Pathogen associated molecular patterns
PC Phosphatidyl choline
PCDA 10,12-pentacosadionylic acid
PE Phosphatidyl ethanolamine
PECVD Plasma enhanced chemical vapour deposition
PINIPAM Poly(N-isopropylacrylamide)
PL Phospholipid
PLC Phospholipase C
POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PRG Photo initiated radical grafting
PSM Phenol soluble modulin
pvl Staphylococcus aureus Panton valentine leukocidin
Pβ Gel/Crystalline mixed bilayer phase
Q Ground state quencher
Q* Excited state quencher
R Radius of curvature
Re Effective molar ratio
RF radio frequency
RL Rhamnolipid
RND Resistance nodulation division family of cation transporters
ROT Staphylococcus aureus repressor of toxin
Rv Vesicle radius
SB2 (E)-2-(((2-(cyclohex-1-enyl)ethyl)imino)methyl)-4-methoxyphenol
Sdr Staphylococcus aureus serine aspartate repeat protein
SEM Scanning electron microscopy
Spa Staphylococcus aureus protein A
spp Species
T Temperature
t Time point
TBSA Total body surface area
TCDA 10,12-Tricosadiyonic acid
TSA Tryptic soy agar
TSB Tryptic soy broth
TSS Toxic shock syndrome
UV ultraviolet
v Acyl chain hydrophobic volume
V Volume of an individual lipid molecule
V Voltage
VOC Volatile organic compound
z Light beam path length in the Z-axis
ZSB2 -(E)-2-(((2-(cyclohex-1-en1-yl)ethyl)imino)methyl)-4-methoxyphenol
Zn Zinc
Γ The average perimeter of open bilayer in membrane plate model
$\Gamma$ Emission rate of a fluorophore
$\gamma_m$ The fractional energy transfer of an electron
$\varepsilon_0$ Permittivity of free space
$\lambda$ Line tension energy of a bilayer
$\lambda_D$ Debye length
$\lambda_{em}$ Wavelength of maximum fluorescence emission
$\lambda_{ext}$ Wavelength of maximum fluorescence excitation
$\lambda_{max}$ Wavelength of maximum absorption
$\tau_0$ Unquenched fluorescence lifetime
$\phi$ Fluorescence quantum yield
Chapter I. Introduction

Section 1.01 Wounds and infection

(a) Prevalence of healthcare associated infection

Healthcare associated infections, (HCAIs), also referred to as nosocomial infections, are characterized as resulting from either direct medical or surgical interventions, as per surgical site infections (SSI’s), or resulting from contact with a healthcare setting [1, 2]. In 2009 it was estimated that HCAIs cost the UK government around £1 billion a year and affected over 300,000 patients in England [3]. Estimates of the cost of HCAIs in the United States and Europe have been similarly large, estimated at anywhere between $5.7 and $16.6 billion for hospital inpatients in the U.S.A [4, 5] and €800 million per year in European hospitals [6].

The majority of the costs of HCAI’s fall on length of stay of patients in ICU’s with money being spent on labour, building space, utilities, equipment, supplies and purchases the hospital must make to sustain the ICU facility [4]. Costs also increase in patients with suspected HCAI’s. A 2003 study in the United States found that while confirmed HCAI’s result in additional costs to health care institutions of $15,275 per patient, suspected cases also demonstrated an increase in cost at $6767 per patient [7]. Although costs will vary between countries, it is not unreasonable to assume that patients in European countries will generate similar costs whether an infection is confirmed or just suspected. As well as the economic cost, the human cost is similarly great. One study in the United states found a six-fold increase in mortality in patients with a confirmed HCAI [8] and a report by the European Science foundation in 2005, suggested a conservative mortality rate of 175,000 deaths per year in Europe, attributable to increased mortality from nosocomial infections [9].

In the UK, the two organisms most commonly associated with HCAIs are *Clostridium difficile*, which is associated with gastrointestinal infections, primarily in the elderly post antibiotic treatment, and methicillin resistant *Staphylococcus aureus* which is estimated to be responsible for around 4% of all blood stream infections and 15.8% of all system infections [10]. Within Europe as a whole, a number of organisms are considered important amongst HCAI’s due to their prevalence in intensive care unit (ICU) acquired
infections. These organisms include *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli* and *Pseudomonas aeruginosa* [11]. These are focused on due to their prevalence (11%, 3%, 7.4% and 8.9% for *S.aureus*, *A.baumannii*, *E.coli* and *P.aeruginosa* respectively), pathogenicity and burden of antimicrobial resistance. Forty two per cent of *S.aureus* infections for example, were identified through the “Hospital in Europe link for infection control through surveillance intensive care unit” (HELICS-ICU) protocol were MRSA’s [12].

The latest report from the European Centre for Disease Control (EDAC) found that the overall prevalence of HCAI’s in acute care settings was 7.1%. The report also goes on to state that more focus is needed on prevention and control of antibiotic-resistant bacteria and the prudent use of antibiotics [2]. According to the European antimicrobial surveillance network (EARS-net) as a general trend. The number of antimicrobial resistant invasive bacterial isolates continues to rise [13]. This however varies greatly from region to region and from organism to organism. Using *S.aureus* as a pertinent example it can be seen that whilst rates of methicillin resistant *S.aureus* decreased in several countries, (Austria, Cyprus, Estonia, France, Greece, Ireland and the UK) over the years 2007 to 2011, rates in other countries have clearly increased (Figure I-1). It is also important to note that while rates in some countries have decreased, the prevalence of MRSA’s in eight out of the 28 European member states is above 25% of total *S.aureus* isolates.
Figure 1-1 Variation in the percentage of MRSA isolates in European hospitals between 2007 and 2011 generated by the European Centre for Disease Controls surveillance system, (TESSy). The prevalence of clinical isolates of MRSA amongst European countries is presented as a percentage of total isolates. Values are banded and colour coded between <1% and ≥50% for individual countries for the years of 2007 (A) and 2011 (B).
Current methods for control of HCAI rates are largely preventative and are based on rigorous surveillance and limitation of spread, best exemplified by Denmark’s hospital admission screening, (search and destroy) policy and adequate guidelines for the treatment of infections in hospitals [14, 15]. Whilst these methods do show efficacy in reducing infection rates, there are also constant front-line battles going on with the emergence of community acquired MRSA’s (CA-MRSA) and the potential for spread to hospitals, the high prevalence, of porcine MRSA’s within farming communities in the Netherlands [16], and the epidemic-like qualities which CA-MRSA infections attained within the United States since the mid 1990’s [17].

Taken as a whole, the evidence demonstrates that HCAI’s, whether suspected or actual, generate a large burden of cost on healthcare systems the world over as well as increasing the mortality of patients. This creates a clear driver for the development of technologies for both the early identification and treatment of nosocomial infections. These could potentially help by a) decreasing the prevalence of such infections occurring and b) by increasing clinician’s ability to diagnose infections. As such the aim of the work presented here was to generate novel methods of identifying and controlling bacterial infections in the wound environment, specifically in paediatric burn wounds. Hopefully, this will allow both the prevention of patients being treated unnecessarily as well as a decrease in mortality and shorter hospital stay for infected patients.

(b) Causes of antimicrobial resistance

As previously stated, antimicrobial resistance is a serious problem in hospital infections which again leads to increases in cost and mortality [18]. One cohort study in 2005 looking at *S.aureus* bacteraemia patients showed an increase in cost of around $7000 between patients with an MSSA infection and those with an MRSA infection [19]. In an attempt to look further into the future of healthcare, a current report in BMJ suggests that the healthcare community may be greatly underestimating the cost of resistance due to antimicrobials being so heavily relied on as a prophylactic treatment and the current lack of investment in developing new antimicrobials [20]. If trends in resistance [21] continue we will eventually run out of drugs to treat patients with. This will mean currently routine procedures, which require prophylaxis for infection prevention, such as hip prosthesis will
become less common and burdens of morbidity and associated cost will increase. Mortality will also increase as patients essentially become untreatable.

The molecular mechanisms by which bacteria generate chromosomal mutation i.e. conjugation, transformation and transduction, (Figure I-2) are common to development of antimicrobial resistance as well as numerous other evolutionary processes which these microorganisms might undergo [22]. As such they are fairly well understood. In order to properly understand the development of antimicrobial resistance however, we need to understand the pressures selective for resistance which are placed on the organisms and how conditions favourable to gene transfer are brought about. These mechanisms are both numerous, complex and show high variability between organisms.
Figure I-2 Modes of bacterial gene transfer. (A) bacterial transformation whereby genetic information released into the environment by a donor cell, either through destabilization of the donor cell’s membrane or active release e.g. as a biofilm structural element, is taken up and incorporated into the genome of a recipient cell. (B) Bacterial transduction where transfer of genetic material is mediated by phage either through acquisition of material during the lysogenic lifecycle or through capsid packing in the lytic lifecycle. (C) Bacterial conjugation where a transposon is inserted into a donor cell plasmid, a mating bridge is formed between donor and recipient cells and the plasmid undergoes rolling circle replication. One of the main conditions required for transfer of antibiotic resistance is proximity. Organisms which take up foreign DNA, by whatever means, and incorporate it into their own genome must be physically close to that source of hereditary material. This is true whether the source of such material is the reservoirs of resistance elements that occur naturally in some organisms [23-25] (the resistome), or if the necessary genes are found in other pathogenic strains. Whilst this seems like an obvious and possibly moot point to make it is an important one. There are a limited number of situations in which antibiotic susceptible bacteria are in an environment suitable for direct genetic transfer from compatible bacteria or phage and it is precisely the environment which can affect horizontal gene transfer. A study performed in 2003 looked at the likelihood of horizontal gene transfer (HGT) between the genomes of eight different organisms and found a positive correlation between gene transfer and genome size, G/C content of genomes, carbon utilisation and temperature as well as salinity, pH and pressure [26].

A potential example of such an environment would be the now highly popularised bacterial biofilm. Naturally occurring bacterial biofilms are usually multispecies and represent highly dynamic colonies of cells embedded within an exo-polymeric matrix which form at either a solid/liquid or a liquid/gas interface [27]. It has been hypothesised that biofilms are highly favourable to HGT due to numerous species existing in close proximity in a relatively stable environment [28]. Conversely it has also been suggested that plasmids involved in HGT promote biofilm formation as prolonged and stable social interaction between bacteria increases the plasmids own success [29]. Given that biofilms and not
planktonic bacteria are now considered to make up the majority of microbial populations; that they can exist in most environments including on both biotic [30] and abiotic [31] surfaces found in wounds; that the structures themselves confer some intrinsic antibiotic resistance [32] and that biofilms can be involved, at least experimentally, in increased rates of transfer of resistance elements [33] they can be seen as a potentially favourable environment for development of resistance. Whilst biofilms are not necessarily the only micro-scale environment in which antimicrobial resistance might arise, they do represent how dynamic microorganisms can be in response to environmental stressors and how environmental factors can collude to ensure that the rise of resistance is more likely.

Antibiotic use or antibiotics present in a microorganism’s environment are widely agreed to be the main selective pressure for development of resistance [34], although this is difficult to prove categorically. As such correlations have been broadly made in several different studies between geographical distribution of antibiotic prescription and variations in the levels of resistance [35-38] In Figure I-3 below, a correlation can be seen between the use of penicillin’s in the community by country and the incidence of penicillin resistant *Streptococcus pneumoniae* [39].
Such relationships between antibiotic use in individuals and antibiotic resistance suggest that one environment in which selective pressures can collude to generate resistance is in the patients being treated with antibiotics. Indeed a 2002 study concluded that exposure to antimicrobial drugs, amongst other things, was one of the risk factors involved in succumbing to a drug resistant infection [40].

Other example environments in which the consistent levels of antimicrobial use over time required to select for resistance are found are agricultural environments and waste effluents. Antibiotics are used in agriculture prophylactically in order to prevent spread of disease amongst a herd as well as being used as growth promoters in some animals. In the European Union use of several antibiotics as growth promoters has been banned. The ban has provided the potential to study the effects of withdrawal of antibiotics on the “de-selection” of resistance. Figure I-4 was taken from review in 2003 which demonstrated the
positive correlation between decrease in the use of antimicrobial growth promoters and decrease in the levels of *Enterococcus faecium* showing resistance to medically important antimicrobials seen since the 1997 ban on antimicrobial growth promoters (AGP’s), like avoparcin, (a glycopeptide with efficacy against gram positives) [41]. The sentiment that antibiotics in animal feed are likely to be a harbour for the development of resistance has also been recently echoed in a 2013 BMJ article [42].

![Graph showing weight of active antimicrobial growth promoters](image)

**Figure I-4** Weight of various active antimicrobial growth promoters used in animal feed per year in Denmark and prevalence of drug resistant *Enterococcus faecium* found in stool samples of healthy animals at slaughter over the same time period. Figure taken from Antibiotics in animal feed and their role in resistance development. *Current Opinion in Microbiology* 2003 [41]

The final source of antimicrobial resistance I will exemplify is that of waste effluent. Antimicrobials and antimicrobial resistant organisms in waste water can be found as run off from manufacturing plants and in sewage waste [43-45]. Whilst the antibiotics themselves generate a necessary selective pressure, the pool of antimicrobial resistance genes (ARG’s) found in mobile genetic elements of already drug resistant organisms within this water supply also supply some of the material required for the development of resistance. A 2011 study looking at effluent from an antibiotic manufacture facility demonstrated that water contaminated with antibiotics promotes resistance genes in.
environmental bacteria, in this particular case a diverse array of mobile fluoroquinolone resistance genes [46]. Another study in 2010 looked at bacterial populations receiving water effluent from an oxytetracycline production plant in China and found that 95% of the downstream isolates contained tetracycline resistance genes [47]. In both cases, contamination of water supplies has been shown to have an effect on the development of resistance. What has not been established as clearly is how or whether these resistant organisms can migrate back into situations of medical relevance. Although contaminated water supplies are potentially problematic, it depends what if any kind of treatment the water source will undergo before being re-introduced to human populations, as well as whether resistance will be maintained in the absence of selective pressures. On this last point there is some mixed opinion, but one recent review suggests that if patterns of resistance are reversible, that reversal will likely be slow [48].

The current picture we have of antimicrobial resistance is that it is a problem that’s not going away, due to increasing use of antibiotics (particularly in the third world) and lack of research into new antimicrobials. As such, any mechanisms developed to detect wound site infections should ideally be rapid and discriminatory, in order to prevent the use of broad spectrum antibiotics in sick patients which could potentially lead to rapid development of resistance in colonising organisms. Also, if any antimicrobial mechanisms are to be put in place, it should be ensured that they do as little as possible to encourage the development of resistance and ideally prevent initial biofilm formation.

(c) Current practices for the treatment of healthcare associated infections and their difficulties.

Working from the assumption that HCAIs are present in acute care settings, current practices for treating such infections vary greatly depending on the patient, the type of infection, the site of infection and the country the patient happens to be in. The most common types of nosocomial infection according to a report in the Lancet in 2003 are, respiratory, urinary and venous or central line catheter with smaller percentages of HCAI’s belonging to soft tissue and wound site infections [49]. The traditional approach to confirmed or suspected HCAI’s in the UK is to initially treat with an inexpensive narrow spectrum antimicrobial agent. If the patient deteriorates or a multi-resistant organism is found the therapy is broadened. Antimicrobial treatment is also coupled with other
treatments when possible, such as drainage of infected fluid from the wound site and debridement of infected tissue.

Numerous studies have highlighted the inadequacies that can be found when treating nosocomial infections [50, 51]. These studies suggest that treatment should be started as early as possible and should target the organism likely responsible for the infection. Studies also suggest that local patterns of antimicrobial resistance and susceptibility in suspected organisms should be taken into account when choosing a treatment option [50].

Lack of early, adequate, antibiotic treatment has been shown to increase patient mortality while early treatment has been shown to decrease length of hospital stay [52-54]. Adequate antimicrobial treatment in these cases is defined as the administration of antimicrobials with demonstrated bacteriostatic or bactericidal activity against the identified etiologic agents of infection. In the study showing increased patient mortality [52], delays in starting treatment were mainly due to delays in writing antibiotic orders. Before orders are made however patients must first meet the diagnostic criteria for the condition they are suspected of being subject to. These criteria are a combination of a clinician’s expert opinion and hospital lab work. Prior to diagnostic criteria being met for an infection, treatment with antibiotics is usually started on an empirical basis. This is due to laboratory tests on appropriate samples commonly taking up to 48 hours to process [55, 56].

De-escalation is the procedure of giving narrow spectrum antibiotics early in a patient’s treatment when an infection is suspected but not confirmed [57, 58]. The process is designed to keep levels of unknown pathogens low whilst preventing the development of resistance to more targeted members of the pharmacological arsenal to which the clinician has access. This manoeuvre is commonly used in that period prior to the receipt of lab results. It is however, not without its own inherent risks. The assumption that sufficient knowledge of the relationship between use and resistance is available in order to tailor regimes of medication to minimise resistance is questionable. Certain antibiotics will have narrow enough activity to avoid generating resistance in problem strains or organisms that a clinician may be worried about. They may however, have a much greater effect on other strains which were outside the scope of concern [59]. In addition, the designation of susceptible or resistant to an organism is also ultimately a clinical definition based on the likelihood of a successful therapy. Use of an “inactive” antibiotic does not prohibit the
possibility of the drug interacting with that pathogen in a way which might generate resistance without our knowledge. Despite criticisms, empirical evidence does lean cautiously toward de-escalation being useful in some therapies [60-62].

Determining the pathogen responsible for an infection or the presence of an infection in the first place has been demonstrated to be complex and time consuming. Time taken to correctly diagnose a patient may be time that he or she does not have and application of inappropriate antibiotics in patients with a suspected infection can potentially lead to the rise of a drug resistant strain, which will be harder to treat and can diminish the chances of a good outcome for the patient. This generates a catch 22 situation. The aims of this project then, in early detection of infection in the wound site, could potentially remove some of the ambiguity from treating suspected infections as well as, if correctly tuned, bringing some clarification to the type of infection present before lab results are returned.

(d) Commonality, aetiology and cost of burn injuries.

The National Burn Care Review published in 2001 [63] estimates that burn injuries are experienced by about 250,000 individuals per year. Around 175,000 of these individuals will visit accident and emergency departments and around 13,000 of them will be admitted to hospital. Around 300 of the individuals will die as a result of their injuries. Patients who survive burn injuries can struggle with increased chances of infection and once their hospital treatment has come to an end, the physical, psychological and social impacts of any scarring they are left with [64, 65].

Primary diagnoses from the 2011-2012 hospital episode statistics show that 13,187 individuals were admitted for burn and/or corrosion injuries during the period. Of this group the majority of individuals were under the age of 14. Figure I-5 shows the variation in burn injuries by age group between the years 2003 and 2012. While the age categories of this initial data are broad, the 15-59 years group is three times the size of the 0-14 years age group. As such we might logically expect the number of patients in the older age range to be three times greater than that in the younger age range. However as we can see the number of patients are very similar in both groups. This suggests that younger individuals are three times as likely to incur burn injury or at least three times as likely to be seen by a health professional as the result of a burn injury. Another important point generated by this data is the temporal trend seen in these two age groups. Between 2003
and 2012 the older age group has more or less seen a levelling off in the number of patients sustaining burn injuries year to year. In the 0-14 years age group however there has been a general increase in the number of patients received with burn injuries. An increase over the decade of around 2000 patients is greater than any other age range.

![Figure I-5](image)

**Figure I-5** Total number of patients presenting with a primary diagnosis of burn or corrosion injury between the years of 2003-2012. Patients are split into four age groups, 0-14, 15-59, 60-74 and 75+ years of age. All data was harvested from hospital episode statistics, admitted patient care, primary diagnosis summaries from the years listed. These reports were downloaded from Health and Social Care Information Centre (www.hscic.gov.uk).

Following recommendations in the 2001 burn care review, the international burn injury database (iBID) was set up in 2005 to collect information from burn centres across the UK. The first report was released in 2008. The report uses data collected from 1987-2007 and demonstrates that the greatest number of burn injuries is in young children ranging from 0-5 years of age (Figure I-6). Although these burns are most numerous, they score lower in severity and complexity [66] than some of the burns presented in the older age groups (25-
44 years of age). The split in age groups between severity and number of burns is likely in part due to the type of burns, a higher percentage of flame burns are seen in adults which will have complexities such as a tracheal burning from hot air inhalation [67, 68], as well as complicating co-morbidities such as respiratory conditions which are not seen in children and higher percentage total body surface areas (%TBSA) burns in adults.

![Figure 1-6 Number of burns of a given total body surface area per age group. Data is taken from the 2008 international burn injury database report which was collected between 1987 and 2007.](image)

The trend in deaths by age group generally shows a positive correlation with burn severity. However, this trend is skewed slightly in the older age groups, where there are a higher number of deaths in patient groups with moderate and moderate/severe burns (Figure I-7). Again, this is likely to be a consequence of greater co-morbidities found in the higher age brackets [69]. It is also important to note the relatively high number of deaths seen in those under the age of two with severe rather than severe/complex burns. This suggests that younger children are more likely to succumb to more simple injuries than their older counterparts.
Figure I-7 Correlation between burn wound severity and morbidity amongst different age groups. Burn classifications are based on simplistic variables of age, total body surface area and length of stay. Based on these factors, the workload they represent and accordingly, the type of facility which will be required to treat such patients, they are classified as moderate severe (requiring treatment in a burn unit), severe (requiring treatment in a burn unit/burn center) and severe complex (requiring treatment in a burn center). Data for the figure was taken from the 2008 international burn injury database report and was collected between 1987 and 2007.

As mentioned before, there is also marked variation in the types of burn injuries experienced by each age group. The most common type of burns experienced by children (0-14.9 years of age) and the elderly (55-120 years of age) are scalds, making up 61% and 38% of total injuries respectively. Whereas, the scalds category only makes up 24% of the burns experienced by adults (15-54.9 years of age), the most common burn here, being flame injuries at 28% (Figure I-8). The report also highlights that the majority of burn injuries for all age ranges, just over 65% of the total, occur in the home and of those, 35% occur in the kitchen [70]. The next most common locality for burn injuries is an industrial work setting that makes up less than 7% of the total number of injuries and which is populated almost entirely by working age adults.
A highly comprehensive report similar to the iBID 2008 report is produced by the American Burn Association. The report covering 2011 to 2012 looks at 183,036 patients admitted to 91 hospitals across 35 states [71]. Similarly to the UK’s iBID report, most burns are seen in children, although the percentage of total cases is much lower at 19%. The report also agrees that the majority of burns (72%) are small (under 10% TBSA) and that the most common aetiologies are scalds (again being most prevalent in under-five’s), and flame burns.

By looking at a paper produced in 2006 by clinicians at the South West Regional Burns Centre at Frenchay Hospital in Bristol [72] it can be seen that the majority of children’s burns actually occur in a much smaller age range than the range used in the 2008 iBID report. Around 80% of those admitted are under the age of five [73]. The paper does agree with the report in that the majority of the cases are small, uncomplicated scalds covering
less than 10% of TBSA. The paper also demonstrates that although the majority of burns are small and of partial thickness, the cost of treatment is still high due to more specialised treatments, including the involvement of specialised services such as plastic surgery, (as recommended in the 2001 burn care review [63] for all cases severe enough to require hospital admission), and specialist skin substitute dressings being required for better outcomes.

The paper looks at three case studies in order to estimate the cost of children’s burns to the NHS. In all cases patients were ≤2 years of age and suffered less than 5% TBSA scald caused by spillage of hot liquids. The average cost of the three patients was £1849 with the majority of the costs being spent on total hospital stay. It is important to note that these estimates cover only direct costs once a child is admitted. Hospital transfers etc. prior to admittance as well as scar management and psychological support, which can continue for years after the injury at considerable expense are not included [74]. It is important to note that none of these cases were treated for infection which would also exacerbate costs.

The large proportion of burn injuries in the UK and elsewhere [75, 76] that occur in children under the age of five, as well as the high initial and long term costs of treatment in these cases make them worthy of study as a demographic. This data coupled with the low incidence of co-morbidities (and therefore complexities), seen in children and the relatedness of a large proportion of cases (a high number of small to medium partial thickness burns), ensured that technologies were developed specifically with paediatric burn wound infections in mind.

(e) Pathology and infections in Paediatric burns.

Burn injuries are primarily a form of necrotic damage generated in tissues through protein coagulation, subsequent cell damage and death as result of exposure to a heat source. While the heat source and the resulting local biochemistry may vary slightly, the macro-scale damage is markedly similar [77]. As previously stated the majority of thermal injuries in children under the age of 5 are scalds. Scalds differ from other types of thermal injury only in that they are caused by moist heat, tend to be shallower partial thickness burns and show intermingled patterns of collagen damage rather than the clear demarcation zones seen in contact burns [78] (Figure I-9).
Partial thickness burns can be classified as either superficial or deep. Superficial partial thickness burns extend through the outermost layer of skin the epidermis, which consists of proliferating keratinocytes at the basal membrane and differentiated suprabasal keratinocytes, and into the superficial layer of the dermis. Deep partial thickness burns extend into the reticular layer of the dermis, which is comprised of a complex extracellular matrix with embedded adipocytes, fibroblasts and macrophages [79] (Figure I-10).

Figure I-9  A scald injury on a child’s hand showing collagen patterning and scar tissue formation. Image taken from NHS burns and Scalds information page (www.nhs.uk).
Burn wounds can generally be broken down into three characteristic zones, the zone of coagulation, zone of stasis and the zone of hyperaemia [81]. The zone of coagulation is found at points of maximum damage. Tissue loss here is irreversible due to the degree of protein coagulation. The zone of stasis is characterised by decreased tissue perfusion, i.e. damage to capillary beds. If tissue perfusion can be increased in the zone of stasis it is possible to prevent damage from becoming permanent. The outermost area of a burn is the zone of hyperaemia. This zone shows increased levels of tissue perfusion and will invariably recover well provided there is no sepsis. The purpose of classifying these zones is to define the level of fluid management to provide adequate resuscitation and to subsequently minimise loss of the zone of stasis to necrosis [82] (Figure I-11).
Burn wounds can also cause some immunosuppressive systemic effects due to the local release of cytokines and inflammatory mediators. Increased levels of T helper-2 lymphocytes and consequent increase in interleukins IL-4 and IL-10, decreased levels of interleukin IL-12 and suppression of splenic T cell proliferation have been demonstrated in animal models [83, 84]. IL-4 has been shown to induce further IL-10 production which in turn is implicated in T helper-1 suppression and subsequent overall immunosuppression [85]. Suppression of splenic CD4+ T-cell populations is reliant on the presence of macrophages and seems to involve an imbalance in regulatory and co-stimulatory signals to which the population is sensitive [86]. The majority of these effects tend to be size dependant and are found to be most prominent in larger burns of 30% TBSA or more [87]. Having said this, at least one study has found that characteristics which seem to be important in immune dysfunction post burn injury, such as a hyperactive macrophage phenotype, are found regardless of injury size [88]. This study also pointed out that although factors that relate to immune suppression were seen, no actual suppression of the splenic T-cell population was observed with the small (6.25% TBSA) burns.

Inadequate care of partial thickness burns can allow progression to full thickness skin loss, a condition that increases morbidity, mortality, risk of infection and scarring. Monitoring
of wound thickness can initially be performed using laser Doppler imaging, however this method is not useful after wound occlusion, at which point measurement of wound depth becomes extremely difficult [89]. Prevention of progression to full thickness burns relies on several factors: prevention of further damage and inflammation, such as that caused by infection, through use of topical antimicrobials; adequate resuscitation which in small partial thickness burns can usually be managed through oral hydration and occlusion of the wound, although for more significant damage I.V. hydration may be required [82, 90]; the use of biologic dressings to promote re-epithelisation. [87] The schematic below shows factors that can lead to conversion to a full thickness burn (Figure I-12).

Figure I-12  Schematic of factors affecting burn wound conversion to full thickness burn. Schematic adapted from The pathogenesis of burn wound conversion. Annals of plastic surgery 2007 [87].
Progression to full thickness burn and the immunosuppressive factors associated with trauma injuries are risk factors associated with infection in patients with burn wounds. As well as these, inhalation injuries and TBSA burns greater than 10% have also been demonstrated as independent risk factors [91, 92]. Burn wounds are commonly debrided soon after initial presentation in order to remove necrotic tissue and burn eschar; this will further decrease infection risk and increase perfusion.

Although burn injuries predispose patients to burn wound site infections, a number of other infection types can be a risk for burn patients. One study in Turkey in 2003 looked at 610 paediatric burns cases admitted over a five year period in the mid to late 1990’s. Infections were identified in 207 patients and 150 of those (72%) were infections of the wound site. The remainder of infections consisted of urinary tract infections (10%), pneumonia (9%) and septicaemia (7%) [93]. Urinary tract infections are commonly associated with microbial colonisation of urinary catheters that may be in place [94], and patients with tracheal or other respiratory damage show higher incidence of pneumonia [95].

Toxic shock syndrome (TSS) whilst being a symptom of an infection rather than an infection itself is worthy of note here due to its high mortality rate, (50% if untreated) [96, 97]. TSS is often misdiagnosed in children due to the majority of criteria for its diagnosis being originally designed for adults [98] and the fact that fever is not a reliable predictor of infection in children[99, 100]. The symptoms of TSS are also difficult to separate from other conditions from which a child may be suffering on primary presentation [101]. The final factor that makes TSS important is its apparent disregard for wound size as it is found commonly in small burns [102].

A number of pathogens are found to be associated with burn wound infections. Table I-I-1 lists these organisms [103]. Historically Streptococcus pyogenes was the primary pathogen causative of burn wound infections. After the development of antibiotic treatments in the 1950’s, S.aureus superseded S.pyogenes as the primary aetiological agent in burn wound infections [104, 105]. Although S.aureus is still a common cause of burn wound infections, P.aeruginosa is currently the main burn wound pathogen [106, 107]. Studies of infectious organisms in children’s’ burns have shown similar patterns to those looking at patients more generally. A 2003 Turkish study found that within their patient cohort 74% of the infections were caused by P.aeruginosa followed by 7% E.coli and 12% S.aureus. Another study from Brazil in 2002 looked at burn wound infections in both adults and
children and found that \textit{S.aureus} was the most common pathogen followed by \textit{P.aeruginosa} [108].

Table I-1: list of pathogenic microorganisms commonly associated with burn wounds. Table was adapted from Burn wound infections, 2006 [103].

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive organisms</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{Staphylococcus aureus}</td>
</tr>
<tr>
<td></td>
<td>\textit{Methicillin-resistant S.aureus}</td>
</tr>
<tr>
<td></td>
<td>Coagulase-negative staphylococci</td>
</tr>
<tr>
<td></td>
<td>Enterococcus spp.</td>
</tr>
<tr>
<td></td>
<td>Vancomycin-resistant spp.</td>
</tr>
<tr>
<td><strong>Gram-negative organisms</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{Pseudomonas aeruginosa}</td>
</tr>
<tr>
<td></td>
<td>\textit{Escherichia coli}</td>
</tr>
<tr>
<td></td>
<td>\textit{Klebsiella pneumoniae}</td>
</tr>
<tr>
<td></td>
<td>\textit{Serratia marcescens}</td>
</tr>
<tr>
<td></td>
<td>\textit{Enterobacter spp.}</td>
</tr>
<tr>
<td></td>
<td>\textit{Proteus spp.}</td>
</tr>
<tr>
<td></td>
<td>\textit{Acinetobacter spp.}</td>
</tr>
<tr>
<td></td>
<td>\textit{Bacteroides spp.}</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{Candida spp.}</td>
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<tr>
<td></td>
<td>\textit{Aspergillus spp.}</td>
</tr>
<tr>
<td></td>
<td>\textit{Fusarium spp.}</td>
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<tr>
<td></td>
<td>\textit{Alternaria spp.}</td>
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<tr>
<td></td>
<td>\textit{Rhizopus spp.}</td>
</tr>
<tr>
<td></td>
<td>\textit{Mucor spp.}</td>
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<tr>
<td><strong>Viruses</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{Herpes simplex virus}</td>
</tr>
<tr>
<td></td>
<td>\textit{Cytomegalovirus}</td>
</tr>
<tr>
<td></td>
<td>\textit{Varicella-zoster virus}</td>
</tr>
</tbody>
</table>

From this information it can be understood that burn wound treatment and the prevention of progression to a full thickness wounds are complex processes which are sometimes at odds with one another e.g. measuring burn wound depth whilst maintaining wound...
occlusion. Infection in this respect is both a risk factor for and a possible outcome of burn wound conversion and will always exacerbate patient outcomes. Considering these factors a system which monitors infection in situ at the wound site could be useful in improving patient outcome, as well as helping clinicians to diagnose difficult conditions such as TSS.

Section 1.02 Understanding microbial pathogenesis

(a) General mechanisms of wound site invasion.

Although wounds will be sterile immediately after thermal injury, they will quickly be colonized by the patient’s normal flora as well as some organisms which commonly cause infection. Wounds will remain colonized until re-epithelisation is complete and closure has occurred. Infectious organisms can be endogenous, exogenous from the immediate environment or can be transferred from healthcare staff. Transmission modes of these organisms can include contact, droplet and airborne spread [109, 110]. Spread of organisms can be limited within an ICU through careful adherence to specific isolation protocols [111].

Some studies have shown that the profile of colonizing organisms changes over time. A 2005 study carried out on 203 patients in a Brazilian hospital demonstrated that S.aureus strains were the primary colonisers, representing 28% of the organism’s isolated from the wound within the first week. By the third week however P.aeruginosa dominated. Fungi (primarily Candida species) were seen to begin colonization within the second week and to reach their peak incidence within the third and fourth weeks [112]. A Turkish study saw similar results with a sample of 51 patients. Coagulase-negative staphylococci and S.aureus were the most prevalent isolates on admission but were superseded by P.aeruginosa isolates over the following weeks. This study also noted an increase in MRSA’s over the total study period (three weeks) [113]. Classification of colonization or infection relies primarily on the bacterial load of the wound. Classically a bacterial load of ≥10^5 CFU/gram of tissue was considered an infected wound. Some studies however, have found that this figure is more indicative of heavy colonisation as opposed to infection [114]. Other methods for classifying bacterial load as infective rely upon biopsies of the wound. These suggest that infection has taken hold when invading organisms have migrated through wound eschar and into the underlying tissue [115].
Burn wound infections can be sub-divided into non-invasive and invasive infection. Non-invasive infection will be localized to the wound area and can be characterized by localized inflammation, purulence, graft or biogenic dressing detachment, raised temperature of >38.5°C and leukocytosis. Invasive infection is characterized by wound conversion, rapid eschar separation, necrosis of wound blood vessels, oedema, inflammation and tenderness at wound edges [116].

At a microbial level, wound site colonisation involves several processes including adherence, out competition of commensal organisms and evasion of local immune factors. Adherence and general sessile behaviour are common mechanisms for colonisation of any new environment in bacteria. As such, organisms usually have an extensive repertoire of receptor specific and non-specific attachment factors. These factors can take many forms such as the multimeric surface pili displayed by *E.coli* and *P.aeruginosa* (type I and IV respectively) [117, 118], high molecular weight autotransporter proteins characterized by *H.influenza*’s HMW1/HMW2 [119] and the anchorless adhesion expressed by some gram positives such as extracellular adherence protein (Eap) in *S.aureus* [120, 121].

Out competition of commensals may not be as important in a burn wound due to the nature of the injury generating an initially sterile environmental niche. However, a study of wounds sustained by soldiers in Iraq in 2004 showed that shortly after initial trauma, wounds would be colonised by less pathogenic antibiotic sensitive gram positives [122]. Topical antibiotics have the potential to change the balance of organisms in a wound environment, by killing of any commensals present in a particular niche. It is also possible that such antibiotics could affect wound healing by destroying commensal organisms that potentiate epithelial cell migration through the stimulated generation of reactive oxygen species in the wound site. Such slowing of epithelial cell migration could allow extra time for pathogenic colonisation of the wound by microorganisms; however this process has only been demonstrated in gut epithelia [123]. A study in 2009 demonstrated that epidermal, (but not dermal), keratinocytes exposed to lipoteichoic acid (LTA) from commensal strains were de-sensitised to inflammatory factors including an RNA released from necrotic tissues [124]. The same de-sensitisation was not seen with a hospital strain of *S.aureus* (hypothesized to be related to specific LTA compositions). The study highlights the effect the native microbiome can have on the tenuous balance between excessive and insufficient inflammation in wound healing. Complete suppression of epidermal inflammation caused by LTA from commensals and possibly pathogens
prevents the body’s recognition of damage and leaves the wound open to infection. Removal of suppression by commensals can cause cytokine cascades and high levels of neutrophil influx resulting in a sustained inflammatory response and poor healing [125].

The aforementioned inflammatory response can often be a mechanism by which bacteria can invade tissues due to the effect of pro-inflammatory cytokines on paracellular permeability and tight junctions [126]. Once invasion has occurred however the same inflammatory response can be extremely detrimental to bacterial survival. Consequently bacteria have developed numerous mechanisms to enable them to subvert, evade and exploit aspects of the host immune response.

Numerous microbes employ immune modulators, compounds which can affect the immune system’s function or recognition of foreign organisms. Lipopolysaccharide (LPS), for example, which is a surface component of a number of gram-negative organisms, is recognized by the complement system, but as it is on the surface of the bacterial membrane, rather than integrated into it, membrane attack complexes are rendered useless. The hypervariability of the outer portion of lipid A, the major component of LPS, means that different strains of the same organism may not be recognized by targeted immune responses [127, 128]. As well as surface bound modulators, organisms can also employ secretory systems such as the cholesterol dependent cytolysin (streptolysin O) found in Streptococcus pyogenes that delivers NAD-glycohydrolase directly into keratinocytes to induce cytotoxicity [129].

Immune evasion in a number of pathogens is characterised by either using host molecules to disguise the invading organism or by varying surface antigens which can signal the pathogen’s presence to the host. S. aureus uses protein A (SpA) expressed on its surface to bind host IgG antibodies by the Fc fragment ensuring that the antigen sensing FAb fragment is displayed outwardly. This function makes cells less susceptible to phagocytosis by neutrophils [130] as well as inhibiting complement fixation by the classical pathway [131]. Sufficient antigenic variation to cloak a pathogens presence is usually achieved in one of three ways: by having multiple copies of a surface antigen all controlled by independent on/off switches; by having one expression locus for the gene of a particular antigen plus numerous silent copies of the gene and consistently switching which copy is expressed; by having a hyper-variable region in the antigen itself [132]. Neisseria species exemplify antigenic variation by employing all three mechanisms.
Neisseria gonorrhoeae and Neisseria meningitidis have numerous opacity proteins all of which are under independent expression controls at the translational level and which can be displayed on their surfaces in multiple combinations during infection [133, 134]. The Neisseria pilus is expressed from the pilE locus. However Neisseria also have a number of silent partial copies of the pilin gene stored at the pilS locus. Alleles from the silent locus can be recombined with the expression locus to ensure that a constantly shifting pilus is expressed [135]. Finally N.meningitidis displays lipo-oligosaccharide on its surface and can display 13 different immunotypes of the molecule by switching terminal sugar residues [136].

Obligate intracellular bacteria such as chlamydia species are well versed in exploiting their hosts. Through the inhibition of apoptosis from within the cell, by blocking cytochrome C release, the bacteria maintain their environment and prevent detection by host immune responses [137]. Salmonella is particularly adept at manipulating its host. When encountering gut epithelial cells for the first time it uses a type III secretion system to inject factors into the cell which induce sustained activation of Akt/protein kinase B, a pro-survival kinase which prevents apoptosis [138]. When encountering macrophages however, the same secretion system is used to inject factors which result in capase-1 activation and the subsequent release of interleukins IL-1β and IL-18 which facilitate cell death.

These examples demonstrate that wound site invasion is not a brute force attack by bacteria on host systems, but the disruption of a subtle interplay of pro and anti-inflammatory responses to a trauma. Invading organisms are well equipped with an arsenal of molecular tools to subvert and neutralise host responses. As well as the effect of invading pathogens, human factors such as overuse of antimicrobials and disruption of normal commensal flora can also upset the inflammatory balance. As such tact and careful thought must be employed in any interventions that seek to protect or detect infection in a wound environment.

(b) Molecular mechanisms of disease progression in S.aureus infections

Staphylococcus aureus is a gram positive coccii, which forms part of the normal human flora. Despite causing infections in humans S.aureus is also a commensal organism most commonly located in the anterior nares [139]. Reports of rates of nasal carriage within
populations vary greatly. However, a number of cross-sectional surveys have reported carriage rates of around 27% since the early 2000’s [140, 141]. A study of the literature on different carriage rates also suggests that nasal carriage corresponds to higher rates of carriage at other sites on the body [142].

Once *S. aureus* has colonized the epidermis, it is, as previously mentioned, tolerated by the epithelial immune response. This continues until it reaches the wound site where it will undoubtedly come into contact with the dermis, a locality where it will be notably less tolerated. In crude terms, the bacterium’s goal at this point is to survive the immune response and colonise further, utilizing any carbon sources which may become available. As such a cornucopia of virulence factors are available to achieve this goal.

Initially *S. aureus* will seek to secure itself in a new niche by means of surface attachment and concealment. The majority of protein adhesins expressed by *S. aureus* belong to the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family of proteins, in that they interact specifically with extra-cellular matrix (ECM) components [143]. *S. aureus* MSCRAMMS are anchored to the peptidoglycan wall of the bacteria by the cell wall sorting motif LPXTG. Anchoring of these proteins is performed by a transpeptidase, (sortase or LPXTGase), which cleaves between the threonine and glycine of the motif, freeing the threonine carboxy terminus to form an amide bond with the peptidoglycan pentaglycine bridge [144, 145]. A 2002 *in-silico* analysis of six *S. aureus* genomes identified 21 genes encoding surface proteins belonging to the LPXTG family, 11 of which had been identified previously [146]. Of the previously identified proteins, the clumping factors ClfA and ClfB, the fibronectin binding proteins FnbpA and FnbpB, the serine aspartate repeat protein SdrE, the plasmin sensitive protein Pls and the collagen attachment protein Cna have known ligands involved in ECM attachment. The attachment mechanisms of these adhesins given in Table I-2 demonstrate the breadth and redundancy in attachment factors available.
Table I-2 list of *S.aureus* surface proteins with ligand interactions known to be involved in attachment.

<table>
<thead>
<tr>
<th>Adhesin</th>
<th>Mechanism</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClfA</td>
<td>Binds to the C-terminus of the γ chain of fibrinogen, recognizing the 4 terminal amino acids [147].</td>
<td>Adherence to fibrinogen containing substances e.g. blood clots or surfaces conditioned with fibrinogen [148].</td>
</tr>
<tr>
<td>ClfB</td>
<td>Binds to the C-terminus of the Aα chain of fibrinogen [149] as well as to the tail end glycine loop region of keratin10 [150].</td>
<td>Adherence to fibrinogen similar to ClfA and adherence to keratin 10 found in the squamous epithelium.</td>
</tr>
<tr>
<td>Cna</td>
<td>B domain of Cna is a jellyroll fold with a groove on its first β sheet, which accommodates the triple helix of many collagen isotypes [151, 152].</td>
<td>Binding to collagen within the ECM.</td>
</tr>
<tr>
<td>FnbpA &amp; FnbpB</td>
<td>A consensus sequence within the D domain of FnBP can bind to fibronectin type 1 modules 4 and 5. Other binding modules are hypothesised [153, 154].</td>
<td>Attachment to immobilized fibronectin, plasma clots and a possible role in epithelial cell invasion through fibronectin dependent bridging to α5β1 host cell integrin’s [155].</td>
</tr>
<tr>
<td>SdrE</td>
<td>Interacts with a glycoprotein of bone, bone sialoprotein [156]. Has also been demonstrated to bind fibrinogen [157].</td>
<td>Mediates attachment to bone and dentine, of particular relevance in cases of osteomyelitis.</td>
</tr>
</tbody>
</table>

Other surface factors such as the previously mentioned Spa can be used primarily to disguise *S.aureus* from immune response rather than to mediate attachment. The majority of surface factors are expressed primarily during the exponential phase of growth [158, 159]. Conversely expression of numerous secreted virulence factors of *S.aureus* is limited to the late exponential and early stationary phases of growth. A two component regulatory system (Fig I-13) the staphylococcal accessory gene regulator (AGR) controls growth phase dependent expression of virulence factors through disruption of another regulatory
molecule, repressor of toxin (ROT) by its own transcriptional product RNAIII. The AGR system is a quorum sensing mechanism which regulates virulence factors on the basis of cell density. Genes on the AGR operon code for an auto inducing peptide, (AIP), a folding and export system which folds the nascent peptide into AIP, a membrane bound receptor of AIP with histidine kinase activity and a phosphorylatable transcriptional regulator which acts on the P2 promoter of the AGR operon as well as the P3 promoter which governs RNAIII transcription [160]

Figure 1-13 Schematic of the two component regulatory system of the accessory gene regulator. The AGR regulon consists of four genes which code for the various parts of the AGR two component self-regulatory systems. AgrD codes for a short nine-residue peptide which is folded into a five-membered thiolactone ring by the export system AgrB. The folded auto-inducing peptide is free to interact with the trans-membrane peptide AgrC which has histidine kinase activity associated with its cytoplasmic C terminus. The kinase will phosphorylate AgrA which will act on both the P2 and P3 promoters up-regulating the agr genes and another regulatory molecule, RNAIII, respectively. Up-regulation of RNAIII allows for the repression of ROT and subsequently greater exo-toxin production. Schematic has been adapted from “Autoinduction and signal transduction in the regulation of staphylococcal virulence” [161]

RNAIII independently down-regulates some genes, such as the protein A gene (spa) and the coagulase A gene (coa), as well as up-regulating others such as α-haemolysin, (hla)
Modulation of ROT is performed through binding to ROT mRNA at its Shine-Dalgamo sequence to prevent translation [165]. As such, a number of virulence, resistance, metabolic and regulatory genes show varying to degrees changes in regulation [166]. Thus, secreted toxins that are repressed by ROT during S.aureus exponential growth phase are produced, once sufficient cell density is reached [166, 167]. These toxins include extracellular proteases (sspB), alpha haemolysin (hla), beta toxin (a sphingomyelinase phosphodiesterase, (hlb), the leukocidins (lukF & lukS which will form gamma haemolysin) and the alpha and beta PSM’s (PSM’s α1, α2, α3, α4 and β1, β2). All of these virulence factors have some activity against cell membranes of eukaryotic cells and have been well described in a review by Dinges et al in 2000 [168]. However a complete understanding of the relevance of these proteins to disease states has not been achieved.

The breadth of virulence factors that S.aureus is able to express and the tight control it displays between colonizing and invasive phenotypes demonstrates how formidable the pathogen is. The lack of toxin production during early growth may represent vulnerability at low cell densities. The number of virulence factors produced also presents an array of possible targets for infection detection.

(c) Molecular mechanisms of disease progression in P.aeruginosa

Pseudomonas aeruginosa is a ubiquitous gram negative organism. Its requirements for survival are minimal and it is able to adapt to a staggering array of environmental conditions. Its ability to thrive in so many environmental niches, as well as its prominent role as an opportunistic pathogen, can be explained, in part, by its large genome (6.3 million base pairs) which contains an abundance of regulatory, catabolism, transport and efflux genes [169]. P.aeruginosa is responsible for around 11-14% of all nosocomial infections, although this rate increases significantly to between 13% & 22% of infections when taking only ICU patients into account [170-173]. This is most likely due to higher incidence of infection in the immune compromised [174, 175].

The large genome of P.aeruginosa has provided the organism with an extensive array of surface molecules that are involved in attachment, immune evasion and the elicitation of host response. P.aeruginosa is equipped with a flagellum which, in addition to various forms of motility allows the bacterium to follow chemotactic gradients [176] and engage in early stage biofilm formation [177], such as primary attachment to numerous epithelial
gangliosides [178]. Attachment of the flagellum to toll like receptors TLR2 and TLR5 can initiate a host inflammatory response [179] which can potentially be useful to the bacterium as an invasion mechanism. Whilst being a potent and useful virulence factor in many regards, the flagella are also highly immunogenic. In this regard *P. aeruginosa* can also generate a-flagellal non-motile mutants although these have generally been isolated in individuals with chronic infections [180].

Numerous type IV pili on the bacterial cell surface mediate attachment to both biotic and abiotic surfaces. Like the flagellum, these structures have a role in attaching to common epithelial glycolipids such asialo-ganglioside M1 [181]. Uncommonly but again in consensus with flagellum function *P. aeruginosa* pili can engage in a type of movement, twitching motility, which has been noted as important in biofilm formation[107].

Other surface virulence factors of *P. aeruginosa* include LPS and alginate. As previously mentioned, LPS has been demonstrated as important in evading immune responses through high variability in its O-antigen [128]. LPS also initiates many pro-inflammatory pathways [182] through interactions with TLR4 [182], and the cystic fibrosis transmembrane conductance regulator [183]. Alginate has multiple roles in protecting *P. aeruginosa* from host responses and antibiotics [184, 185]. However these functions are most effective in the alginate-overproducing mutant, mucoid phenotype, which are found primarily in cystic fibrosis infections.

As would be expected from such a prolific organism the list of secreted *Pseudomonas* virulence factors is equally prodigious, including small molecules and enzymes. In addition to simple secretion, *P. aeruginosa* also has method for the injection of a number of effector proteins across eukaryotic cell membranes. The type III secretion system is a pilus like structure which can inject one of four known exo-toxins (Table I-3) into host cells by creating pores in the cell membrane [186]. The injected toxins invariably affect the normal intracellular signalling machinery of the host but also require intracellular co-factors to be effective.
Table I-3 the mode of action of effector (Exo) toxins of the *Pseudomonas aeruginosa* type III secretion system.[186]

<table>
<thead>
<tr>
<th>Effector</th>
<th>Mode of action</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExoS</td>
<td>GTPase activating protein activity and ADP ribosyl transfrease activity</td>
<td>Disruption of normal cytoskeletal organization and modification of membrane lipids.</td>
</tr>
<tr>
<td>ExoT</td>
<td>GTPase activating protein activity and ADP ribosyl transfrease activity</td>
<td>Disruption of normal cytoskeletal organization; inhibition of wound repair</td>
</tr>
<tr>
<td>ExoU</td>
<td>Phospholipase</td>
<td>Rapid cell death</td>
</tr>
<tr>
<td>ExoY</td>
<td>Adenyl cyclase</td>
<td>Disruption of normal cytoskeletal organization; inhibition of bacterial uptake by cells; increased endothelial permeability.</td>
</tr>
</tbody>
</table>

The role of some enzymes excreted by *P. aeruginosa* such as alkaline protease and protease IV is currently only clear in certain infection types such as cystic fibrosis or corneal infections. For other enzymes however, their role in infection is clearer. Elastase for example is known to disrupt the epithelial barrier through the destruction of tight junctions. This leads to increased epithelial permeability and neutrophil recruitment [187, 188]. The haemolytic phospholipase C attacks membrane lipids, affecting cell integrity, as well as having a function in the suppression of oxidative bursts [189]. Exotoxin A is a ribosyl transferase which, as well as its role in inhibiting elongation factor-2, thus causing cell death, has also been shown to depress host response to infection [190, 191].

*P. aeruginosa* also produces a few small molecules which may not be traditionally classified as toxins but which still have demonstrable effects in the progression of infection and are worth a mention. For example the blue pigment pyocyanin shows a number of effects on immune function such as increasing IL-8 expression, inducing apoptosis in neutrophils and impairing neutrophil infection response [192, 193]. Other examples are the rhamnolipids, small surfactant molecules originally thought to be involved in swarming
behaviour [194]. Now, however there is evidence that rhamnolipids are involved in effecting the barrier function of epithelial cells [195].

Much like *S.aureus*, the array of virulence factors displayed by *P.aeruginosa* is impressive. Whilst the information here is merely a sample of *P.aeruginosa*’s virulence capabilities, it demonstrates, again, a high number of potential targets for a detection system. The number of virulence factors produced by both *P.aeruginosa* and *S.aureus* demonstrate the care which must be taken when choosing a particular factor to ensure adequate expression, and thus detection, across a range of clinically significant strains.

Section 1.03 Current and developing methods of infection detection and control in burns.

(a) Current methods of organism detection and treatment *in-vivo.*
As has been previously stated that burn wound infections are problematic due to their capacity to delay healing, cause further scar formation and their potential to cause more serious complications [196-198]. Current clinical determination relies on more than one factor to define an infection. This requirement for multiple clinical determinants is necessary due to the complex nature of classifying infections *in-vivo.* The main diagnostic tool in identifying infection is the clinician’s knowledge and expert opinion. Whilst clinicians are generally highly skilled, identifying infection still relies on a certain amount of guesswork. In the highly time dependent cases of suspected infection this can leave patients vulnerable.

Guidelines for defining infection in a patient vary greatly from country to country and necessarily between infection types. In the United States, sources such as the Infectious Diseases Society of America (ISDA) provide guidelines on the majority of infections. In the United Kingdom, these guidelines are provided by the National Institute for Health and Care Excellence (NICE). In both cases, the guidelines are drawn up by a board of experts. In the case of burn wound infections in Europe, guidelines are provided in the document “Identifying Criteria for Wound Infection” [199]. Diagnosis is based on the Delphi process. Delphi is a consensus of criteria from experts based on clinical indicators of infection in a particular wound type. Criteria for infection are scored by individual experts on a scale from 0-9 in order of importance. These scores are then collated and averaged.
with scores below 4 being discarded. For partial thickness burns, symptoms scored as most indicative of an infection (Table I-4) were cellulitis (inflammation of dermal tissue) and Ecthyma gangrenosum (lesion in the skin surrounded by inflammation, usually necrotic). Other factors considered as signs of infection are discoloration inflammation, haemorrhagic lesions, malodour and changes in the dimensions of the wound. As well as these mostly visual observations of the patient’s state, passive observations of temperature and heart rate will also be taken.
Table I-4 List of criteria for the identification of partial and full thickness burn wounds as determined using the Delphi process. Criteria which ranked from 8-9 are considered diagnostic of infection, these are listed in red. Criteria which ranked 6-7 are considered indicative of infection and cause for further observation, these are listed in blue. Criteria which ranked 4-5 are listed in green and are considered to be more subtle indicators of infection. The table was adapted from “Identifying criteria for wound infection”[199].

<table>
<thead>
<tr>
<th>Partial Thickness Burns</th>
<th>Full Thickness Burns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulitis</td>
<td>Black/dark brown focal areas of</td>
</tr>
<tr>
<td>Ecthyma gangrenosum</td>
<td>Discolouration in burn</td>
</tr>
<tr>
<td>Black/dark brown focal areas of</td>
<td>Cellulitis</td>
</tr>
<tr>
<td>Discolouration in burn</td>
<td>Ecthyma gangrenosum</td>
</tr>
<tr>
<td>Erythema</td>
<td>Erythema</td>
</tr>
<tr>
<td>Haemorrhagic lesions in subcutaneous tissue of burn wound or surrounding skin</td>
<td>Haemorrhagic lesions in subcutaneous tissue of burn wound or surrounding skin</td>
</tr>
<tr>
<td>Malodour</td>
<td>Increased fragility of skin graft</td>
</tr>
<tr>
<td>Spreading peri-burn erythema (purplish discolouration or oedema)</td>
<td>Loss of graft</td>
</tr>
<tr>
<td>Unexpected increase in wound breadth</td>
<td>Spreading peri-burn erythema (purplish discolouration or oedema)</td>
</tr>
<tr>
<td>Unexpected increase in wound depth</td>
<td></td>
</tr>
<tr>
<td>Discolouration</td>
<td>Sub-eschar pus/abscess formation</td>
</tr>
<tr>
<td>Friable granulation tissue that bleeds easily</td>
<td>Unexpected increase in wound breadth</td>
</tr>
<tr>
<td>Sub-eschar pus/abscess formation</td>
<td></td>
</tr>
<tr>
<td>Increased fragility of skin graft</td>
<td>Friable granulation tissue that bleeds easily</td>
</tr>
<tr>
<td>Increase in exudate volume</td>
<td>Malodour</td>
</tr>
<tr>
<td>Increase in local skin temperature</td>
<td>Oedema</td>
</tr>
<tr>
<td>Loss of graft</td>
<td>Opaque exudate</td>
</tr>
<tr>
<td>Oedema</td>
<td>Rapid eschar separation</td>
</tr>
<tr>
<td>Onset of pain in previously pain-free burn</td>
<td>Rejection/loosening of temporary skin substitutes</td>
</tr>
<tr>
<td>Opaque exudate</td>
<td></td>
</tr>
<tr>
<td>Rejection/loosening of temporary skin</td>
<td>Secondary loss of keratinised areas substitutes</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary loss of keratinised areas</td>
<td></td>
</tr>
</tbody>
</table>
Defining infection by a series of symptoms is clinically useful in beginning a treatment. However it leaves much to be desired in terms of variability of symptoms between injuries and the problem that infection may be fairly well developed by the time some of these symptoms are noticed. Clinical characterization of infection also does little to determine the organism responsible for infection.

Whilst biochemical analysis of the patient’s blood will be somewhat routine, microbiological analysis, other than a nasal swab to identify potential MRSA on entry to the health care facility, is generally only performed when there is concern. This can leave the patient temporarily at risk.

When wounds are subjected to analysis, the most common method of sampling is the use of a wound swab. Less frequently used, now that debridement of the wound is commonplace, is tissue biopsy sampling [200, 201]. Wound swabs are simple and can thus be performed longitudinally throughout a patient’s care pathway. Such swabs are used to generate quantitative cultures which can be useful in identifying organisms implicated in the infection. There are doubts however, in their ability to predict bio-burden of the wound due to large variations in bacterial count between cultures [201, 202]. Despite this there is some evidence that semi-quantitative methods can differentiate between wound colonisation and infection [203]. The sample method will ultimately rely on the type of wound and the condition of the patient.

As well as determining which organisms are present in the wound, the susceptibility of those organisms to antibiotics is also important in choosing a treatment. Traditional broth dilution minimum inhibitory concentration (MIC) assays are most commonly used to determine the efficacy of antimicrobials. MIC determination is often automated and is coupled with disc diffusion and agar dilution for conformation. Whilst aiding in the determination of antimicrobial resistance in planktonic cells, these assays do not give any information on the susceptibility of organisms in a biofilm state. Biofilms are fundamentally different in their physiology and susceptibility in comparison to their planktonic counterparts [204]. Due to the poor understanding of antimicrobials ability to reach infections in burn wounds and their inability to penetrate eschar, topical antimicrobials are more commonly used in control of infection [205, 206].

Commonly used methods for identification of both infections and the organisms responsible for them highlight several areas that could be improved. Some empirical
evidence of microorganisms presence at the point of diagnosis may be useful in informing a clinician’s decision to treat, especially as treatments, like infections, can slow wound healing and affect patient outcome. Early evidence of a microbe’s presence, before clinical indicators are demonstrated, has the potential to speed up the treatment process and improve outcomes. Distinguishing between groups of organisms early on would also allow for treatment to begin before the time consuming process of microbiological testing. All of these changes to the treatment of patients would be small but have the potential to increase survival rates, improve patient outcomes and potentially lower overall treatment costs.

(b) Emerging methods of infection detection.

There a number of technologies both in-vivo and ex-vivo currently being developed to fulfil the task of rapid bacterial detection and discrimination in wounds. Approaches to the problem are numerous and the majority of these are in their nascent stages. Techniques attempt to detect several different aspects of a wound, which would signal infection. These can include directly sensing the organism, sensing a by-product of the organism, a change in the environment in response to a pathogen or sensing the host’s response to the pathogen.

Significant progress has been made in adapting what were previously considered analytical chemical techniques to the problem of in-vivo pathogen detection. In particular, the area dubbed “volatiletomics”, the field of identifying organisms by their associated volatile organic compounds (VOC’s) offers promise. One such technique is mass spectrometry (MS) which can be used to determine the molecular identity of complex mixtures of compounds from the weight of their ions fired at a detector [207, 208]. A paper published in early 2013 used secondary electrospray ionization-MS to detect volatile compounds produced by P.aeruginosa and S.aureus in an infected mouse model [209]. The model used multiple biomarkers to detect the organisms as well as to distinguish between two strains of P.aeruginosa. The use of more than one biomarker in this study presents a considerable step forward for the field where it was previously noted that not enough variation in the selection criteria was a downfall [210, 211]. A current limitation to this technique is the size and cost of the required equipment. Whilst it may be feasible to have MS devices in hospital labs, having one at the bedside of each ICU patient is currently not. Despite this
work has been done on real time, quantitative discrimination of bacterial isolates using selected ion flow tube MS [212]. Work has also been performed looking into storage of samples for later analysis [213].

Electro-chemical methods also provide some candidates for bacterial detection, such as one that is being developed to, again, identify VOC’s [214]. Due to its chemical nature, this sensor, which is based on polyanaline nanoparticles, senses only ammonia, which may limit its current application, given the previously mentioned problems associated with single biomarkers. The method does however represent a low cost technique for sensing of microbial associated VOC’s, provided additional sensors can be incorporated. It is also useful in that the sensor can be incorporated into fabrics, eliminating the need for a collection method.

A method not yet at the device stage, using a combination of magnetic nanoparticles and cyclic voltammetry is being developed at the University of Michigan. This method employs magnetic polyanaline nanoparticles to bind to and separate two bacterial species. The presence of the target cells then interrupts current on a screen-printed electrode signifying the presence of the target organism [215]. This suffers from similar problems to the previous example in that it is currently a highly organism specific system which would require some development for use with other pathogens. A detection technique being developed at the Texas A&M University gets round the issue of organism specificity by using bacteriophage as part of an ex-vivo detection mechanism. This mechanism relies on the phage dependent invasion of bacterial cells triggering an ion cascade which can then be detected using a small scale capacitor [216]. While this device does have potential as a small, high throughput, portable solution to the sensing problem its high specificity may also provide its key limitation. Bacteriophage are known to be specific to not just species but strains, and the bacterial sensitivity to phage is not binary [217].

A number of combination enzymatic/electrochemical or colourmetric approaches have also been taken to the problem. One such approach is based on the use of enzyme-linked gold nanoparticles [218]. β-galactosidase is electrostatically linked to gold nanoparticles with quaternary ammonium head groups. The electrostatic linkage inhibits the enzymes activity but is disrupted when the cationic nanoparticle interacts with a bacterial cell surface. The disruption of the linkage frees the β-galactosidase to interact with a chromogenic substrate that is used for signal detection. This method is a useful rapid analysis tool for the
presence and quantification of bacterial cells. It doesn’t, however, provide a method of identifying or distinguishing between bacterial species. Some ELISA’s have also been developed to determine the presence of specific organisms in a wound environment [219-221]. While this technique is a significant step forward, until a way is found to test and distinguish multiple organisms at one time, its efficacy will be limited. In regards to this limitation one group has demonstrated that it is possible to perform this type of ELISA assay in a microfluidic device thus allowing for multiple samples to be assayed at the same time [222]. The cost of multiple antibodies however may make this approach prohibitively expensive.

Finally, a group in Sheffield has developed a method of bacterial detection based on antimicrobial fragments. The system relies on antimicrobial fragments (polymixin B or Vancomycin), covalently attached to collapsible hydrogel polymers, (PNIPAM), with an incorporated fluorescent dye, (ethidium bromide) [223]. Upon attachment of the antibiotic fragment to a bacteria, the gel collapses releasing the dye. The approach is useful in both signaling bacterial presence somewhat quantitatively and also lowering the number of viable bacterial cells in the wound. Like all approaches there are some drawbacks. While the Polymixin-B modified polymer only uses the binding portion of the antibiotic, the Vancomycin version the antibiotic is whole and does have the potential to generate resistance. Also the fluorophore in use would need to be changed due to its toxicity in humans.

Numerous methods of microbiological detection are being developed which utilize innate attributes of the microbes they are detecting. However there are potential flaws in each technique. The issue not addressed in any example listed above is the detection of specifically infective organisms. The targets for detection are all microbial in origin but rely upon biomarkers which may not be present at sufficient levels, or at all during an infection. As previously discussed, microbial organisms within a specific wound environment are not necessarily indicative of an infection. This lack of infective specificity warrants another look at the problem of microbial detection.

(c) Current and emerging wound site prophylaxis.

The use of topical agents to treat burn wound infections has been demonstrably important in fighting infection before it becomes entrenched. Although topical antimicrobials are
much better understood in terms of their pharmacodynamics in the burn wound [205, 224] (penetration of eschar, definable local concentrations etc.) treatment is still limited to presentation of infection to prevent the development of resistance [225]. While this is important in the protection of the patient, the understanding that wound colonisation is normal rather than the deviation and the well documented evidence on the effect of abiotic surfaces on increasing bioburden in wounds should make us proactive in our terms of microbial limitation [226]. Our aim, therefore, must be to prevent drug resistant biofilms forming in the first place, either by lowering the bacterial load through killing, or by preventing attachment to a surface or both. All of these ideas were recently presented in a review concerning effective treatment of antibiotic resistant infections [227]. Meeting these goals can be achieved through the use of antimicrobial coatings which lower bioburden and display a low propensity for the development of resistance.

There are a number of different surface coatings for use in medical environments that are currently in various stages of testing. However all of these coatings vary in their efficacy and their ease of construction. Whilst a number of these models fare well in in-vitro testing of antimicrobial efficacy they often prove less effective in clinical testing.

A common type of coating is based on silver metal ions. Modes of toxicity for silver, (along with other metal ions) involve disruption of numerous intracellular pathways, and disruption of the proper functioning of surface proteins. Numerous metal ions are required at carefully maintained levels within both eukaryotes and prokaryotes, and are found bound as components of metalloenzymes, cofactors to other proteins. Changes in metal ion concentrations can affect a diverse range of biological functions through genetic regulation [228]. The toxicity of metal ions in biological systems is likely due to a combination of genetic effects, redox cycling of organic compounds to create superoxide species [229], and disruption of normal metabolism [230]. The antimicrobial activity of silver is suspected to be dependent upon silver atom’s ability to interact with DNA to prevent unwinding at replication [231].

A number of silver coating methods have been attempted in laboratory settings including ion beam deposition [232] or plasma deposited silver [233], silver containing hydrogels [234] and materials impregnated with silver nanoparticles [235]. Currently there are four main silver coatings on the market. Acticoat is a series of wound dressings that are coated with the Silcryst™ nanocrystalline silver coating used on various wound dressings which
releases silver ions into the wound site upon wetting, (either with wound exudates, or if moistened). AQUACEL® is a silver coated wound dressing which absorbs wound exudates and bacteria and again kills by the release of silver ions for a period of up to 7 days. Contreet is a similar product, which also absorbs wound exudates and releases silver ions. The fourth type Urgotul® is a dressing which releases the compound silver sulphadiazine into the wound site for 2-7 days. While a number of these dressings are used regularly in the treatment of chronic and acute wounds there have been few trials published demonstrating their effectiveness in a clinical environment. For example a trial comparing Acticoat™ to silver sulfadiazine cream in the treatment of superficial or partial-thickness burns saw a reduction in mean healing times for patients treated with Acticoat™ [236]. However there was no difference between the two treatments in terms of requirement for skin grafting or rates of infection. Another study looked at mixed aetiology delayed healing wounds in 619 individuals in several different countries [237]. Participants in the study received either a “local best practice treatment” (which ranged from antimicrobial products to normal gauze dressings), or a Contreet foam dressing for a 4 week period. The study determined that there was a 16% greater decrease in wound area in the Contreet treated patients. Treatments however were only carried out for 4 weeks when the majority of chronic ulcer wounds, (82.5% of the study group), take around 12 weeks to heal. Wound areas were generally larger at the beginning of the study than those found in the Acticoat™ trial. Some studies have also started to identify the problems of toxicity associated with products containing nanoparticles such as Acticoat. A 2006 review found that while Acticoat has anti-inflammatory properties in burn wounds and shows no in-vivo toxicity, in-vitro toxicity has been found which should lead clinicians to be cautious in its use [238].

An alternative to silver coated products are those coated with antibiotics or antiseptic agents. Two such dressings currently available are the Framitulle™ dressing containing the antibiotic framycetin sulphate, (an aminoglycoside), and 3M™’s Tegaderm™ dressing which is impregnated with the antiseptic Chlorohexadine Gluconate. These dressings have the benefit of known bactericidal activity. Catheters impregnated with chlorhexidine-silver sulfadiazine, or minocyclin and rifampin are also available. A study into chlorhexidine-silver sulfadiazine coated catheters found that they were more effective at preventing microbial colonisation of the catheter surface compared to untreated catheters in a study of 793 patients over a two year period [239]. However, although there was a difference
between the two treatments the results were not significant. A study was also carried out to determine the efficacy of the minocycline and rifampin coated catheters. The study looked at 147 treated and 151 untreated central venous catheters over a seven day period. The study showed an 18% increase in colonisation on the untreated catheter compared to the treated model, which was significant [240]. In this particular study resistance to the antibiotics used was not seen to develop. However, the trial was only continued for a short period, whilst urinary catheterisation can be maintained for months at a time. The main problems associated with antibiotic impregnated catheters are the development of resistance strains of bacteria, short half-life of the antibiotic [241], and difficulty in attaching the compound whilst maintaining its functionality in an industrially viable manner.

Each of these coating types has unique properties which make them desirable in the development of antimicrobial medical devices. However there is still a requirement for coatings that can both prevent attachment of microbes and reduce their numbers over a long period of time with low levels of resistance developing.

Section 1.04 Aims and objectives

In the broadest sense the intent of this project was to develop nano-materials which could aid in the detection and treatment of bacterial infections in paediatric burn wounds. More specifically the initial concept was to develop a lipid based nanocapsule which could be incorporated as part of a wound dressing. Such devices could then potentially be used as a diagnostic aid by clinicians.

Nanocapsules were envisioned to contain molecules designed to signal the presence of infection, in the first instance the fluorescent dye 5,6-carboxyfluorescein which can be quenched through concentration dependant means. The nanocapsules, being constructed of lipids would respond to haemolytic membrane interacting toxins produced by bacteria by releasing their contents. Such dye release would act as indicator of bacterial infection.

In order to decrease the likelihood of bacterial infection in a wound environment an antimicrobial coating which could be incorporated into wound dressings would be
developed. Such a coating would provide passive protection against microbes ideally preventing attachment to dressings and subsequent biofilm formation.

From these quite disparate aims several key objectives have been identified. Each objective and its relation to the project aims are discussed in some detail below.

(a) **Identification of factors involved in nanocapsule disruption**
Attempts must be made initially to identify which clinically relevant organisms are capable of disrupting the nanocapsules and thus releasing their dye payload. This investigation will be performed by screening the secretome (in the form of the supernatant by-product of bacterial growth), of a variety of organisms against a prototype capsule.

Once relevant organisms have been identified two notions must be determined simultaneously. The first is the factor or factors produced by these organisms which are specifically responsible for nanocapsule disruption or lysis. Several methods will be employed to resolve an answer to this question. These include: Identification of secreted, lipid active molecules produced by each organisms through literature searches; determination of the involvement of individual lytic factors through the use of synthetic examples and specific knockouts; determination of the presence of lytic factors by chemical means in lytic and non-lytic mutant strains of particular organisms.

Secondly the prevalence of these lytic factors amongst clinical isolates of each organism must be determined. This can be achieved through screens of a significant number of isolates and the application of statistical methods to confirm prevalence. This data may also be cross referenced with that found in relevant scientific literature.

(b) **Determination of the sensitivity of nanocapsules to identified lytic factors**
In order to determine how clinically relevant such a nanocapsule detection system can be, it is important to understand its sensitivity to identified stimuli. Knowledge of the susceptibility of prototype capsules to individual lytic agents however is not enough. A comparison also needs to be made between the susceptibility of the capsules and key host
cells to both the lytic agents in question and the total toxic output of a particular species. In this way the applicability of such a vesicle system can be clarified.

To identify these susceptibilities, prototype nanocapsules and a human T-cell line will be challenged against purified or synthetic versions of identified lytic agents. Concentrations corresponding to 50% lysis will then be identified. Similarly, the lytic response of T-cells and nanocapsules will be tested against the secretome of the microbes in question in a dose dependant manner. The sensitivity of the nanocapsules relative to the T-cells will enable us to gauge the applicability of the system.

(c) Potential for nanocapsule tuning

As well as potentially being able to detect the presence of infective organisms through the toxins and lytic agents they produce, the nanocapsules have the potential to aid clinicians further in distinguishing between organisms. Narrowing down the organisms involved could potentially be performed at the point of capsule lysis by exploiting inherent differences in lytic factors mechanism of action.

Lytic actions of membrane active toxins generally rely on the interaction between the toxin and the membrane itself. As such changes in the membrane bilayer components can alter the capacity of the toxin to lyse.

Alterations such as variations in cholesterol concentration or acyl chain length of primary lipids will be made to the bilayer of the prototype vesicle. The effect that these changes have on toxin interaction will then be tracked by observing variations in the lytic capacity such changes impose. By varying a number of bilayer components it should be possible to clarify the role each plays in regards to a specific toxin and to tune sensitivity.

(d) Antimicrobial coating development

Antimicrobial coatings will be developed using an organometallic compound previously synthesised by Dr Charlotte James (a previous member of Dr Toby Jenkins Biophysical Chemistry research group). Surface attachment will be attempted through two industrially applicable methods: low temperature inductively couple plasma deposition and radical photo-initiated grafting.
In order to optimise coating efficiency numerous parameters of each method will be varied and compared. Within the plasma deposition method plasma pulsing and inert gas inclusion will be investigated as methods for increasing deposition efficiency. In the UV grafting method, level of UV exposure, photo-initiator concentration and graft compound concentration will all be investigated as methods for the control of surface attachment.

Evaluation of graft efficiency will be determined through classical chemical analysis of both the surface and the surface leachate in an aqueous environment. This analysis will primarily consist of Infrared spectrophotometry, UV/visible light spectrophotometry and atomic absorption spectroscopy.

Microbiological methods will also be employed to assess the success of grafting methods. These will consist primarily of comparisons between the antimicrobial activity of the initial organometallic (as determined from the minimal inhibitory concentration against clinically relevant organisms), and the antimicrobial activity of the modified wound dressing. A modified *E.coli* zinc biosensor will also be employed to assess the presence of free zinc in leachate from the treated fabrics.

## Section 1.05 Conclusions

The picture generated from the variety of sources I have presented above is that acute infection of paediatric burn wounds is a problem with no simple solution. Whilst we can treat infection with antibiotics, growing resistance amongst both hospital and community acquired isolates is a prevalent problem. Additionally the topical antimicrobials which seem to be most effective in wounds with significant eschar can threaten proper wound healing thus effecting patient recovery. A potential solution to these problems is to treat a patient only once an infection has been confirmed and with antibiotics relevant to the invading organism.

In order to fulfil these criteria the ability to detect infection at its onset as well as a capacity to determine the identity of the infective organism will be required. As such in the following chapters I have proposed the development of a nanoscale detection system designed to mimic the effect of haemolytic bacterial toxins on the membrane of eukaryotic cells. As such the system will consist of dye encapsulating lipid nanocapsules. These capsules will be tested for their susceptibility to common bacterial pathogens. In order to
ascertain our aptitude in detecting a variety of toxins the formulation of the nanocapsules will be altered and the lytic susceptibility of these various formulations to toxins of known efficacy will be determined.

Finally in order to limit wound-dressing colonisation, which has been demonstrated to decrease the required infective dose of certain organisms, a novel antimicrobial coating will be developed. This coating will be generated on non-woven polypropylene fabrics, and its ability to prevent bacterial attachment and growth will be classified.

Section 1.06 References

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42. David W, David GSB: Does adding routine antibiotics to animal feed pose a serious risk to human health? BMJ 2013, 347.


221. Messmer TO, Martinez J, Hassouna F, Zell ER, Harris W, Dowell S, Carlone GM: Comparison of two commercial microimmunofluorescence kits and an enzyme immunoassay kit for detection of serum immunoglobulin G antibodies to


Chapter II. Materials and methods

Due to the broad range of methods and techniques utilised in this body of work this Chapter will be split into the following subheadings:

- Chemical synthesis
- Microbiological methods
- Biochemical assays
- Analytical methods
- Data processing

Any methods listed under these headings will fall broadly in line with the Chapter by Chapter layout of this thesis. The Chapter, under which these techniques were used, will also be listed.

Unless otherwise stated all chemicals and solvents, of HPLC grade, were sourced through Sigma Aldrich; lipids used were obtained from Avanti polar lipids; Sephadex NAP-25 columns used in vesicle purification were obtained from GE healthcare; 96 well microtitre plates were obtained from Fisher Scientific; and biological materials such as bacterial growth medium and pre-poured 5% sheep blood agar plates were supplied by Oxoid.

Section 2.01 Chemical Synthesis

Chemical synthesis in these terms will encompass both soft synthesis such as the production of lipid bilayer vesicle as well as synthesis of compounds in the case of the zinc Schiff base used and generation of materials such as grafted fabrics.
(a) Vesicle/Nanocapsule synthesis

Used in the generation of vesicles for work carried out in Chapters IV and V

All vesicles used were composed of mixtures of phosphocholine (PC) lipids, phosphoethanolamine (PE) lipids, cholesterol, purchased from Avanti Polar lipids and 10,12-tricosanodonic acid (TCDA), which was purchased from Sigma Aldrich. Lipids and other vesicle components were dissolved individually in chloroform to a concentration of 100 mM/m^3 and stored at -20 °C prior to use. Components were combined immediately prior to vesicle synthesis.

The vesicles were synthesized in a similar procedure to that described by Olson and Hunt in 1979 [1]. Stocks of the various components used in each vesicle type were mixed to a total volume of 100 µl. 200 µl of chloroform was added and the mixture was subsequently dried under nitrogen to form a lipid film. The film was rehydrated using 5 ml of 50 mM [5,6]-carboxyfluorescein prepared in HEPES buffer to pH 7.4. The rehydrated sample was heated to a temperature of 75°C for a period of 10 minutes after which it was vortexed briefly to re-suspend the lipid film. Once cooled to room temperature the sample underwent three freeze-thaw cycles under liquid nitrogen. The lipid sample was extruded under nitrogen (10-15 bar of pressure), through a double layer polycarbonate membrane of pore size 100 nm. The extruder used was a Liposofast LF 50 (manufactured by avestin), which was maintained at a temperature of 60°C. The extrusion process was performed a total of 5 times per sample. Removal of un-encapsulated fluorescein dye was performed by passing the vesicle mixture through a NAP-25 sephadex column. The columns were washed with 10 mL of HEPES buffer to remove the 0.15% Kathon CG/ICP biocide prior to use. Vesicles were eluted using HEPES storage buffer, the non-fluorescent fraction was collected. Nascent vesicle solutions were stored a 4°C overnight before cross linking of the incorporated TCDA. Cross linking of the vesicles was performed using a UVP CL-1000 cross linker at energy of 1600 µJ/cm^2 with the major component at 254 nm. After crosslinking vesicles were stored at 4°C in the dark until use. Vesicles remain stable in these conditions for a period of up 20 days [2]. An overview of the synthesis process with the final vesicles incorporated into an agar gel is shown in Figure II-1.
(i) **Buffer solutions for use with vesicles**

Aqueous buffer solutions were used in the storage and synthesis of vesicles. These solutions were made up according to the compositions given below. The chemicals were weighed using a HR120g Orion analytical balance (manufactured by A&D) with a minimum resolvable mass of 0.1mg. All solutions were made up using ultra-pure deionised water from a Thermo Scientific Barnstead easy pure II purification system. After mixing components buffers were autoclaved at 121°C and 15psi to sterilize and then stored at 4°C until use.
Table II-1 the components and quantities of materials used in making HEPES buffer pH 7.4. HEPES buffer was used for storage, dilution and elution of vesicles.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
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</tr>
<tr>
<td>NaCl</td>
<td>4.680 g</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.168 g</td>
</tr>
<tr>
<td>HEPES</td>
<td>1.7895 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.219 g</td>
</tr>
</tbody>
</table>

Table II-2 The composition of 50mM 5(6)-carboxyfluorescein solution pH7.4.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
<td>100 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.0585 g</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.5405 g</td>
</tr>
<tr>
<td>HEPES</td>
<td>0.2387 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.0285 g</td>
</tr>
<tr>
<td>5(6)-CF</td>
<td>1.8789 g</td>
</tr>
</tbody>
</table>

(ii) Vesicle mixtures

DPPC53 vesicles are used in Chapter IV, other vesicle types are all used in Chapter V

Several vesicle types were synthesised in this body of work. Lipids used in vesicle synthesis were all purchased from Avanti polar lipids with the exception of TCDA which was purchased from Sigma Aldrich.

All vesicle mixtures were generated from 100mmol dm$^{-3}$ stocks mixed to a final volume of 100µl. As such lipid proportions are written as percentage volumes. Lipids used in all experiments were as follows.
Table II-3 List of Lipids with structures, used in vesicle synthesis.

<table>
<thead>
<tr>
<th>Lipid name</th>
<th>Short name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>CHO</td>
<td><img src="image" alt="Cholesterol Structure" /></td>
</tr>
<tr>
<td>10,12-tricosanyonic acid</td>
<td>TCDA</td>
<td><img src="image" alt="10,12-tricosanyonic acid Structure" /></td>
</tr>
<tr>
<td>1,2-dimiristoyl-sn-glycero-phosphocholine</td>
<td>DMPC</td>
<td><img src="image" alt="1,2-dimiristoyl-sn-glycero-phosphocholine Structure" /></td>
</tr>
<tr>
<td>1,2-dipalmitoyl-sn-glycero-phosphocholine</td>
<td>DPPC</td>
<td><img src="image" alt="1,2-dipalmitoyl-sn-glycero-phosphocholine Structure" /></td>
</tr>
<tr>
<td>1,2-distearoyl-sn-glycero-phosphocholine</td>
<td>DSPC</td>
<td><img src="image" alt="1,2-distearoyl-sn-glycero-phosphocholine Structure" /></td>
</tr>
<tr>
<td>1,2-dimiristoyl-sn-glycero-phosphoethanolamine</td>
<td>DMPE</td>
<td><img src="image" alt="1,2-dimiristoyl-sn-glycero-phosphoethanolamine Structure" /></td>
</tr>
<tr>
<td>1,2-dipalmitoyl-sn-glycero-phosphoethanolamine</td>
<td>DPPE</td>
<td><img src="image" alt="1,2-dipalmitoyl-sn-glycero-phosphoethanolamine Structure" /></td>
</tr>
<tr>
<td>1,2-distearoyl-sn-glycero-phosphoethanolamine</td>
<td>DSPE</td>
<td><img src="image" alt="1,2-distearoyl-sn-glycero-phosphoethanolamine Structure" /></td>
</tr>
</tbody>
</table>
The following lipid mixtures were used.

Table II-4 List of vesicle types used and % volumes of their constituents.

<table>
<thead>
<tr>
<th>Vesicle short name</th>
<th>Mixture (% volumes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC53</td>
<td>53% DPPC 2%DPPE, 20% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>DMPC 53%</td>
<td>53% DMPC 2%DMPE, 20% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>DSPC 53%</td>
<td>53% DSPC 2%DSPE, 20% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>0% Cholesterol</td>
<td>73% DPPC 2%DPPE, 0% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>10% Cholesterol</td>
<td>63% DPPC 2%DPPE, 10% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>30% Cholesterol</td>
<td>43% DPPC 2%DPPE, 30% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>40% Cholesterol</td>
<td>33% DPPC 2%DPPE, 40% cholesterol 25% TCDA</td>
</tr>
<tr>
<td>50% Cholesterol</td>
<td>23% DPPC 2%DPPE, 50% cholesterol 25% TCDA</td>
</tr>
</tbody>
</table>

(b) Synthesis of Zinc Schiff base ZSB2

ZSB2 used as a surface coating agent in Chapter VI

A zinc containing Schiff base (ZSB2), was originally synthesised by Dr Charlotte James, (a previous PhD student in the Jenkins lab) in a two-step process. 2-hydroxy-5-methoxybenzaldehyde was dissolved in 30 ml of methanol, 2-(1-cyclohexenyl)-ethylamine was subsequently added the mixture was refluxed for two hours at 60°C. This resultant ligand (E)-2-(((2-(cyclohex-1-en1-yl)ethyl)imino)methyl)-4-methoxyphenol was redissolved in a further 30ml of methanol. Triethylamine and zinc chloride were added and the reaction refluxed for four hours at 60°C. The resulting solution was allowed to cool and the solvent was removed under vacuum. The product was washed in hot hexane to remove remaining reactants. The compound was redissolved in dichloromethane and dried with magnesium sulphate which was removed through vacuum assisted Büchner filtration.
Solvent was again removed by rotoevaporation and the product was allowed to crystallise affording -di-(E)-2-(((2-(cyclohex-1-en1-yl) ethyl)imino)methyl)-4-methoxyphenol Zinc.

Figure II-2 Structures of the SB2 ligand and ZSB2. (A) Chemical structure of the ligand (E)-2-(((2-(cyclohex-1-en1-yl)ethyl)imino)methyl)-4-methoxyphenol (B) the crystal structure of the zinc Schiff base -di-(E)-2-(((2-(cyclohex-1-en1-yl) ethyl)imino)methyl)-4-methoxyphenol Zinc. The crystal structure was determined by Dr Andrew Johnson.

Characterisation of the Zinc Schiff base ZSB2 (5-Methoxysalicylaldehyde and 2-(1-Cyclohexenyl)ethylamine as seen in Figure II-2, B is as follows:

FT-IR: FT-IR peaks (cm⁻¹): 3075 (C-H, aromatic), 1664(C=C), 1620(C=C aromatic), 1256 (C=N), 1194 (C-O), 815 (C-H aromatic)

NMR: 1H NMR (250 Hz) (ppm): 1.7 (s, 2H, CH₂) 1.84 (m, 2H, C₆H₁₀), 1.99 (m, 2H, C₆H₁₀) 2.21 (d, 2H, CH₂), 3.77 (s, 3H, CH₃) 4.10 (d, 2H, CH₂), 5.34 (d, 1H, CH) 6.7 (m, 4H, aromatic), 8.1 (s, 1H, -CH).

13C NMR (75 MHz) (ppm): 22.5, 24.1, 26.4, 28.2, 32.2, 34.4, 55.1, 114.0, 114.9, 115.9, 125.3, 137.1, 144.9, 155.2

Crystal structure (as determined by Dr Andrew Johnson department of Chemistry,
University of Bath): The zinc Schiff base ZSB2 complex can be seen to be monomeric most likely due to steric hindrance generated by the para-methoxy group and the relatively large 2-(1-Cyclohexenyl)ethylamine. The complex has two ligands to one zinc metal center (Figure II-2 B).

(c) Generation of ZSB2 coatings on Non-Woven Polypropylene fabrics

Development of antimicrobial coatings Chapter VI

All coatings were generated on non-woven polypropylene fabrics purchase from Boots LTD. In both plasma and UV grafting procedures fabrics were coated with a 10mmol dm$^{-3}$ methanol solvated form of the ZSB2 compound. In the case of some of the UV grafting procedures coating took place during the treatment time. Both plasma and UV grafted samples were generated and used on the day of the experiment.

(i) Plasma Deposition

The plasma reactor used by the Biophysical Chemistry Research Group was constructed with the aid of Luis Duque from the Max Plank Institute of Polymer Research, Mainz, Germany. The reactor chamber consists of a borosilicate cylinder 30cm in length with a diameter of 12.5cm. At one end of the chamber are a monomer and a gas inlet valve whilst the other is attached to an Edwards RV5 vacuum pump via a nitrogen Dewar. A coiled electrode spans the length of the reactor chamber. The system is powered from the mains electricity supply, which is converted to an RF signal of the required wattage using a Coaxial Power Systems Ltd RFG100 radio frequency generator. From here the RF signal is fed to a manual matching unit by a 2mm copper wire, and then to the reactor electrode. The matching unit is used to match the impedance of the electrode to the bulk impedance and to adjust for any reflectance of the signal. From the reactor electrode the signal is then passed to a TGP110 10MHz pulse generator from Thurlby Thandar Instruments. The pulse generator is used to modify the duty cycle of the signal, which is visualised using an Iso-Tech IDS706 60MHz oscilloscope. The circuit is completed through the return of the signal from the pulse generator to the RF generator.
A swatch of non-woven polypropylene measuring 200cm² was cut from a larger sheet and immersed in 10ml of a 10mmol dm⁻³ ZSB2 methanol solution before being placed on a glass sheet to dry. Plasma grafting was performed by placing the coated fabric over a glass hanger down the centre of the reactor chamber. The chamber was sealed ensuring both inlet valves were closed. The nitrogen Dewar was filled in order to lower the pressure of the system and the vacuum line was opened. Argon gas at a flow rate of 2sccm was fed into the reactor at all times during the reaction. Pressure in the reactor chamber was monitored using an ITR 200 digital pressure gauge. The pressure was adjusted to between $2 \times 10^{-5}$ and $2 \times 10^{-6}$ bar. Before the power was turned on the duty cycle, if any is required, was adjusted using the pulse generator and oscilloscope, and the RF generator was switched to continuous wave, (CW), regardless of duty cycle. When the RF generator was
switched on the required wattage was set on the generator, the matching unit was then used to adjust the incident and reflected wattages before switching the output to pulse, (if a duty cycle other than CW is required). All depositions were carried out for a 30 minute period where the pressure, along with the incident and reflected power, were monitored for the duration of the experiment.

After the 30 minute period, the RF generator was switched off. The vacuum line was closed and pressure was returned to the system using a bleed valve. The fabric swatch was then carefully removed from the reactor and cut into the sections required.

(ii) **UV grafting**

UV grafting was performed using a Dymax 5000-EC 400watt UV flood lamp. Fabric samples were coated with a mixture of 10mmol dm$^{-3}$ ZSB2 and 100µmol dm$^{-3}$ benzophenone (dissolved in methanol), or just benzophenone in the case of pre-treatment experiments, before graft initiation. Samples were then placed in a glass tank with a spectrosil® quartz lid which was in turn placed in the flood lamp enclosure. The tank was flooded with nitrogen for 1 minute before initiation of the graft. All grafts were performed for five minutes with a total energy of ~7J/cm$^2$. In the case of pre-treatments which were performed on 3cm diameter swatches, the graft was stopped at the desired time point and the solvated ZSB2 was added as quickly as possible before resuming the graft procedure. Post treatment, samples were left under nitrogen for 30 minutes before testing.

(iii) **Washing of Fabric Swatches**

Washing of treated fabrics was performed in order to determine the retention of both the compound and antimicrobial efficacy. Washing steps fell into two categories either a gentle or a harsh wash. In the gentle procedure swatches were soaked in 500µl of PBS 2% DMSO per cm$^2$, (5ml for a 3cm X 3cm swatch), for 10 minutes before rinsing in Fresh PBS. In the Harsh wash cycle swatches were sonicated in 500µl methanol/cm$^2$ for 10 minutes. After sonication swatches were rinsed in methanol and then rinsed in PBS. In both cases washed fabrics were dried completely before any microbiological tests were performed.
Microbiological methods

All manipulation of cultures or materials to be used in experiments took place under sterile conditions using a ScanLaf Mars pro class II laminar flow cabinet. Materials were handled using appropriate aseptic techniques in order to maintain sterility. Growth media for all *P. aeruginosa* experiments was Luria-Bertani, (LB), broth or LB agar. Growth media for all *S. aureus* cultures was Tryptic soy broth (TSB) or tryptic soy agar. *S. epidermidis* was grown on TSB and *E. coli* *K. pneumoniae* and *A. baumannii* were grown on LB. In the case of *E. coli* MC1061 zinc free media was created according to the recipe below.

In all experiments overnight broth cultures were generated from inoculation of culture media with single bacterial colonies from stock plates. Cultures were grown for 18 hours at 37°C shaking at 250rpm before subculture into 10ml of fresh media. Cultures were allowed to grow for 4 further hours shaking at 37°C before being centrifuged at 10000g in an Eppendorf 5414 D microfuge for 3 minutes and subsequently re-suspended in fresh media. Optical density of the re-suspended culture was measured using a Thermo Electron Helios γ UV/Vis spectrometer OD$_{600}$ was then adjusted to 0.01 (~$10^5$ CFU/ml referred to as experimental inoculum) before inoculation of experimental samples.

(a) Microtitre plate assays

Several types of biological assay including, minimum inhibitory concentration (MIC), fluorescence response to bacterial growth and/or supernatant and GFP::RNAII production assays were performed using either a bmg labtech Omega FLUOstar or bmglabtech Omega SPECTROstar microtitre plate reader. Both readers were equipped to measure UV/vis spectra from 200-800nm and to measure fluorescence using spectral filters. Unless otherwise stated costar 96 well round bottom microtitre plates were used for all experiments. The lids of plates were treated with 5ml of a 0.05% solution of triton X-100 in ethanol prior to any test in order to prevent the occurrence of condensation during an experiment [3]. In all vesicle fluorescence measurements positive and negative controls consisting of either 10µl of HEPES storage buffer or 10µl of 0.1% triton X-100 dissolved in PBS were added to each plate. Band pass excitation and emission filters allowing light
at 485nm and 520nm respectively were used in fluorescence experiments with either [5,6]-carboxyfluorescein or GFP. Unless otherwise stated the gain for fluorescence experiments was set to 650. Spectral characteristics of fluorescence excitation and emission filters can be seen in below.

(i) **Vesicle lysis and growth assay**  
**Used in determination of lytic species Chapter IV**

In vesicle fluorescence assays 50µl of vesicle suspension and 130µl of fresh media were added to each test well of a plate. 20µl of the experimental inoculum was added to each test well and the plate was run in the reader at 37°C, shaking at 250rpm for 18 hours. During this time period measurements of absorbance at 600nm and fluorescence were made every 10 minutes.

(ii) **Vesicle lysis by supernatant**  
**Used in lytic species determination and *S.aureus* and *P.aeruginosa* screen strains Chapter IV**

18 hour cultures of desired strains were centrifuged for 10 minutes at 10000g to pellet bacterial cells. The resulting supernatant was then filtered through a 0.1µm syringe driven filter to remove remaining cells. 150µl of the filtered supernatant was then either added to 50µl of vesicles neat or a dilution series of the supernatant was generated and vesicles were treated with 150µl of each dilution. Measurements of vesicle lysis were again made using the 485nm excitation filter and a 520nm emission filter. Measurements of fluorescence were made every minute over a two hour period.

(iii) **Effect of bacterial growth on fluorescence**  
**Chapter IV**

20µl of experimental inoculum was added to microtitre test wells containing 140µl of sterile growth media and 20µl of 250µmol dm⁻³ [5,6]-carboxyfluorescein to give a final CF
concentration of 25µmol dm$^{-3}$ dual absorbance fluorescence measurements were made every 10 minutes for 18 hours.

(iv) **GFP::RNAIII dual absorbance fluorescence measurements**

*Used in determination of quorum sensing involvement in lysis Chapter IV*

In the pGFP::RNAIII measurements of GFP production 20µl of the experimental inoculum (containing 10µg/ml of chloramphenicol for maintenance of the plasmid) was added to 180µl of TSB containing 10µg/ml of chloramphenicol. The plate was then incubated for 18 hours and fluorescence and absorbance were measured as above. The gain for measurements was set to 1400 due to the lower fluorescence seen from GFP in comparison to CF50

(v) **MIC Assay**

*Used to determine antimicrobial efficacy of ZSB2 compounds in Chapter VI*

Stock solutions of 10mM ZSB2 and 10mM SB2 were made in either in either LB or TSB 2% DMSO. 200µl of these solutions were subsequently mixed with 19.8ml of LB each. These stock solutions were used to create a dilution series, in 2 separate 96 well plates with 3 repeats of each concentration for each compound. 3 columns were made up with controls, (each repeated 3 times). A zinc acetate dilution series (50× final concentration) was made in sterile H2O due to poor solubility in DMSO. The controls were media only and media 2% DMSO. The outside wells of each plate were filled with sterile dH$_2$O, to compensate for evaporation.

20µl of the experimental inoculum was added to each of the wells in both the 96 well plates, excluding blanks. Plates were read in a shaking plate reader incubated at 37°C for 18 hours. Absorbance measurements at 600nm were taken every 5 minutes for an 18hr period. The plate was shaken at 250rpm in between reads. Growth curves from the reading were plotted and the integrals were taken to generate MIC curves.

MICs for the two biosensor strains were performed using zinc free M9 medium (per dm$^3$:
33.9 g Na$_2$HPO$_4$, 15 g KH$_2$PO$_4$, 2.5 g NaCl, 5 g NH$_4$Cl, 2 mM MgSO$_4$, 0.5 mmol dm$^{-3}$ CaCl$_2$) supplemented with 1 mg L$^{-1}$ thiamine, 0.2% (w/v) glucose, 0.2% (w/v) acid.
hydrolysate of casein, 100µg ml⁻¹ ampicillin and 10µg ml⁻¹ tetracycline. Experimental temperature was maintained at 30°C which has been deemed to be the optimal temperature for these organisms [10].

(b) Generation of GFP transformants
pGFP::RNAIII transformants were used in Chapter IV to correlate quorum sensing and toxin production

Strains in which plasmid pRNAIII::GFP were to be electroporated into were grown in Brain-Heart Infusion medium, (Oxoid) overnight and washed in ice cold sterile water three times before electroporation. pRNAIII::GFP was first electroporated into RN4220, recovered using a GeneJET mini plasmid prep kit (Fermentas). Isolated plasmid was then electroporated into selected strains. Successful electroporation was classified as growth of strains on chloramphenicol plates (10 µg ml⁻¹) and isolation of plasmid.

(c) Synergistic haemolysis assay
Used in determination of PSM involvement in vesicle lysis Chapter IV

The presence of delta haemolysin is conventionally determined by the synergistic lysis assay, however it has recently been determined that the PSMs are also involved in this synergistic effect [4, 5]. The Presence or absence of phenol soluble modulins/delta haemolysin was determined by streaking the beta – haemolysin positive S. aureus RN4220 strain on 5% sheep blood tryptic soy agar plates (Oxoid). The test strains are streaked perpendicular to this strain and any enhanced zones of haemolysis where the delta lysin overlaps with the beta – haemolysin zone is scored positive for PSM/delta haemolysin production

(d) Colony counting assay based on the Japanese Industry Standard (JIS) 1902
Used to determine the antimicrobial efficacy of ZSB2 fabric treatments

100µl of experimental inoculum (1ml for a 3cm diameter circular swatch), was added to plasma treated or UV grafted polypropylene. Three swatches of fabric were used for each test condition. Three untreated swatches of fabric were also included in each experiment.
All of the swatches were placed in individual 50mm Petri dishes before being wrapped in Parafilm and placed in a static incubator at 37°C for 18 hours.

Post incubation the swatches were removed from the incubator. Swatches were individually washed in sterile 0.9% saline to remove any excess culture before being placed in 50ml falcon tubes containing 20ml of 0.9% saline. Each tube was then vortexed 5 times for 5 seconds each time using an Autovortex SA6 (Stuart Scientific), in order to remove attached cells from the fabric. A dilution series was created ranging from the original vortexed saline solution, down to a dilution of $10^{-6}$ for each sample. Agar plates were inoculated with 100µl of each of these dilutions and incubated overnight at 37°C. Plates were removed from the incubator and those with colony counts between 30 and 300 were recorded. Counts below 30 may result from spontaneous formation and would not be accurate; at counts above 300 it becomes less likely that colonies have been formed from individual cells[6]. From colony counts CFU/ml were determined for the original swatches by multiplying up by the required dilution factor.

**Determination of the presence of free zinc using the biosensor E.coli MC1061(pSLzntR/pDNPzntAlux)**

Chapter VI

A previously reported method was used in order to determine if the antimicrobial efficacy of the zinc Schiff base compound was a result of free intracellular zinc [10]. The method relies on a biosensor strain of *E.coli* which carries two plasmids. The first one, pSLzntR, encodes a zinc responsive regulatory protein (ZntR). The second, pDNPzntAlux, codes for the genes involved in luciferase production (lux). The lux genes are located downstream of a ZntR responsive promoter (ZntA). As such bioluminescence will increase with increasing free zinc ($Zn^{2+}$), concentration. The construction and validation of this strain of *E. coli* was originally reported by Ivask et al [7].

The zinc-responsive *E. coli* strain was grown overnight at 30 °C, shaking at 250 rpm, in M9 minimal medium of the composition described using the same strain in the MIC assay. 500 µL of this culture was sub-cultured in fresh M9 media (with supplements as before) for 4 h until exponential phase was reached ($OD_{600} \sim 0.4–0.6$). The culture was then diluted to an $OD_{600}$ 0.1. 100µl aliquots of culture were then added to individual wells of a 96-well
plate (Flat clear bottom, black, Greiner), which also contained dilutions of either Zn acetate or the zinc Schiff base ZSB2 (100µl per well). Plates were incubated at 30 °C (without shaking) and bioluminescence measured using a FLUOstar Omega plate reader (BMG Labtech) at 2hr and 4hr.

(f) **Live/Dead staining**

**Performed to examine surface killing of ZSB2 treated fabrics Chapter VI**

100µl of experimental inoculum was deposited on each of four fabric swatches measuring 1cm²; two UV graft treated, two untreated. Of the 2 swatches coated using the 3 minute UV pre-treatment one had been exposed to a gentle wash cycle and the other to a harsh wash cycle. All swatches were placed in individual Petri dishes before being wrapped in Parafilm and placed in a static incubator at 37°C for 4hrs. After 2 hours one of the untreated samples was removed and exposed to 70% ethanol. A solution of live/dead stain was made using an Invitrogen LIVE/DEAD BacLight L13152 staining kit. Pre-measured quantities of SYTO9 and propidium iodide were rehydrated in 5ml of Millipore dH2O.

After four hours had elapsed the swatches were removed from the incubator and individually washed in 5ml of sterile 0.9% saline solution. 20µl of the LIVE/DEAD mix was added to each swatch, the swatches were left to stain in total darkness. After an hour the swatches were washed in 5ml of 0.9% saline under low light conditions before being visualised using a Nikon eclipse 90i confocal epifluorescence microscope.

**Section 2.03  Biochemical assays**

(a) **Orcinol rhamnolipid detection**

**Detection and quantification of rhamnolipid from bacterial supernatant Chapter IV**

The orcinol assay was used to detect the presence of the glycolipid rhamnolipid in supernatants of *P.aeruginosa*. The assay functions through acidification of the rhamnolipid rhamnose groups to methyl furfural which react with orcinol to form a coloured substrate.
Glycolipid was separated from 5ml of filtered 18-hour supernatants of the desired strains by extracting twice with 60ml of diethylether. Solvent was removed from glycolipid samples through drying under nitrogen flow. Once completely dry, samples were rehydrated in either 1ml or 5ml of phosphate buffered saline to generate 1× and 5× concentrated samples respectively. 100µl of the rehydrated sample was added to 900µl of 53% H₂SO₄. The acidified sample was heated at 80°C for 30 minutes. Once cooled to room temperature the absorbance of the sample was measured at 421nm, absorbance values were subsequently compared to a standard of R-95 rhamnolipid purchased from Sigma Aldrich in order to generate values of rhamnolipid concentration in µg/ml [8].

(b) PSM and Rhamnolipid vesicle assay

Used to investigate the interaction of synthetic PSMs and R-95 rhamnolipids with vesicles Chapter IV and V

50µl samples of vesicles were exposed to various concentrations of R-95 rhamnolipids or synthetic PSMs dissolved in PBS. Samples had a total volume of 200µl. Fluorescence response from the samples was measured for a period of 2 hours. 60 minute values were subsequently normalised between a positive control (0.1% triton X-100) and a negative control (HEPES buffer).

(c) Congo red spectral shift assay

Used to detect aggregation of PSMα2 at high experimental concentrations Chapter V

Congo red, (Sigma Aldrich) was dissolved in 90% PBS 10% ethanol to a concentration of 100mM and vortexed. In order to remove micelles of the dye the solution was filtered through a 0.1µm polycarbonate membrane. The concentration of the filtered dye was determined against a standard concentration gradient. The filtered dye was added 1:10 to concentrations of PSMα2 dissolved in 90% PBS 10% ethanol ranging from 0.1µmol dm⁻³ to 90 µmol dm⁻³. Absorbance spectra of the Congo red/PSMα2 samples were measured and observed for characteristic spectral shifts. Treated samples were subsequently centrifuge to pellet dye peptide complexes and give a quantative measure of amyloid formation [9].
Section 2.04 Analytical techniques

(a) FT-IR of Fabric swatches

Examination of ZSB2 treated fabrics surface chemistry Chapter VI

FT-IR measurements were performed using a PerkinElmer spectrum 100 FT-IR spectrometer. The FT-IR crystal was cleaned with isopropanol before use and blanked against air. 32 scans at a resolution of 2 cm⁻¹ were taken. From these scans a concatenated plot was produced.

(b) Measurement of Relative Fluorescence

Semi quantative method for detection of surface bound ZSB2 after washing Chapter VI

In order to determine if ZSB2 was retained on fabrics post washing and if UV treatment cycles had an effect on the level of attachment, relative levels of fluorescence were measured. The fluorescence spectra and stoke shift of the compound was determined by taking a fluorescence profile of a 1µm solution of ZSB using a Perkin Elmer LS50 B Luminescence Spectrometer. In order to measure the relative fluorescence of the coated fabric, 3cm diameter swatches treated under the various UV graft cycles and subsequently exposed to the harsh methanol wash cycle. Swatches were then placed into a 6 well tissue culture plate ensuring that correct orientation of the swatches relative to one another was maintained. The fluorescence of the swatches was determined using the bmglabtech FLUOstar Omega plate reader. The reader’s excitation and emission filters were set at 355 nm and 460 nm. The machine was programmed to measure the fluorescence of each point in a 6×6 matrix for each well, the gain was auto adjusted to display the highest value as 90% of the theoretical maximum before the experiment.
(c) Scanning electron microscopy

Observations of UV grafted polypropylene samples Chapter VI

Polypropylene swatches UV grafted with ZSB2 were placed in a desiccator for 18 hours in order to dry. On removal from the desiccator individual threads of the treated fabrics were adhered to sticky carbon film before being sputter coated with gold to a thickness of 2-4nm. The polypropylene surface was then visualised using a JEOL-SEM6480LV scanning electron microscope with a beam energy of between 10-20kv.

(d) Energy dispersive X-ray spectroscopy

Elemental analysis of UV grafted polypropylene samples Chapter VI

Similarly to SEM investigations UV grafted swatches were dried overnight in a vacuum desiccator. After 18 hours fibres were adhered to sticky carbon film before being sputter coated with carbon to a thickness of 2nm. The polypropylene surface was similarly interrogated using a JEOL-SEM6480LV scanning electron microscope with a beam energy of 10kv. Data was analysed using an Oxford INCA X-ray analyser.

(e) Retention of plasma deposited compound

Study of the leaching of ZSB2 from UV treated gentle wash fabrics Chapter VI

The molar extinction coefficient of the ZSB2 compound was determined by creating a dilution series of ZSB2 of known concentration in PBS with 2% DMSO. An absorbance spectrum of each dilution in the series was taken using the fluorescence plate reader. From these spectra, the $\lambda_{\text{max}}$ of the compound was determined to be 387nm. Concentration was plotted against absorbance for each of the dilutions and $y = mx + c$ was determined. The retention of the ZSB2 compound on fabric treated under various UV graft conditions was compared to an un-grafted control through absorbance measurements of the fabric leachate over a five day period through.
Section 2.05 Data processing

All data analysis was performed using Microsoft Excel and Originlab 9 pro graphical analysis software. Dose response fits of fluorescence change over PSM concentration ranges were generated using the inbuilt hill equation within Origin. Weighting of the fits was based on experimental error. The only constraint put on the data was that start values must not be below zero.

Fluorescence data was normalised to the equation:

\[ \frac{(F_t - F_0)}{(F_{max} - F_0)} \]

Where \( F_t \) the sample fluorescence at a specific time point, \( F_0 \) is the fluorescence value of the negative control and \( F_{max} \) is the fluorescence value of the positive control. In order to prevent error propagation in the normalised standard error of the mean the following adjustment was employed:

\[
\left( \sqrt{\frac{F_t}{F_{max} - F_0}} \right) \times \left( \frac{F_t}{F_{max} - F_0} \right) \times \left( \frac{F_0}{F_{max}} \right)
\]

Where \( F_t^- \), \( F_0^- \) and \( F_{max}^- \) are the errors of the sample fluorescence, negative control and positive control respectively.

Section 2.06 References


7. Ivask A, Rolova T, Kahru A: A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing. *BMC biotechnology* 2009, **9**:41.


Chapter III. Phospholipid vesicles as a method for detecting bacterial pathogenesis.

Section 3.01 Introduction

(a) Biosensors: considerations and mechanisms.

Detection of pathogens in wound environments can be performed in several different ways utilising either direct or indirect methods. The forms of interaction between a pathogen and a putative detection system can generally be placed into one of four classes (Figure III-1). Direct interactions are the result of close coordination between the organism itself and the sensor through generalised non-specific attachment and binding, or through a tethered moiety on the organism’s surface and the sensor. These close association sensors can take the form of changes in the incidence angle of a surface plasmon, or an increase in capacitance in an inductive sensor (in the case of generalised binding), or complexation of surface components with specific antibodies (in the case of recognition of surface moiety’s). The other three classes of pathogen probe interaction are grouped as indirect methods, as direct communication between organism and sensor are not required. The first such indirect method is interaction between excreted or secreted pathogen derived molecules and the chosen sensor. Such compounds could be secreted pathogen associated molecular patterns (PAMP’s) commonly associated with immune recognition, toxic compounds such as LPS, or more classical toxins such as α toxin from S.aureus or super antigens such as toxic shock syndrome toxin (TSST). The major caveats to secreted molecule detection are diffusion of the molecule to the sensor, and specificity of the sensor. If sensors are too specific, this limits detection; if they are too general the risk of false positives is increased. The second indirect method would be designed to focus on changes which pathogens might impart on their environment. Increases in CO₂ caused by increased respiration rate can cause acidification of an aqueous environment; similarly changes in temperature or dissolved oxygen concentration are measurable parameters which could be employed for use in a sufficiently sensitive device. Finally, sensors could be designed to detect the response of the host organism to a pathogen; again through the release of specific molecules or a change in the wound environment. Whilst such a downstream sensor does reduce the risk of false positives due to the detection of
environmental colonisation rather than an actual infective state, it runs the risk of lacking any specificity, as a detectable host response can be very general.

Figure III-1 General targets for pathogen sensor system in wound environments. (A) Direct interaction between sensor and pathogen; (B) Recognition of exogenous pathogenic marker; (C) Recognition of a pathogen mediated environmental change; (D) Recognition of a host response to a pathogen.

Each of the possible systems described above has their own pros and cons. The system in this study takes the form of lipid bilayer nanocapsules, encapsulating a quenched fluorescent dye. The concept is that interaction between secreted bacterial toxins and the bilayer will cause the vesicle to lyse or collapse, releasing the payload and generating a visible colorimetric response. A schematic of this type of response is shown in Figure III-2. In this respect the bilayer mimics membrane damage, which eukaryotic cells experience due to interactions with bacterial membrane active agents.
Figure III-2 The proposed mode of action of the vesicle based sensor system. (1) Bacteria produce numerous lytic agents which are free to interact biosensor vesicles. (2) Interactions between toxins and biosensors can occur in a number of ways: (a) membrane active enzymes such as phospholipases can directly affect the lipids which make up the bilayer destabilising the capsule; (b) toxins can generate pores or channels in the bilayer creating an osmotic imbalance with the potential to collapse the capsule as well as causing dye leakage, (depending on the pore size); (c) Amphipathic molecules can integrate with the bilayer lipids generating disorder and bilayer stress leading to the eventual collapse of the vesicle. (3) Once the bilayer has been destabilised and disrupted, de-quenching of the encapsulated dye occurs and this can be observed through measurements of fluorescence.

The use of the vesicle bilayer as the effective sensor generates both problems and opportunities as whilst the composition of the bilayer allows for a high degree of tunability, the simplicity of such bio-mimicry means that bacterial toxins will likely not be the only trigger for the system. Those bacterial toxins that will act as triggers for the system are also likely to be numerous. As such, it is important to identify which molecules trigger the system and in what quantities, in order to gauge how the system will work outside of a lab setting.

(b) Aims and Objectives

The aim of work reported on in this chapter was to test susceptibility of an adapted biosensor, based on a partially cross-linked lipid bilayer nano-capsule, encapsulating a fluorescent dye, 5,6-carboxyfluorescein (CF) to a variety of clinically relevant organisms. Once key organisms to which the capsules were susceptible were identified, the next goal was to determine the mechanism or mechanisms from which this susceptibility arose. Understanding of such mechanism(s) would be key to the tuning and design of the second generation of smart capsules.

To meet the first objective of determining organisms to which the capsules proved sensitive, vesicles were tested against both supernatant and cells of a variety clinically derived organisms. Not only did this allow for the identification of S.aureus, S.epidermidis
and *P. aeruginosa* as organisms of note in regards to capsule lysis but it also enabled identification of appropriate methods for the determination of differences in lytic capabilities between organisms.

To determine the relevance of organisms which had proved lytic screens of a number of *S. aureus* and *P. aeruginosa* strains were performed. What followed from this was an in-depth comparison between known of each organism’s haemolytic capacities and their vesicle lytic capacities. The comparison was made through a barrage of classical microbiological tests, biochemical tests, strain transformation, lysis rate analysis and literature searches.

Ultimately the goal of Organism determination and relevance, toxin determination and mechanism identification was successful. Whilst the identification of mechanisms was not 100% certain owing to the lack of availability of certain toxin mutants, the collected evidence suggests predictions were accurate.

**Section 3.02 Response of vesicles to common burn pathogens.**

**(a) Haemolytic activity of common burn pathogens.**

In order to gauge the general applicability of the system, an assessment of the organisms which the system would be susceptible to, and the degree of susceptibility to each of those organisms, was required. An initial investigation was made using clinical isolates of a number of organisms which are relevant to burn wound infections (See Table I-I-1). Strains were initially screened for their ability to generate zones of lysis on blood agar plates (Figure III-3). This was then compared with the strains potentials to lyse 53% DPPC 20% cholesterol vesicles (DPPC53) vesicles in an 18hr plate assay.
Figure III-3 Heamolysis induced by the growth of various bacterial clinical isolates. (A) *E.coli* Dh5α, (B) *P.aeruginosa* PA01, (C) *S.aureus* USA300 LAC, (E) *S.epidermidis* 12228, (F) *K.pneumoniae* BC01150, (G) *A.baumannii* BC0777. Images (D) and (H) show magnified views of numbered plates.

The organisms initially investigated were *E.coli*, *P.aeruginosa*, *S.aureus*, *S.epidermidis*, *K.pneumoniae*, *A.baumannii*. The *E.coli* used was a laboratory strain DH5α whose toxicity has been largely attenuated [1, 2]. As such its inclusion was as a negative control. Whilst *S.epidermidis* is not a common burn pathogen, it is a common commensal organism which is associated with chronic infection especially in immune compromised patients [3]. Observations of the blood agar plates (Figure III-3) allows for discrimination between β (total), α (partial) or γ (lack of) haemolysis [4] demonstrated by different organisms. *S.aureus* and *P.aeruginosa* show the greatest of clearing around growth with a return to the base colour of the medium and an increase in agar transparency, which is indicative of β-haemolysis, (yellow arrows Figure III-3). It should be noted however that clear lysis by *P.aeruginosa* was seen only around areas of heaviest growth. This could be due to relatively “weaker” lytic species, fewer lytic species, a rate dependant effect (rate limited enzymatic action), or potentially a quorum effect. It is known from literature that *S.aureus* produces several lytic exotoxins which could be responsible for the haemolytic actions seen (these are listed in Table III-1). *P.aeruginosa* on the other hand produces a number of toxins but only two main lytic species, haemolytic phospholipase C and the glycolipid, rhamnolipid (RL) [5, 6].

Discolouration of the media surrounding colonies was seen with *S.epidermidis* and *K.pneumoniae* (Purple arrows Figure III-3). Green/brown discolouration of blood agar is
synonymous with \( \alpha \) haemolysis an effect caused by the interaction of hydrogen peroxide, produced by the bacteria, with haemoglobin to form methaemoglobin. Neither \( K.\text{pneumoniae} \) nor \( S.\text{epidermidis} \) however produces hydrogen peroxide. Media discolouration may simply be due to nutrient depletion of the agar. No change in agar appearance was seen on the \( E.\text{coli} \) or \( A.\text{baumannii} \) plates. \( S.\text{epidermidis} \) is known to produce phenol soluble modulins, which have been demonstrated as having some haemolytic activity, but which are more commonly associated with biofilm detachment [3]. \( K.\text{pneumoniae} \) is known to produce LPS and toxin complexes which may have a role in inflammation or metabolism modulation, but no haemolytic species have been identified [7, 8]. \( A.\text{baumannii} \) produces a number of toxins involved in immune modulation but has also been shown to produce phospholipases, (phospholipases C & D), interestingly however, these phospholipases have been demonstrated as haemolytic to horse but not sheep blood [9, 10]. The differences in lytic capacity of \( S.\text{aureus} \), \( P.\text{aeruginosa} \) and \( E.\text{coli} \) can perhaps be seen more clearly when all three organisms are displayed on one plate (Figure III-4).

![Figure III-4](image-url) 5% sheep’s blood agar plate showing the haemolysis produced by \( S.\text{aureus} \), \( P.\text{aeruginosa} \) and the lack of haemolysis by \( E.\text{coli} \).
(b) Lytic capacity of common burn pathogens towards phospholipid vesicles

The haemolytic behaviour of the clinical isolates was compared with their ability to lyse DPPC53 vesicles through measurement of fluorescence response over a 17 hour period (Figure III-5).

Figure III-5 Growth and lytic capacity of clinical bacterial isolates as measured through changes in growth medium turbidity and normalised fluorescence. (A) Growth as measured by optical density at 600nm. (B) Vesicle lysis as determined by normalised fluorescence.

Primary observations of the fluorescence response from all organisms show distinct separation into two separate groups. The normalised fluorescence values of *E.coli*, *A.baumannii* and *K.pneumoniae* do not extend much above those of the blank (vesicles and uninoculated media only). *S.aureus*, *S.epidermidis*, and *P.aeruginosa* on the other hand show fluorescence values roughly equivalent with those shown by the positive control after 17 hours (data was normalised with the lowest negative control value representing 0, and the highest positive control value representing 1).
These results are largely what would be expected given the haemolytic activity demonstrated by each of the organisms. *S.epidermidis* represents something of an oddity, in that it showed no obvious haemolysis on blood agar, but vesicles did respond to the organism. This demonstrates that the correlation between haemolytic activity and vesicle lysis is not necessarily straightforward. It may therefore be possible to use such a vesicle system to detect the presence of pathogenic organisms (other than those tested here) which also show no haemolytic activity. There appears to be some correlation between growth phase and lysis in all three lytic strains. Such a correlation requires confirmation through further experimentation, however it would make sense in respect to *S.aureus* and *S.epidermidis*, with regards to the toxin regulating function of the accessory gene regulator (AGR)[11]. Whilst the correlation between growth and toxin production is less well defined for *P.aeruginosa*, there is a strong correlation between virulence and quorum sensing in this organism [12].

(c) The effect of bacterial cultures on carboxyfluorescein fluorescence

The peculiar pattern of the initial fluorescence peak, subsequent drop, and final fluorescence increase seen with both Staphylococcus species seems to be characteristic of staphylococci and has seen in numerous experiments with a variety of *S.aureus* strains. As of yet its cause has not been identified. The initial increase varies in degree between different experiments. It is observed even after extensive washing of exponential phase cultures used as the inoculum for experiments, suggesting that it is unlikely to be caused by excess toxin. Lack of variation in fluorescence response with differing starting concentrations of cells suggests that either cell surface proteins do not play a role in the peak’s occurrence, or that the peak is caused by very low level interactions with cells.

There are a number of potential interactions between elements of the biological environment to be measured and either the fluorophore itself or some aspect of the measurement process which could lead to the development of such aberrations. Measurements in biological systems can be particularly problematic due to their dynamic nature. The use of multiple types of biological media for example can cause a few issues. Firstly the different chemical compositions of different media will likely have different spectral properties, absorbing different amounts of incident and emitted light. Whilst this effect can generally be compensated for through normalisation of measurements through
inclusion of positive and negative controls, differences over time caused by bacterial growth (or through variations in secreted products when utilising bacterial supernatant rather than cultured cells), can complicate measurements further.

The different chemistries of the different media can also affect quenching of a fluorophore differently, i.e. a greater quencher concentration or a greater number of quenchers in one media type may cause greater attenuation of fluorescence as compared to another media type. The polarity of the solvent in which the fluorophore is solvated can also affect the intensity of fluorescence signal received. Generally increased polarity of a solvent leads to greater fluorescence intensities as well as red shifted emission maxima [13].

Solvatochromic effects are generally due to lowering of the energy barrier required for excitation [14] [15]. While such media based effects cannot be immediately discounted, in this case it would seem unlikely that they are wholly responsible for the observed variation. The different media used for the Staphylococcus species as compared to the other organisms tested might account for the some of the mid exponential phase fluorescence drop off seen. However, internal controls would eliminate the initial observed high fluorescence.

Turbidity of biological samples caused by cell growth in the media will also affect the intensity of the fluorescence signal received from the sample due to both quenching and light scattering effects [16]. In a microplate fluorometer excitation light will be focused into the centre of a microplate well and emitted light will be collected at a fixed angle 45° from the incidence beam. The cross-section of the incidence and emission region is considered the optically active zone as it is common to both. The intensity lost to scattering in a 2 dimensional slice of the optically active region with a thickness \( d \) is proportional to both the concentration of scattering particles, \( h \) and \( d \) itself.

\[
\frac{dI}{I} = - (\alpha_A + \alpha_S) h \, dz
\]  

(1)

Where \( \alpha_A \) and \( \alpha_S \) are the wavelength dependant attenuation coefficients caused by absorption and scattering of light respectively and \( z \) is the beam path length. Intensity of
the excitation light \( (I_{ext}) \) along the total path length, (within the well) can be obtained through the integration of:

\[
I(z) = I_{ext} \exp\left[ (-\alpha_A - \alpha_S)hz \right]
\]  

(2)

If the scattering/absorbing particles in the well are associated with a number of fluorophores which have quantum yields of \( \phi \) then the intensity of emitted light reaching the detector \( (I_{em}) \) can be written as:

\[
I_{em} = CI_{ext}n\hbar\phi e^{-ah}
\]

(3)

Where \( \alpha \) is the grouped attenuation functions of both the incident and emitted light and \( C \) a constant of the instrument [17]. From these equations we can see that an increased degradation of excitation and emission intensities is directly related to the number of and attenuation characteristics of absorbers and scatters. Equation 3 also demonstrates that the emitted light is related to the degree of scattering of the excitation beam both in terms of excitation beam attenuation, (and subsequent emission attenuation) and excitation beam broadening creating a larger optically active region. These concepts are useful in explaining the complexity that scattering particles can introduce into a fluorescence system (Figure III-6).
Figure III-6 Schematic of the effect of scattering particles on excitation and emission beams in a microplate fluorometer. The striated blue region represents the incidence light into the microtitre well along the path length $z$. Dashed lines coming out from the light path show the potential for scattering into the optically active region a process which can alter the excitation signal given constant incidence intensity. The emitted light which is generated in all directions (green circle), will encounter similar problems of scattering.

It is possible then that the observed fluorescence decrease for both *Staphylococcus* species in Figure III-5 is caused by light scattering during bacterial growth. Taking this train of thought a step further it is possible that the subsequent increase in the late exponential/stationary phase of growth could be as a result of gradual CF release. As the extra-vesicular concentration of CF increases quenching by scattering processes is gradually negated. Under this assumption however it is still unclear why this would occur only when staphylococci are used although it indicates inherent differences in scattering generated by staphylococcal as opposed to pseudomonal cells for example [17].

The mid exponential phase decrease could also potentially be accounted for by pH dependant effects which can have a profound effect on fluorescence depending on the sensitivity of the fluorophore in question. In the case of fluorescein both fluorescence and absorbance spectra show broadening at lower pH values as well blue shifting of the absorbance maxima (Figure III-7 F). These variations are caused by differently ionised forms of the molecule. Both the phenol and carboxyl groups of fluorescein are susceptible to protonation at low pH and de-protonation at high pH. Above pH 9 the molecule is in its dianion form, at around pH 5.5 the monoanion form dominates with the phenol group being protonated [18]. Around pH 3 the neutral form of the molecule dominates but is in equilibrium with a lactone form, at lower pH a cation is generated [19]. Neither the cation
nor the neutral form of fluorescein is fluorescent, the monoanion is weakly fluorescent but the majority of the fluorescence response is due to the dianion form even at low pH as demonstrated in Figure III-7.

![Diagram showing pH effects on fluorescein ionisation and spectral characteristics](image)

Figure III-7 Effect of pH on fluorescein ionisation and spectral characteristics. (A, B, C and E) show the dianion, monoanion, neutral and cation forms of the molecule respectively. (D) shows the lactone form of the molecule with which the neutral form is in equilibrium at around pH3. (F) Demonstrates how excitation and emission spectra change with pH. (F) was adapted from the Invitrogen website [http://goo.gl/RG1eD3](http://goo.gl/RG1eD3) (20/09/2013).

Whilst there are many structural derivatives of fluorescein, 5,(6)-carboxyfluorescein included, which will shift absorption and emission maxima the spectral response to pH variations remains broadly the same. A factor of staphylococcal metabolism (increased CO₂ production for example) could therefore potentially lower the pH of the growth media leading to a decreased quantum yield.

pH alongside solvent polarity could also generate some of the effects seen through shifting of the absorbance maximum. This change in absorbance maximum can generate variance in measurements of fluorescence intensity as values are not generated from fluorescence spectra but from measuring the intensity of emission allowed through a band pass filter. The graph below shows the absorption and emission profile of 5,(6)-carboxyfluorescein under idealised conditions versus the spectral properties of the filters used in all measurements of CF fluorescence intensity.
Stokes shift = 22nm

As can be seen in Figure III-8 the band pass filters (indicated by the shaded peaks on the graph), cover a small number of wavelengths with a relatively high level of transmission. If the $\lambda_{\text{max}}$ of CF absorption shifts due to any of the above mentioned solvachromatic effects then there is the potential that the observed fluorescence will increase or decrease accordingly. This can generate difficulties when pH or other physical parameters of the solvent in which the fluorophore is dissolved are not consistent. This is the case with biological medium used in the above experiments and as such may represent another source of the observed variation or may at least compound other effects of pH change etc.

Whatever the cause, the decrease is almost certainly as a result of the fluorophores interaction with the bacteria or bacterial by-products rather than the vesicles, as variations in fluorescence are seen when the bacteria are grown in the presence of the dye only (Figure III-9).
A comparison of the response of the control organism *E.coli* and the two lytic organisms *P.aeruginosa* and *S.aureus* shows clear variation despite the size of the experimental error bars. Both *E.coli* and *P.aeruginosa* show increases in fluorescence which approximately coincide with growth, in the case of *E.coli* the increase occurs once growth has reached stationary phase; *P.aeruginosa* on the other hand generates the a peak during early exponential phase. There does not appear to be a correlation between a specific absorbance value and fluorescence increase, as increases begin at an absorbance value of around 1.1 for *E.coli* and at 0.1 for *P.aeruginosa*. Additionally it seems unlikely that processes, such as cellular clumping, which would lower the number of suspended particles in the media whilst increasing the average particle size would result in the fluorescence increase without a corresponding absorbance decrease. This is not to say that
turbidity as a whole is not involved in generation of the fluorescence peak, as absorbance was measured only at a single wavelength and the two organisms are likely to have very different absorbance and scattering profiles. *P. aeruginosa* for example produces the blue-green pigment pyocyanin ensuring scattering of blue wavelengths responsible for CF excitation would be high. This combined with the large size of *P. aeruginosa* cells in comparison to the 600nm wavelength at which turbidity measurements were made ensures high levels of scattering in the forward direction. Factors such as these for example could form the basis of a hypothesis as to why fluorescence increases differ between *P. aeruginosa* and *E. coli*. They are for the moment however, just that, as the complexity of interactions in such a measurement defies clarification of the causes of fluorescence variation.

The response of the fluorophore to *S. aureus* differs in that a decrease in fluorescence is seen with growth of the organism. As with *E. coli*, the fluorophores response occurs late in exponential phase around the onset of stationary phase. This is more in keeping with the response we would expect whereby the fluorescence excitation beam is broadened through scattering induced by the sample. This scattering subsequently affects the number of emitted photons reaching the detector. The following return to “normal” levels of fluorescence is less easily explained again as clumping is not apparent from absorbance measurements.

Looking at Figure III-9(D) a comparison of the two controls, CF with either LB or TSB, can be made. An immediate observation is of the relatively higher levels fluorescence seen in TSB as compared to LB. Although the values of the two controls are somewhat muddled by the cross over in their errors toward the end of the experiment, there is distinct difference between them for around the first 12 hours. The higher intrinsic levels of fluorescence seen in TSB may in part be responsible for the higher fluorescence seen at the beginning of the vesicle experiments with both *S. aureus* and *S. epidermidis* in Figure III-5(B). The initial similarity in fluorescence values regardless of media may be explained by low levels of CF leakage at this time point as demonstrated by the blank. The variance that differing fluorescein concentrations exert on the effect can be seen clearly by performing the experiments shown in Figure III-10 with differing CF concentrations (data not shown). Comparing the blanks to their respective organisms, it is observed that the decrease in fluorescence resulting from growth with *S. aureus* causes a negative deviation from the TSB control whilst *E. coli* shows a positive deviation. *P. aeruginosa* demonstrates
something of a positive deviation from the LB control; however this deviation is never outside the interaction of errors of the two data sets, making its relevance unclear. The dynamic nature of bacterial cultures coupled with the complexity of scattering and absorbance effects on fluorescence values makes determining how bacterial growth is affecting fluorescence very difficult. Effects especially for *S. aureus* are quite clear but understanding their nature may prove problematic. Difficulty in determining the degree to which these effects affect experiments with vesicles stems from the problem of determining the exact concentration of CF present in the media at any one time during vesicle experiments. However, as a lytic response can be clearly identified above the blank in Figure III-5(B), they are unlikely to cause problems in the functioning of the vesicles within a medical device, though confounding of experimental data is possible.
(d) The lytic potential of burn pathogen supernatant towards phospholipid vesicles

In order to ensure that lytic behaviour displayed by the organisms was neither induced nor repressed by the presence of the vesicles, their contents or the buffer used vesicles were also exposed to supernatant from 18 hour cultures of each of the organisms (Figure III-10).

![Fluorescence response of vesicles exposed to supernatant from 18 hour cultures of test organisms.](image)

Figure III-10 Fluorescence response of vesicles exposed to supernatant from 18 hour cultures of test organisms. (A) Fluorescence values generated from the interaction of 18 hour supernatant from each strain with vesicles after a 60 minute period. (B) The fluorescence response over time generated by exposure of vesicles to 18 hour supernatant from the different organisms.

The overall pattern of strains that were lytic to the vesicles remained the same. Lower levels of fluorescence are seen from the three lytic organisms; this could be due to lower toxin production due to the change in growth method (microtitre plate Vs 15ml tube), loss
of lytic species through centrifugation and filtration of the sample, or possibly, that something discretely associated with the cells tested also aids in lysis. This last possibility, or at least the possibility that bacterial cells have a direct effect on fluorophore release, can to some extent be inferred by the rate data associated with the experiment (Figure III-10 B). Starting fluorescence values for the three lysis negative strains are similar in both the supernatant and whole cell culture experiment and yet over 120 minutes fluorescence from the *E.coli* inoculated plate (Figure III-5B), increases by around 0.1 normalised units, fluorescence from the supernatant plate (Figure III-10A), on the other hand increases by around 0.03, a roughly three fold difference. This difference in the lysis negative organisms suggests that something other than exo-toxins can have an effect on fluorophore release. The plateauing effect seen from the lysis positive organisms suggests that the lower fluorescence is not simply due to a rate dependant factor, whereby values would be higher if the experiment had been allowed to continue for a longer time period.

Complications from turbidity generated through the use of cell cultures, rather than supernatant in experiments, as well as the roughly similar difference between culture plates and supernatant measurements for both lysis positive, (0.29 ±0.07) and lysis negative, (0.28, ±0.06) organisms, ensured the use of supernatant from 18 hour cultures in the majority of the following experiments on bacterial lysis. Additionally, although three organisms were identified as lytic to vesicles, it was decided that only *S.aureus* and *P.aeruginosa* would be taken on for further testing due to their greater relevance to burn wound infections.

Section 3.03  Lytic potential and molecular mediators of lysis of *Staphylococcus aureus*

(a) Screen of *S.aureus* strains for vesicle lysis.

In order to gain an understanding of the commonality of lytic behaviour of *S.aureus*, supernatant was collected from 18 hour cultures of 100 different clinically relevant isolates of *S.aureus* from a well described strain collection[20, 21]. A mixture of strains isolated from either community (CA, 32 strains) or hospital acquired (HA, 48 strains) invasive infections or nasal carriage strains (NC, 19 strains) taken from healthy individuals within the same community were tested (Figure III-11). Supernatants from strain RN6390B which
is known to have accessory gene regulator (AGR), activity and an isogenic AGR knock out strain, RN6911, were also included in the screen due to the perceived requirement for *S.aureus* growth to reach stationary phase before the onset of lysis.

**Figure III-11** Screen of clinically relevant isolates of *Staphylococcus aureus* against DPPC53 vesicles. Strains from the strain library of Dr Ruth Massey. Each column represents the normalized fluorescence response achieved from exposure of vesicles to 18 hour supernatant of an individual strain. Column values are the mean of six measurements, (two biological repeats each performed in triplicate), error bars represent the SEM. The mean of all samples is represented as a solid line at a value of 0.832 normalised fluorescence units, the dashed line at 0.290 highlights two standard deviations below the mean which was chosen as the cut off for distinction between lysis negative (below 2 stdev) and lysis positive strains (above 2 stdev).

A normalised fluorescence value two standard deviations below the mean value of all samples was chosen as the cut off between lytic and non-lytic, (shown as a dotted line in Figure III-11). All values collected were either well above or well below this separator. As can be seen from the graph 8 strains fell below the separator value, not inclusive of the AGR knockout, thus 92% of the strains tested were lysis positive. Interestingly the AGR knockout also showed fluorescence values consistent with lack of lysis, suggesting that the molecular species involved in vesicle lysis may be AGR dependant.

Of the lytic strains tested, variation was observed in fluorescence values obtained from individual strains. Analysis of this variance between the three isolate categories, (CA, HA
or NC) showed no difference in the mean fluorescence value of the three groups, (average value was 0.9 normalised fluorescence units in all three cases). Similarly using Fisher’s exact test to compare the actual number of lysis negative and positive strains between the three isolate types to the expected gave a $p$ value of 0.9 demonstrating no correlation.

The 8 lysis negative strains identified from the screen strain (Figure III-11), as well as the AGR positive and AGR negative strains RN6390B and RN6911 were grown on blood agar plates in order to further assess the correlation between haemolysis and vesicle lysis Figure III-12.

Figure III-12 Blood agar plates of strains identified as lysis negative in vesicle screen. Plates show: (A) *S. aureus* RN6390B AGR positive control, (B) *S. aureus* RN611 isogenic AGR knockout of RN6390B, (C) *S. aureus* MSSA 71, (D) *S. aureus* MRSA 378, (E) *S. aureus* MSSA 442, (F) *S. aureus* MRSA 455, (G) *S. aureus* MSSA 511, (H) *S. aureus* MSSA 707, (I) *S. aureus* MSSA 3005, (J) MSSA 15981. Zone of haemolysis can be observed around individual colonies of *S. aureus* RN6390B.
Zones of haemolysis can be clearly seen around colonies of the AGR positive *S.aureus* strain RN6390B. This clearing of the agar is missing from the isogenic AGR knockout RN6911 and the other eight *S.aureus* strains which gave a negative result for vesicle lysis. This result adds further evidence to the possibility that the molecular species responsible for haemolysis is also responsible for vesicle lysis.

(b) The AGR system and vesicle lysis

![Diagram showing the AGR system and vesicle lysis](image)

Figure III-13 Cell density dependence of AGR regulation of toxins. (A) *S.aureus* cells produce background levels of AIP which bind to agrC on the cell exterior. (B) AIP stimulation of agrC is insufficient to elicit repression of ROT. (C) *S.aureus* cell density increases and greater AIP production allows for agrC activation and subsequent agrA phosphorylation. (D) High levels of phosphorylated agrA ensure high levels of P3 transcription and subsequent ROT repression by RNAIII. As a result toxin production is increased. (E) A schematic of the relationship between cell density and quorum sensing.

As previously mentioned (Figure I-13Schematic of the two component regulatory system of the accessory gene regulator., the accessory gene regulator is a quorum based system whereby cells produce an extracellular peptide AIP, which interacts with the AgrC/AgrA two component signalling system in order to illicit a response in terms of RNAIII.
production, and subsequent down regulation of toxin repression. This shift toward toxin
production is tightly controlled by the availability of AIP, which in turn is related to cell
density in a specific environment [22]. AIP concentrations reach levels required for toxin
production either through growth to a certain cell concentration i.e. the classical in-vitro
relationship between growth phase and toxin production [23] or through growth in a
volume constrained environment in which diffusion of AIP is limited [24](Figure III-13).

The difference in haemolysis and vesicle lysis between the AGR positive and AGR
negative strains seen in Figure III-11 and Figure III-12 furthers the idea that AGR is
involved in regulating the lytic species seen in both assays. In order to clarify this
correlation, an overnight culture of S.aureus USA300 LAC (which has been sequenced and
is known to have AGR activity [25-27]) was diluted to 3 different concentrations which
were used as starting inoculum in an experiment measuring both bacterial growth as a
function of light scattering, and vesicle lysis as increase in normalised fluorescence units
Figure III-14.
Figure III-14 The effect of starting inoculum of *S. aureus* on time of fluorescence response using phospholipid vesicles. (A) Normalised fluorescence response of vesicles to *S. aureus* LAC cultures of different starting concentrations. (B) Growth of *S. aureus* LAC cultures of different starting concentrations as measured by optical density.

Clear separation and ordering of the three different starting inocula can be seen in the exponential region of the growth curves displayed in Figure III-14 (B), a pattern which is mirrored in the fluorescence response curves (A). Using the grid lines as a guide, it can be seen that increases in fluorescence values for each of the inoculum concentrations roughly coincides with the transition from exponential phase growth to stationary phase, which is consistent with what is known of the quorum sensing function of the AGR system. The vesicle response to growth of *S. aureus* LAC can also be compared to LAC transformed with an RNAII::GFP reporter plasmid (Figure III-15).
A pattern of fluorescence response to growth similar to that of Figure III-14 is seen in Figure III-15. Again, delay in fluorescence, which in this case is due to GFP production, is seen between the three different starting inocula, and again the onset of fluorescence response aligns approximately with the arrival at stationary phase of the individual cultures. In this experiment the onset of exponential phase growth occurs more rapidly than in the vesicle lysis experiment (Figure III-14) and optical densities obtained are higher despite the same optical densities being used for the starting inocula. In this experiment, vesicles without encapsulated CF were used due to the absorption and emission filters required for measurement of vesicle lysis and GFP being the same. This may therefore point to an inhibitory effect of CF on bacterial growth.

In order to test this concept further, attempts were made to transform the strains identified as lysis negative with the pRNAIII::GFP plasmid [28]. Transformation by electroporation was only possible however in two of the eight strains, (511 and 15981). As a control, the strain showing the highest fluorescence response in the strain screen (MSSA 591) was also transformed with the plasmid (Figure III-16).
As can be seen from Figure III-16 above, whilst all *S. aureus* strains transformed with the reporter plasmid grew, only LAC and the lysis positive strain MSSA 591 showed any GFP production. These three experiments together to suggest an AGR/RNAIII regulated factor as the agent responsible for vesicle lysis.

### (c) *S. aureus* lytic species regulated by AGR

Considering the relatively robust correlation between the timing of vesicle lysis in respect to bacterial growth and what is known of the cell density dependence of AGR based quorum sensing, an investigation was made into the effect of lytic toxins, known to be regulated by AGR (Table III-1), on vesicle lysis [29, 30].
### Table III-1 List of haemolysins and lytic agents produced by *S. aureus*.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Mode of action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alpha toxin</strong></td>
<td>Formation of heptameric pores in cell membranes leading to lysis from osmotic imbalance and cell dysfunction through Ca(^{2+}) influx.</td>
<td>[31]</td>
</tr>
<tr>
<td><strong>Beta haemolysin</strong></td>
<td>A sphingomyelinase</td>
<td>[32]</td>
</tr>
<tr>
<td><strong>Gamma haemolysin</strong></td>
<td>A two component toxin system which forms cation specific pores in cell membranes through hetero-oligomer assembly. Responsible for lysis of several immune cell types part of the leucocidin family.</td>
<td>[33]</td>
</tr>
<tr>
<td><strong>Panton-valentine leukocidn</strong></td>
<td>Also a two component system very closely related to gamma haemolysin which mainly acts on neutrophils.</td>
<td>[34]</td>
</tr>
<tr>
<td><strong>Delta toxin</strong></td>
<td>A small amphipathic peptide reported to destabilise membranes through a surfactant like mode of action.</td>
<td></td>
</tr>
<tr>
<td><strong>Phenol soluble modulins</strong></td>
<td>A series of small amphipathic peptides highly similar to delta toxin. The mode of action is unknown but due to their structural similarity is thought to be similar to that of delta toxin.</td>
<td>[35]</td>
</tr>
</tbody>
</table>

The study was carried out using a number of strains with specific toxin functionalities knocked out (Table III-2) in order to assess the role that individual toxins might have in vesicle lysis. Initially a comparison was made between the different toxin mutants and RN6390B as a functional control.
Table III-2 List of *S.aureus* toxin knockout strains used in this study.

<table>
<thead>
<tr>
<th><em>S.aureus</em> strain</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN6390B</td>
<td>NTCC8325 cured of three prophages</td>
<td>[36]</td>
</tr>
<tr>
<td>RN6911</td>
<td>RN6390B Δagr::tetM</td>
<td></td>
</tr>
<tr>
<td>8325-4</td>
<td>Lab strain NCTC8325 cured of three prophages</td>
<td>[37]</td>
</tr>
<tr>
<td>DU1090</td>
<td>8325-4 Δhla::Em’</td>
<td>[38]</td>
</tr>
<tr>
<td>DU5719</td>
<td>β-haemolysin –ve Phage 42E lysogen of 8325-4</td>
<td>[39]</td>
</tr>
<tr>
<td>LAC (USA300)</td>
<td>Community acquired MRSA strain</td>
<td>[40]</td>
</tr>
<tr>
<td>LAC Δpvl</td>
<td>LAC ΔlukF/S-PVL::spcm</td>
<td>[40]</td>
</tr>
<tr>
<td>LAC Δhld</td>
<td>LAC hld deletion mutant; hld start codon changed from ATG to ATT</td>
<td>[40]</td>
</tr>
</tbody>
</table>
Strains RN6390B and RN6911 (columns 1 and 3 in Figure III-17) again represent AGR functional and non-functional strains and are included as controls for the AGR mediated lytic activity of *S. aureus* against vesicles. The second column in Figure III-17 represents the lytic activity of RN6390B supernatant which was heated to 80°C for 1 hour prior to its exposure to vesicles. Heat treatment was primarily used as a method for the inactivation of the heat labile gamma haemolysin, due to no knockout of gamma haemolysin being immediately available; however alpha and beta toxins are also heat labile under these treatment conditions [41, 42]. Gamma haemolysin is an important virulence determinant found to be present in between 57%, (of 141 isolates) and 99%, (of 309 isolates), of isolates from clinical infections [43, 44]. Gamma haemolysins are bi-component toxins formed from two peptide components, an S-class component consisting of either HlgA or HlgC and the F-class component HglB [33]. These toxins then come together in membranes to form hetero-oligomeric pores consisting of all components. Retention of
lytic activity of the heat treated supernatant of RN6390B suggests that gamma haemolysins are unlikely to be involved in the lytic effects of *S. aureus* on vesicles. This is complemented by the membrane specificity which is required for binding of gamma haemolysin subunits before pore formation. A 2009 study demonstrated that monomer subunits are much more likely to attach to membranes when phospholipid head groups are exposed [45]. However, this requires short chain length lipids of 13 carbons, whereas the primary lipid used in the construction of vesicles, DPPC, has an acyl chain length of 16 carbons making monomer attachment unlikely.

Columns 4, 5 and 6 of Figure III-17 show the effect of *S. aureus* laboratory strain 8325-4 [46, 47] and two of its isogenic mutants with genes for alpha haemolysin, (DU1090 Δhla [48]), and beta haemolysin, (DU5719 Δhlb [49]), removed respectively. Alpha haemolysin is similar in structure and sequence to gamma haemolysin and functions through the formation of heptameric pores in membranes. At low concentrations, alpha haemolysin requires a specific receptor for membrane binding, but at high concentrations subunits are able to bind directly to phospholipids [31, 50]. It has also been demonstrated that alpha haemolysin is capable of binding to and mediating lysis in micron size protein free vesicles constructed from naturally derived phosphatidyl choline’s [41, 51, 52]. These factors made investigation into the lytic potential of alpha haemolysin against vesicles a necessity. The lack of difference between the wild type and the mutant suggest a lack of involvement from alpha. This is likely due to the lack of sphingomyelin in the vesicles which has been reported as a requirement for alpha haemolysin heptamerisation and subsequent lysis [51].

Beta haemolysin is a sphingomyelinase that is particularly active against erythrocytes; this is presumed to be due to their high sphingomyelin content [53]. Beta haemolysin has some substrate plasticity in that it will also hydrolyse lysophosphatidylcholine, albeit at a much slower rate than sphingomyelin, however it shows no activity against phosphatidylcholine phosphatidylethanolamine or phosphatidylserine. As such, beta haemolysin was not suspected to be active against the vesicles. Indeed no significant change in activity was seen between the wild type strain and the beta knockout.

Whilst the lack of difference in levels of lysis was less expected from the alpha knockout, there are significant differences between the vesicles used in this experiment and those used in experiments where the lytic activity of alpha was previously observed. The most obvious variation is in the constituent lipids of the various vesicles. In previous
experiments vesicles were generally made of combinations of naturally derived lipids such as egg or soya bean phosphatidyl choline, bovine sphingomyelin etc. and cholesterol [51, 52]. Substances such as egg phosphatidyl choline tend to consist of mixtures of different phosphatidyl choline molecules with varying chain lengths and saturations, and subsequently different effects on bilayer packing and membrane fluidity. A recent study has demonstrated the lipid and phase specificity of alpha haemolysin which demonstrates preferential binding to sphingomyelin, as opposed to phosphatidylcholine, rich domains within membranes, as well as an affinity for the liquid disordered phase (usually brought about through higher temperatures and lower cholesterol contents) [54]. The lack of sphingomyelin and the relatively high cholesterol content (20%) in the DPPC vesicles used in these experiments would likely affect the ability of monomers to bind to the membrane. Furthermore, the TCDA content of the membrane would likely retard any activity alpha may have against the DPPC vesicles due to the limitations it imposes on diffusion of both bilayer components and inserted monomers[55].

Columns 7, 8, and 9 in Figure III-17 convey the lytic activity of S.aureus USA300 LAC and two knockout strains missing genes for Panton-valentine leukocidin (LAC Δpvl), and delta toxin (LAC hld) respectively.

PVL is another bi-component toxin similar in its mode of action to gamma haemolysin. Like gamma, formation of membrane pores by PVL is dependent on two peptides, LukS-PV and LukF-PV, to make up the pore subunits [56]. It has also been noted that it is possible for LukF-PV to form a highly haemolytic pore subunit with the gamma peptide HlgA [57]. Unlike gamma haemolysin the PVL locus is much less common being found in between 33%-36% of clinical isolates [58, 59]. Structural studies of LukF-PV have demonstrated a possible phosphatidylcholine binding site in the proteins rim domain [60] and site directed mutagenesis studies have demonstrated that alterations in this region lower the membrane binding association constant by around 20 fold [61]. Studies using artificial membranes have demonstrated that PVL toxins are able to bind to artificial membranes, (in this case mixed cholesterol phosphatidylcholine small unilamellar vesicles) and form β-barrel structures however this did not result in an increase in permeability to the vesicle contents [62]. The lack of lytic efficacy of the PVL toxins against artificial membranes therefore agrees with the lack of measurable difference in lysis between the wildtype LAC strain and the Δpvl mutant used here.
Delta haemolysin is a small amphipathic peptide 26 amino acids in length, which is the translational product of RNAIII, the regulator of ROT. The peptide has been demonstrated to have differing effects on phospholipid vesicles based on concentration. One face of the peptide is largely hydrophobic. In solution it is thought that the hydrophobic surfaces of individual peptides associate with one another to form tetramers at concentrations above 2µM, larger structures are suggested at higher concentrations [63]. Experiments exploiting the intrinsic fluorescence of delta haemolysins tryptophan residue have demonstrated that upon interaction with a lipid bilayer, the residue becomes buried, suggesting relaxation of the tetrameric structure seen in solution in to a stable structure at the membrane water interface [63]. This structure can subsequently cause membrane leakage or destabilisation [64, 65]. The consistency of lytic action of the delta knockout in comparison to the LAC wildtype was an oddity in that delta, unlike some of the other candidates for vesicle lysis, is known to act against both bilayers of a single lipid constituent [64] and those containing cholesterol. Due to its ability to assemble in solution and its preliminary interaction with the bilayer surface rather than the bilayer proper, it was assumed that the diffusional constraints imposed by TCDA would also have a limited effect on its action.

The lack of difference between any of the strains tested and their isogenic knockouts led to the assumption that vesicle lysis involved either multiple lytic species or species were involved which had not yet been tested. In the first case the reason for the lack of difference in supernatants from mutants, as opposed to their wild type counterparts would be due to the lytic response being saturated at low toxin concentrations. In order to determine the validity of these concepts dilutions were made of 18 hour supernatant of each of the wild type strains and their isogenic mutants. It was hypothesised that if one of the lytic components from the wild type strain was not present in the mutant strain then increasing dilution would cause lytic activity to decrease more rapidly in the mutant.
Figure III-18 Comparison of the effect of dilutions of 18 hour supernatant from wild type and mutant strains on vesicle lysis. (A) and (B) compare the effect of supernatant dilutions from neat (1) to a 1000:1 ratio (1E-3) with TSB using strain 8325-4 and mutants and USA 300 LAC and mutants respectively. (C) Illustrates the difference between USA 300 LAC and its Δhld mutant in more detail over a dilution range from neat (1) to a 1:100 dilution (0.01). Red arrows on (B) and (C) highlight differences between the mutant and wild type strains.

As can be seen from Figure III-18  B and C, the decrease in fluorescence occurs over a narrower range of dilutions for LAC Δhld than wild type LAC. Some variation outside of the standard error of the mean are also seen in both Δhla and Δhlb mutants (Figure III-18 C). It is possible that these two haemolysins have a low level interaction with the vesicles. However due to the variation being located around one concentration point and requirements of activity from the literature differing from the bilayer criteria of the vesicles, it seems more likely to be variation brought about by some aspect of the experiment. Of the lytic species listed in Table III-1, only for the phenol soluble modulins (PSM’s) were no knockout strains available.
(d) Phenol soluble modulins as vesicle lytic agents of *S.aureus*

*S.aureus* PSM’s consist of two groups of peptides: the relatively short (~20 amino acids) \( \alpha \) PSM’s, and the longer (~40 amino acids) \( \beta \) PSM’s. Of the two groups, the \( \alpha \) PSM’s bear remarkable sequence similarity to delta haemolysin [66] and are thought to have similar mechanisms of lysis [67]. As transcription of the PSM loci is governed directly by AgrA [35] their role in vesicle lysis would fit comfortably within the AGR mediated model of lysis theorised. In order to test the theory that PSM’s have a role in vesicle lysis, vesicles were exposed to synthetic forms of delta toxin and PSM alpha 1 and PSM beta 1 over a biologically relevant concentration range and observed for fluorescence increase.

![Figure III-19](image.png)

*Figure III-19 Response of vesicles to varying concentrations of delta toxin (top), PSM \( \alpha 1 \) (middle) and PSM \( \beta 1 \) (bottom).*

As can be seen from Figure IV-15, vesicles exhibit a lytic response upon exposure to each group of the PSM’s as well as delta. Variations in activity of all the PSM’s will be discussed further in Chapter V. In order to further clarify the role of PSM’s in vesicle lysis the CAMP test was utilised.
Synergistic haemolysis between lysis negative \textit{S.aureus} strains and \textit{S.aureus} RN4220.

Whilst the lack of GFP production from lysis negative strains in figure demonstrates that RNAIII is not being produced, it does not rule out the production of PSM’s due to their direct regulation by AgrA. It has previously been demonstrated that a common test for synergistic haemolysis, also called the CAMP test, is able to identify synergistic haemolysis generated by PSM’s through the use of either $\alpha/\beta$ PSM or delta haemolysin knockouts as well as combinations of these \cite{67}. Synergistic haemolysis can be seen between both USA300 LAC and its $\Delta hld$ knockout counterpart, albeit to a reduced degree in the latter. No synergistic haemolysis (indeed no haemolysis of any kind) however is seen with any of the vesicle lysis negative strains identified in Figure III-11. This both further corroborates evidence that PSM’s are involved in vesicle lysis (based on their absence in non-lytic strains) and demonstrates that whatever mutation is generating a non-lytic phenotype in the strains identified as such, is likely above the RNAIII regulatory level and is more likely involved in the AGR system itself.

\textbf{Section 3.04 Lytic potential and mediators of lysis of Pseudomonas aeruginosa}

As with \textit{S.aureus}, a number of \textit{P.aeruginosa} strains (78), were screened against vesicles to determine their lytic potential. Strains used were kindly donated by Professor Mark Enright. All strains were either from chronic (48) or acute (30) wound site infections;
details other than this however are limited. Experiments were performed as previously, by exposing vesicles to 18 hour supernatant from anoxic cultures. All experiments involved in the *P. aeruginosa* screen strain were performed by Maisem Laabei.

A greater number of strains showed low levels of lysis/low level fluorescence than in the previous strain screen involving *S. aureus*. Due to this, the separation between “lytic” and non-lytic strains was in this case identified arbitrarily as half of the maximal fluorescence achievable. Under this selection criterion, 22 of the chronic strains and 26 of the acute were found to be lysis positive and 26 chronic and 4 acute were found to be lysis negative. The two groups of isolates, those from chronic and those from acute infections were subjected to Fisher’s exact test to determine if there was a difference in the number of lysis positive and negative strains between them. A *P* value of 0.00494 was returned, demonstrating that the greater number of lysis negative strains in the chronic isolate group was highly significant. In order to determine if this result was an effect of the arbitrarily defined separation between lysis positive and negative strains, harsher selection criteria were applied where by the separation value was set to 0.2 normalised fluorescence units. Under these criteria the number of lysis negative chronic and acute strains became 23 and 3 respectively the adjusted fishers exact *P* value became 0.000808. Whilst it is possible that this high level of significance could be muddled by other factors such as commonality.
in specific strains genetic background, the effect is nonetheless striking. The virulence factor responsible for vesicle lysis must first be determined before conclusions on this difference can be drawn.

The four highest lysis positive and four lowest lysis negative *P. aeruginosa* strains from the acute and chronic isolate groups were chosen to determine a) which factors were involved in vesicle lysis, and b) if there was variation in the expressed levels of these factors between the two groups. Initially strains were examined for variations in haemolysis using sheep blood agar (Figure III-22).

Figure III-22 Growth of acute and chronic, vesicle lysis positive and negative strains of *P. aeruginosa* on sheep blood agar. (A) Lysis positive acute isolates, clockwise from top quadrant strains shown are PA44883, PA44981, PA45107, and PA45445. (B) Lysis positive chronic isolate, clockwise from top quadrant strains shown are PA00856, PA00887, PA00889, and PA00935. (C) Lysis negative acute isolates, clockwise from top quadrant strains shown are PA45076, PA45100, PA45122, and PA45394. (D) Lysis negative chronic isolates, clockwise from top quadrant strains shown are PA00261, PA00844, PA00918, and PA00929.
Whilst the expected pattern of vesicle lysis negative and lysis positive strains exhibiting as negative or positive for haemolysis was found to be true amongst the chronic isolates tested (Figure III-22 B & D), the pattern did not hold up quite so well for the acute isolates. The lysis positive acute strain PA44981, (Figure III-22 A, yellow star), shows no haemolysis on the blood agar plate, conversely the lysis negative acute strains PA45100 and PA45394, (Figure III-22 C, green stars), show varying degrees of lysis. *P. aeruginosa* is known to commonly produce two haemolytic agents, a phospholipase C and the glycolipid RL[28]. The organism also contains two acyl-homoserine lactone quorum sensing systems, the LasI-LasR and RhlI-RhlR systems the former being hierarchically above the latter. This is not a simple hierarchy however, as activity of the RhlI-RhlR system has been shown only to be delayed by LasI-LasR mutation [68]. A number of studies have investigated the genes regulated by these systems [69-71] and have demonstrated that the haemolytic phospholipase C and RL genes are positively regulated by both systems. Both compounds are also regulated by numerous environmental factors [72]. Whilst the RL production genes, rhlAB belong specifically to the rhl regulon, phospholipase regulation is less well understood. The systems regulating both of these haemolysis factors are therefore highly complex but it is important to recognise that regulation is much more disparate than that seen in *S. aureus*, in that mutation in one of the quorum sensing genes will not necessarily result in a non-haemolytic phenotype due to the haemolytic activity of RLs and phospholipase being independent of one another [73, 74]. Bearing this in mind, the apparent contradicting behaviour in regards to vesicle lysis and haemolysis seen by strains PA45100 and PA45394 may be due to mutation in one of the haemolysin genes rather than in a global regulator which is the more likely case in the other haemolysis negative strains. This then suggests that only one of *P. aeruginosa*’s haemolysins is responsible for vesicle lysis. The lack of haemolysis generated by the lysis positive acute strain PA44981 may be indicative of low levels of haemolysin production to which the vesicles are sensitive but the haemolysis assay is not.

*P. aeruginosa* produces three varieties of phospholipase C one of which is haemolytic (PLC-H) and the other two which are non-haemolytic (PLC-N and PLC-B [75, 76]). PLC-H has shown a high affinity for phosphatidylcholines and sphingomyelins as well as much lower affinity for a number of other phospholipids, (phosphoserine, phosphoglycerol etc) [77]. PLC-H has also been demonstrated by confocal microscopy to interact with giant unilamellar vesicles consisting of phosphatidylcholine, phosphatidylethanolamine and
sphingomyelin in equimolar ratios. In these experiments PLC-H was shown to interact slowly, (over a period of minutes), with the vesicles, binding in a random fashion and gradually fluidifying liquid ordered phases [78]. Like other proteins with a complex tertiary structure, PLC-H is heat labile; RLs on the other had are not. These factors were exploited in an experiment whereby vesicles were exposed to supernatant from the four lysis positive chronic isolates of \textit{P.aeruginosa} which had either been harvested and left at room temperature for an hour before exposure to vesicles, or which had been heat treated at 80°C [75, 79] for an hour prior to vesicle exposure.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Response of DPPC53 vesicles to supernatant from vesicle lysis positive acute isolates of \textit{P.aeruginosa} which were either incubated at room temperature or at 80°C for an hour prior to the experiment.}
\end{figure}

All of the heat treated samples show a slight reduction in normalised fluorescence values as compared to the samples incubated at room temperature; only in the case of PA00856 and PA00887 did these values come outside of the bound of the SEM. A two way T-test was employed for both sets of data and in each case the difference between 25°C and 80°C was found to be non-significant, (PA00856 \(p=0.657\), PA00887 \(p=0.720\)). The fact that a
decrease at 80°C is seen across the board may therefore be an experimental artefact. The lack of significant change between the heat treated and room temperature samples points towards RL as the agent in *P. aeruginosa* mediated lysis.

RLs are amphipathic glycolipids consisting of a rhamnose head group attached to a 3-(hydroxyalkanoyloxy)alkanoic acid (HAA) tail. There are generally two main categories of RL produced by *P. aeruginosa*, mono- and di-RLs that have one or two headgroups respectively [80], however up to 25 congeners have been identified [81]. Experiments using large unilamellar vesicles have demonstrated that RL can cause the permeabilisation of artificial bilayers at a 1:1 lipid to RL ratio [82]. To determine if RLs have an effect on DPPC53 vesicles, commercially purified RL extracted from *P. aeruginosa* supernatant (R-95 purchased from Sigma-Aldrich) was added to a sample of vesicles at a concentration of 50µg/ml (Figure III-24).

**Figure III-24** Fluorescence response of DPPC53 vesicles after exposure to triton X-100, (positive control), HEPES buffer, (negative control) and R-95 rhamnolipids.

Figure III-24 shows a positive fluorescence response to R-95 RL’s on par with the positive control. Having determined that it was possible for RL to lyse vesicles an experiment was devised to test the theory that lysis negative *P. aeruginosa* strains were RL deficient. The test consisted of a comparison of vesicle lysis by the positive and negative chronic and acute strains to RL production by the orcinol method. This method involves the solvent extraction of RLs from culture supernatants and subsequent heating in the presence of sulphuric acid and 1,3-dihydroxy-5-methylbenzene, (orcinol). The acidic conditions cause the hydrolysis of the rhamnose groups to methyl fufural which reacts with orcinol to form a coloured substrate which can be measured spectrophotometrically at 421nm [83]. This
method of detection and quantification requires the use of a standard against which the RL content of a sample can be determined from its absorbance. The commercially available RL’s R-95 was used as a standard (Figure III-25).

![Graph showing absorbance of RL standard concentrations at 421nm after application of the orcinol assay. Points represent concentrations of between 5 and 100µg/ml of R-95 RL increasing in increments of 5µg/ml. Fitting of the data was performed using OriginLab pro 9. The limit of detection of the assay (LOD) and the limit of quantification (LOQ) are represented as a solid and a dashed line respectively.]

The comparison of lytic capacity and RL production of the strains was performed using single batches of 18 hour supernatant from each of the tested strains. For the vesicle testing, supernatant was diluted with TSB, and vesicles were exposed to concentrations from neat to a 95% dilution. Fluorescence measurements were made every minute for a two hour period. A time point of 60 minutes was arbitrarily chosen as the earliest point at which fluorescence values had plateaued after exposure to supernatant for all dilutions. These values were then plotted. For the orcinol assay, glycolipids were extracted from 5ml of supernatant twice in diethylether. The solvent was removed and the resultant RL’s were dissolved in either 1ml (5 times concentrated), or 5ml (standard strength solution) of PBS. A 5 times concentrated sample was include ensuring orcinol values could be generated above the limit of detection/quantification of the assay. Both samples were then mixed

![Graph showing absorbance of RL standard concentrations at 421nm after application of the orcinol assay. Points represent concentrations of between 5 and 100µg/ml of R-95 RL increasing in increments of 5µg/ml. Fitting of the data was performed using OriginLab pro 9. The limit of detection of the assay (LOD) and the limit of quantification (LOQ) are represented as a solid and a dashed line respectively.]

**Figure III-25** Absorbance of RL standard concentrations at 421nm after application of the orcinol assay. Points represent concentrations of between 5 and 100µg/ml of R-95 RL increasing in increments of 5µg/ml. Fitting of the data was performed using OriginLab pro 9. The limit of detection of the assay (LOD) and the limit of quantification (LOQ) are represented as a solid and a dashed line respectively.
with a solution of 53%H$_2$SO$_4$, 0.19% orcinol in a 1:10 ratio of sample to orcinol solution and heated at 80°C for 30 minutes after which time the absorbance of the samples was measured at the $\lambda_{max}$ of 421nm. Data obtained from acute isolates is shown in Figure III-26 and data for chronic isolates is shown in Figure III-27.

Figure III-26 Lytic potential and RL production of acute isolates of *P. aeruginosa*. The response of DPPC vesicles to supernatant from lysis positive (A) and lysis negative (B) acute isolates of *P. aeruginosa*, as well as the RL production from both lysis positive and negative strains (C). Rectangular intersects on (A) represent EC50 values in the relationship between supernatant concentration and fluorescence for individual strains. Intersects are ranked (1, 2, 3) in ascending order of their X values. RL values in (C) were attained using the orcinol method from samples extracted from identical supernatants to those used in (A) and (B). Extracts were measured at either base concentration or at 5 times concentration. The limit s of detection (LOD) and quantification (LOQ) as determined from the blank value are represented in (C) as a solid and dashed black line respectively. Similarly to (A) orcinol rhamnolipid values are ranked from 1-4 from highest to lowest rhamnolipid values.
Figure III-26 and Figure III-27 (A) and (B) once again demonstrate the difference in vesicle lysis between lytic and non-lytic strains. Dilutions in (A) of both figures also highlight differences in the lytic capacity between the strains of each of the groups. All supernatant dilution data is fit to a dose response curve in order to determine an EC50 value (effective concentration required to achieve half maximal vesicle lysis), for each of the strains. These values are represented as coloured intersects. Interestingly the acute strain showing the lowest EC50 is PA44981, which was also the strain that showed no haemolysis in Figure III-22. This then can be taken as evidence in favour of the hypothesis of low levels of haemolysin production, and may demonstrate the sensitivity of vesicles to RL’s.

When comparing the supernatant dilutions A and B of Figure III-26 to results of the orcinol assay C, generalised agreement between the two data sets can be seen. The ranking of supernatants from the most (1) to least (4) lytic are in agreement with the equivalent rankings from the orcinol assay from highest to lowest RL production across both the 1 and 5 times concentrated samples. Similar rankings were not made of the lysis negative dilutions as due to the low normalised fluorescence values attained; any EC50 values would likely be representative of half the maximum of vesicle leakage rather than half the maximum of vesicle lysis. In only one of the lysis negative strains (PA45100), was an RL value above the limit of quantification observed. This value was however only seen in the 5 times concentrated sample. Standard concentrations of all acute lysis negative supernatants showed RL values below the limit of quantification a result that is in agreement with the lack of lysis seen in Figure III-26 (B). Interestingly strains PA45100 and PA45394 both showed some degree of haemolysis on blood agar plates Figure III-22 and both also show the highest RL levels in Figure III-26 (C).
Lytic potential and RL production of chronic isolates of *P. aeruginosa*. The response of DPPC vesicles to supernatant from lysis positive (A) and lysis negative (B) chronic isolates of *P. aeruginosa*., as well as the RL production from both lysis positive and negative strains (C). Rectangular intersects on (A) represent EC50 values in the relationship between supernatant concentration and fluorescence for individual strains, EC50 is derived from a dose response fit of individual data series. Intersects are ranked (1, 2, 3 and 4) in ascending order of their X values. RL values in (C) were attained using the orcinol method from samples extracted from identical supernatants to those used in (A) and (B). Extracts were measured at either base concentration or at 5 times concentration. The limits of detection (LOD) and quantification (LOQ) as determined from the blank value are represented in (C) as a solid and dashed black line respectively. Similarly to (A) orcinol rhamnolipid values are ranked from 1-4 from highest to lowest rhamnolipid values.

Chronic, lysis negative normalised fluorescence values (Figure III-27 (B) and (C)) agree well with values from the orcinol assay in so much as fluorescence values do not reach above 0.1 and while one of the orcinol values reaches above the limit of quantification, the limit is still within the samples error. The lysis positive values (Figure III-27 (A)) also show broad agreement with their orcinol counterparts in that all RL values, bar the 1X concentration for PA00887, are higher than the limit of quantification. Separation between
either intersects in (A) or concentration values in (C) are less clear than in Figure III-26. Separation between the orcinol value for PA00887, which also shows a clearly separated intersect in (A), is obvious at the 1X concentration however at the 5X concentration there is cross over with RL production of the more lytic PA00856. Also in contrast to the lysis response the 5X RL content of PA00935 appears lower relative to the other lysis positive strains than the supernatant dilution would suggest. It is difficult to suggest a reason for these differences then other than a flaw in the method.

In order to demonstrate a direct link between level of vesicle lysis and amount of RL produced an attempt was made to quantify RL production from vesicle lysis data through the position of EC50 in each of the individual strains. A standard curve was constructed by exposing the DPPC53 vesicles to a concentration range of R-95 RL and taking fluorescence values after an hour. Normalised fluorescence values for each concentration were then fit to a dose response curve and an EC50 value of 23.2µg/ml was obtained.

![Dose-response curve](image)

*Figure III-28 RL R-95 standard for the vesicle lysis assay. The standard was generated using R-95 RL, the orcinol and was fitted to a dose response curve, EC50 is marked as a rectangular intersect.*
Under the assumption that RL congener output from all *P. aeruginosa* strains is similar, or at least the all congeners have the same lytic activity, the EC50 was used as a benchmark for RL quantification based on vesicle lysis. If the EC50 of RL against DPPC53 vesicles remains constant then EC50 of each strain can be assumed to have the same RL value. RL values for undiluted supernatant can therefore be determined adjusting the value of 23.2 µg/ml based on its current position on the X axis. Table III-3 Shows RL values in µg/ml as calculated either from the vesicle lysis or the orcinol assay.

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This slightly more in depth comparison highlights the disparity between orcinol and fluorescence estimates of RL content of supernatant. Estimates based on fluorescence all show higher values than those generated from orcinol data. Several factors could be affecting both estimates. Primarily assumptions based on the activity of different RL congeners and the production profile of these RL forms from different strains. The congener profile of the R-95 standard could also have an effect on the values determined by each method. For example, assuming there is no difference between different RL forms in terms of vesicle lysis, orcinol values would still vary due to the chromophore generation being a result of interaction of orcinol with the RL rhamnose head group. If there is a higher ratio of mono- to di-RL in one strain as compared to another, this will manifest as a
difference in RL quantity. Disparity between the two sets of data also prevents any conclusions of the relative toxicity of acute and chronic *P. aeruginosa* isolates.

The data above does not absolutely demonstrate that RL’s are the only causative agent of lysis in DPPC53 vesicles, however it does provide numerous correlations which are highly suggestive of that conclusion; particularly in the lack of RL observed by the orcinol method in the majority of lysis negative supernatants. The data certainly demonstrates that RLs are involved in vesicle lysis. In regards to the screen strain (Figure III-21), the involvement is interesting as it hints at variations in toxicity between *P. aeruginosa* infections from chronic and acute infections and given the myriad regulatory systems involved in the control of RL expression may represent a global genetic change. In terms of burn wounds, the association of RL production with acute isolates is useful due to the expected infection type of burn wounds.

Section 3.05  Conclusion

The results of this chapter have demonstrated that the developed DPPC53 vesicles are sensitive to at least three clinically relevant microorganisms, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Comparisons between the effect of whole cell cultures and cell supernatants on the vesicles confirmed the hypothesis that the molecular species responsible for lysis were primarily secreted products rather than cell surface factors. This comparison also highlighted the potential complications which the use of whole cell cultures can cause in making accurate fluorescence measurements.

On further screening of two of the target organisms (*S. aureus* and *P. aeruginosa*) some concept of species wide interactions was gained. In the case of *S. aureus* vesicles showed very broad susceptibility to clinical isolates with few strains being identified as non-lytic. With *P. aeruginosa* an apparent division was observed between strains identified as coming from acute or chronic infections, where acute strains were generally more lytic. This division was confirmed using a Fishers exact test.

In regards to both organisms lytic factors were identified as surfactant like haemolytic agents, all of which were related to the respective species quorum sensing systems. Seven lytic factors were identified in *S. aureus* all of which are regulated by the accessory gene
regulator. The seven factors consist of the six phenol soluble modulins (PSM’s) and δ haemolysin all of which are short (~ 20-40aa) amphiphilic α-helical peptides. One lytic factor was identified from *P. aeruginosa*, the glycolipid rhamnolipid. Although rhamnolipid is considered one factor it is likely that *P. aeruginosa* produces several different congeners of this molecule.

In terms of meeting the overall project aim to develop a diagnostic tool for paediatric burn wound infection, the relation between quorum sensing and vesicle lysis in each organism suggests that the molecules responsible for lysis are likely to be good indicators of acute infection [84, 85]. The author suggests this due to the requirement for good communication between bacterial cells in order to alter their growth state and infect successfully. If the observed lytic factors were not linked to intercellular communication in some way the likelihood of triggering a lytic response whilst bacteria were in a pre-infective state would be increased. This would be problematic in a diagnostic sense as prior to true infection the bacteria may pose no threat to their host. Thus if a positive signal were generated from the vesicles during this period it would likely be counter-productive in the treatment of the patient, generating some of the problems previously described in the introduction to this thesis. Conversely then quorum related lysis provides a good indicator of the transition of bacterial pathogens to an infective state.

Only three of the five organisms tested in this study met the requirements of vesicle lysis. However, this does not preclude sensing of other potentially pathogenic organisms especially given the prevalence of small surfactant like molecules produced especially by environmental organisms. Additionally, while screen strains used in the above study were small they provide some idea of how relevant the vesicle as a biosensor concept could be. The relatively high number of *S. aureus* strains and acute *P. aeruginosa* strains which displayed lysis suggests a relatively good coverage in terms of infection detection. Finally, knowledge of the specific molecules involved in lysis means the applicability of the system can also be gaged on literature knowledge of the retention of these molecules in infective isolates and their relation to infection lethality [86, 87]. Knowledge of the lytic agents involved also allows for adaption of the vesicle design to either increase or broaden sensitivity, a subject that will be addressed in the next chapter.

While all the overall aims of this thesis have not yet been addressed in their entirety some applications of the developing technology have already become apparent. For example in
the case of both *P.aeruginosa* and *S.aureus* the vesicle system can be used to detect the both the presence of the indicated toxins and when these toxins are produced temporally. As such the vesicles can be used as a method to rapidly screen for these toxins under different conditions or within different strains without the requirement to process cultures or initiate lengthy reagent based methods. Particularly in the case of *P.aeruginosa* use of the vesicle system to detect rhamnolipid production is preferable to other lengthy methods involving either crude isolation or the use of thin layer chromatography [83].

While the vesicle system may not be able to distinguish between the different toxins produced by *S.aureus* another potential arises when considering the mechanism by which these toxins are regulated. Although the vesicle system has been demonstrated as sensitive to δ haemolysin the fact that it is also, if not unsurprisingly, sensitive to the PSM’s may prove more useful in terms of potential applications. As production of PSM’s is regulated directly by a component of the AGR system (AgrA), rather than the downstream RNAIII, the system potentially has use as an indicator of the functionality of the quorum system within various *S.aureus* strains. Traditionally determination of whether the AGR system is intact would be performed either through transfection of a reporter gene or through quantitative RNA analysis both of which are laborious process’s. This technique however holds promise as a rapid analytical method for detection of AGR functionality [88].

### References


Chapter IV. Tuning phospholipid vesicles to bacterial toxins.

Section 4.01 Introduction

Lipid bilayers are the well-known structures that form the basis of biological membranes providing barriers and the compartmentalisation required for life. Lipid bilayers consist of a sheet of glycerophospholipids assembled with their non-polar acyl chains interacting in a manner that excludes water or other polar solvents. The outer surfaces of the membrane are made up from the exposed polar headgroups. Phospholipids self-assemble to form bilayers and other structures depending on how energetically favourable each manifestation is. Many factors can affect the free energy of lipids in solution, and coax them into new forms. Lipid to solvent ratio, lipid shape, including surface area of head group and hydrophobic volume, temperature and solvent type are all involved. By altering physical components of the bilayer we can alter the vesicles stability and sensitivity. In light of this, this chapter describes an investigation which was made into the activity of the phenol soluble modulins produced by \textit{S.aureus}, and the rhamnolipids produced by \textit{P.aeruginosa} in respect to variations in bilayer constituency.

Section 4.02 Aims & Objectives

One of the goals of this study was bio-mimicry. By mimicking cell membranes a sensor can be developed which would respond to bacterial toxins in a similar manner to a cell membrane. As the haemolytic agents produced by our target organisms have been identified, the focus of this chapter was to establish how sensitive the vesicles were in comparison to a eukaryotic membrane. Once determined, parameters of the synthetic bilayer could be altered in order to tune the sensor. Tuning could be performed either to make detection more discrete or to discriminate between lytic agents.

In order to establish the relative sensitivity of the developed sensor to the haemolytic agents described in chapter IV, vesicles were exposed to either synthetic (in the case of \textit{S.aureus}), or purified toxins (in the case of \textit{P.aeruginosa}). Sensitivity was determined through analysis of the dose response of vesicles to a variety of concentrations of toxin in
order to identify the effective concentration required to lyse 50% of the vesicle population. 
A eukaryotic cell line was then challenged with the toxin and the results were compared.

With activities of toxins against the DPPC53 vesicles established as a base line, 
observations were then made to establish the effect that changes in the bilayer composition 
could have on the lytic activity of the individual toxins. Such studies allow for both the 
tuning of vesicles to create a highly sensitive system, as well generating the potential for 
toxin specific sensors. Tuning could potentially expand the utility of such a sensor system 
to multiple pathogen detection, or for detection of variation within pathogens. The 
changes implemented included variations in the cholesterol content of the bilayer and 
variations of the acyl chain length of the bilayer lipids. Again effects that these changes 
would have on the lytic activity of our target toxins were assessed through changes in dose 
response as compared to the DPPC53 standard.

These investigations were ultimately aimed at creating greater confidence in the 
applicability of our system as a biosensor. Additionally, the hope was to identify 
improvements which could be made to the vesicle system through bilayer modification.

Section 4.03  Activity of bacterial toxins against natural and synthetic bilayers

Knowledge on the efficacy of the membrane active toxins identified in the previous 
chapter (Staphylococcal PSM’s, delta haemolysin and the Pseudomonal RLs), varies 
considerably as tests have predominately focused on single toxins with a myriad of 
bilayers. All of the identified molecules are known to be amphipathic in nature and to 
have surfactant like qualities. However, which bilayer factors limit the activity of 
interactions of the toxins is unknown. In order to identify the differences bilayer 
components can impart on lytic activity, it is first important to understand how a standard 
(DPPC53), vesicles will respond to individual toxins.

(a) Structure and mode of action of staphylococcal PSM’s
The PSM’s and delta toxin produced by S.aureus are known to be small alpha helical 
peptides [1]. The degree of alpha helicity in individual peptides may be open to question
however, due to the use of trifluoroacetic acid in previous circular dichroism experiments. This compound is known to impart alpha helicity to peptides [1]. The mechanisms of action of these peptides have been best studied in delta toxin, which was identified earliest, the PSMs were not identified until 2009[2]. Studies of delta toxin have suggested different modes of membrane permeabilisation at different concentrations. At low concentrations it has been suggested that delta toxin forms antiparallel dimers which lie with their hydrophobic faces into the membranes at the interface between acyl chains and hydrophobic headgroups [3]. This is a model which has been suggested previously for numerous other small amphipathic alpha helical peptides [4]. Two modes of membrane permeabilisation have been suggested at low peptide concentrations. The first involves the formation of pores in the membrane through the formation of octameric or oligomeric channels [5] which would then be cation selective due to negatively charged residues of the peptides making up the walls of the channel lumen [6, 7]. Indeed efflux of Ca\textsuperscript{2+} from granulocytes has been observed after exposure to low concentrations of toxin (0.15-15µg/ml) [8]. Whether this effect would allow for the passage of CF is unclear due to the molecules size; however osmotic changes could lead to vesicle collapse. Secondly, low concentrations of delta toxin have been shown to cause gradient efflux of CF from nanometre size POPC vesicles at the same rate as lipid flip flop in the vesicles, (i.e. translocation from one leaflet of the bilayer to the other [9]). These observations have led to the proposal of a “sinking raft” model of membrane permeabilisation, where the toxin will translocate from one leaflet of the bilayer to the other, after small aggregates cause local stress in one leaflet. The temporary membrane disruption is coupled with dye efflux and will occur until peptide concentrations are balanced between leaflets [10]. At high peptide concentrations (125:1 lipid: peptide), it has been demonstrated that delta toxin can cause large multilamellar DMPC vesicles to form smaller unilamellar structures as well as nanometre size discoidal structures[11]. Again this effect does not seem to be specific to delta toxin, and has been seen in other small amphipathic peptides such as melittin [12], despite the two having disparate primary structures. These three concentration dependant processes lead up to vesicle collapse which ultimately is likely due to increases in bending energy imposed by delta toxin on the membrane [13] (Figure IV-1).
Figure IV-1 Schematic of PSM production and modes of membrane permeabilisation. Modes of membrane permeabilisation depicted are based on research performed with delta toxin. (1) Infection of a eukaryotic wound environment with *S. aureus*. (2) Growth of *S. aureus* and concurrent release of quorum signals, (AIP). (3) release of amphipathic PSMs/delta in response to quorum signals. (4) Integration of toxins to membrane. (5a) Sinking raft mechanism of membrane disruption (low concentration); (i) peptides attach to membrane outer leaflet and cause localised stress; (ii) Increase in outer leaflet stress allows for peptide translocation across bilayer with concurrent transient pore formation and vesicle contents release; (iii) peptides reach inner leaflet of bilayer, the translocation process continues until outer and inner leaflet peptide concentration has reached an equilibrium. 
(5b) An illustration of pore formation by peptides with release of Ca$^{2+}$ ions and potentially CF. The concept of pore formation is largely based on theoretical models. (5c) High concentrations of peptide cause positive bending stress on the bilayer resulting in its rupture and the formation of lipid discs.

The α-PSMs are similar to delta haemolysin both in both their primary and secondary structure, although as mentioned previously, this second point may require some clarification. β-PSMs show greater variation in that they are much larger at ~40 amino acids rather than ~20 seen in the α peptides. Both the α and β share the amphiphillicity of delta to some extent, however the degree to which the peptides display either hydrophobic or hydrophilic characteristics could potentially have a bearing on their interaction with, and solubilisation of membranes, bearing in mind their tendency to embed parallel to the membrane. Wenxaing diagrams [14], which can be used to visualise the amphipathic
character of helical peptides, were generated from the amino acid sequences of each PSM and delta toxin [2] using a web server based platform [15].

The Wenxaing diagrams are based on the conical projection of the helix from a surface so the schematic view is end-on to the peptide, with C-terminal residue at the centre of the spiral, and the N-terminal residue at the spiral's outermost point. The diagrams clearly show the amphipathic character of delta haemolysin, highlighting the separation between the hydrophobic (red residues) and hydrophilic (blue residues) faces of the peptide. Whilst most clear for delta, the separation of faces can also be seen in all of the α-PSMs. In general it appears that a larger portion of the surface of the α-PSMs consists of
hydrophobic residues. PSM’s α1 and α2 show nearly identical hydrophobic character while α3 and α4 seem to show slightly increased hydrophobic regions although this would require clarification through determination of hydrophobic/hydrophillic inclination angles [16]. The physically larger β-PSMs show less obvious duality of character with only very narrow bands of hydrophobic residues persisting along the peptides entire length. Although the β’s, (like α1 and α2), show very similar hydro-phobic/philllic profiles, a glutamine residue (Q) at position 13 of β2 generates a disruption of the hydrophobic band which is not seen in β1.

Other factors such as peptide helicity and residue charge may also have an effect on the peptides interaction with membranes although due to the proposed mechanism of lysis of delta, it is likely that there is a high requirement for a consistent hydrophobic face to ensure stable surface binding. It also seems likely that this mode of surface interaction is common across the PSMs.

(b) The activity of the Staphylococcal PSMs against DPPC53 vesicles

A dose response model of the activity of toxins against prototype DPPC53 vesicles was applied in order to gauge variations in lytic activity between the toxins. The model was applied based on the assumption that vesicle lysis is not an all or nothing response. This postulation was grounded in an understanding of the mechanism of action of delta toxin. It is understood that at higher concentrations (above 2 µmol dm$^{-3}$), delta toxin will assemble in solution in a geometric fashion in relation to its concentration [17]. It was also assumed that bilayer destabilisation by the peptides would require them to be sequestered by the lipids in a permanent fashion or at least that the rate of peptide release from peptide-lipid complexes would be low when compared to the rate of lysis. As such toxins will not accumulate in the membranes of all vesicles in a homogenous manner, which would allow for a rapid lysis of all vesicles simultaneously at a specific toxin concentration. Instead the aggregation of toxin will ensure uneven temporal distribution of vesicle lysis. As such the free CF concentration in solution will change over time relative to the degree of vesicle lysis. As can be seen from Figure IV-3, the quantum yield of CF is heavily influenced by concentration. At high concentrations up to 50mM, quantum yield is influenced by CF’s self-quenching effect. This is thought to result from a combination of the formation of dimer pairs of the dye, which may be non-fluorescent due to greater delocalisation of aromatic electrons and collisional quenching of monomers by the dimer pairs [18-20]. At
lower concentrations (below 0.5µmol dm$^{-3}$), the quantum yield will relate simply to the dilution of the dye.

The encapsulated concentration of CF is 50mM which generates the desired quenching effect on the dye allowing for the on/off signal desired of the sensor. Upon lysis of a vesicle the encapsulated dye is diluted into a relatively large volume of fluid. The dilution is such that the concentration will be below the peak fluorescent yield seen in Figure IV-3 which balances between dilution and quenching. As such if vesicles are lysed in the

Figure IV-3 Change in fluorescence intensity as a response to 5,(6)carboxyfluorescein concentration based fluorescence quenching. (A) Shows a dilution series of CF concentrations from 50mmol dm$^{-3}$ to 5µmol dm$^{-3}$. (B) Shows fluorescence values for this same concentration demonstrating the characteristic bell curve seen as the samples go from high concentration low fluorescence to low concentration low fluorescence. (C) Again shows the same concentration range under blue light, thus highlighting the fluorescence effect.
heterogeneous manner suspected by the PSM’s, the quantum yield of a sample will start low and gradually increase over time as more vesicles are lysed. The permanent sequestration of the toxins will ensure that at a given toxin concentration the rate of lysis will at some point plateau providing a stable fluorescence value which can be used to assess the dose dependant response of vesicles to toxins.

This preliminary investigation into the efficacy of synthetic versions of each of the peptides was made using DPPC53 vesicles (Figure IV-4). The vesicles were exposed to concentrations of the αPSM’s and δ from 0.1μmol dm$^{-3}$ to 10μmol dm$^{-3}$. Due to relatively low levels of lysis at the maximum concentration of this range, the vesicles were exposed to a slightly increased range of concentrations of the βPSM’s reaching a maximum of 50μmol dm$^{-3}$. After exposure, vesicles were incubated with the toxins for 2 hours at 37°C, with measurements of fluorescence being taken every minute. Values shown in Figure V-3 are representative of data at the 60 minute time point for each toxin and concentration. Sixty minutes was chosen as fluorescence readings had plateaued by this point and no more increases were observed.
Figure IV-4 Preliminary investigation into the effect of delta haemolysin, alpha and beta PSMs against DPPC53 vesicles. (A), (B) and (C) show the response of DPPC53 vesicles to delta haemolysin, the α-PSMs and the β-PSMs respectively. The fluorescence values shown were taken after exposure of the vesicles to the indicated peptide concentration for a period of 60 minutes. Peptide concentrations from 0.1µmol dm^{-3} to 10µmol dm^{-3} were used for delta haemolysin and the α-PSMs. For the β-PSMs a concentration range from 0.1µmol dm^{-3} to 50µmol dm^{-3} was used. (D) shows the EC50 value (effective concentration of toxin required to reach a fluorescence value half that of the maximum), of each of the peptides tested against DPPC53 vesicles. EC50 values were determined through fitting of the normalised fluorescence Vs Toxin concentration data to a dose response curve using OriginLab 9.

(i) Differences in EC50

A cursory glance at Figure IV-4 highlights that the key difference between the delta/αPSM’s and the βPSM’s is the concentration range over which vesicles show a lytic response. As previously mentioned the concentration range over which the βPSM’s were evaluated was larger than that of the α-PSMs; while the normalised fluorescence value of all α-PSMs had more or less plateaued at a concentration of 10µmol dm^{-3}, values for the β-PSM’s were still increasing. These differences can be clearly observed in Figure IV-4(D), which displays the EC50 (concentration required for 50% of maximum lysis) values of each of the PSMs and delta. Internal differences within groups can also be seen. The EC50
of β1 is at least an order of magnitude higher than values seen for any of the αPSM’s. β2 shows a reduced EC50, in comparison to β1, with a value of around 4µmol dm⁻³ which is much closer to the values seen from the α-PSMs. There is also a degree of internal variation within the αPSMs, for which delta will be included for simplicity’s sake. PSM α2 demonstrates the lowest EC50 value (i.e. the lowest amount of peptide is required for vesicle lysis). This is closely followed by PSM α3, and then α1, and delta, which are within one another’s error range. Values for the first 4 α-PSM’s are all below 1µmol dm⁻³. Contrary to this, PSM α4 gives a relatively high EC50 of around 1.2µmol dm⁻³.

(ii) Differences in Fluorescence maximum

Whilst EC50 is an important metric to consider in the interaction of the PSMs with vesicles, it is not the only one that counts. Also important is the maximum normalised fluorescence value, which each of the peptides generate. For example, while the EC50 values for PSMα1 and delta haemolysin are nearly identical, there is a difference in the maximum fluorescence; each obtains of 0.162 normalised fluorescence units. While these variations are interesting from the standpoint of identifying the most lytic species produced, they may be particularly important when considering the quantities of toxin produced in a real wound environment, and hence the variability which can be imposed on vesicles.

Maximum fluorescence reached will also become important in situations where detection of fluorescence by eye becomes difficult. Although an objective measure, it can be assumed that a sufficiently low fluorescence value would essentially be recognised as a negative response in clinical setting (if it is to be judged by eye). Vesicles which show high sensitivity but have a low maximum when exposed to a particular toxin are essentially useless. While highly sensitive, the sensitivity is unrecognisable without specialist equipment. This is due to the EC50 having no bearing on the value of maximum fluorescence reached. Based on this reasoning, a cut-off fluorescence value was decided, below which the sensor response would be deemed to be negative. This value was arbitrarily decided through observation of fluorescence of vesicles treated with PSMα1 (Figure IV-5).
Due to the decrease in visible fluorescence below a concentration of 1µM, a normalised fluorescence value between the corresponding 0.62 and 0.37 concentrations was chosen. The obvious cut off value between on/off sensing therefore seemed to be 0.5 normalised fluorescence units.

(iii) Fluorescence drop off at high toxin concentrations

Another point, which can be important when comparing toxin efficacy, is that maximum fluorescence does not always correlate with the highest toxin concentration used. For example in two of the α-PSMs shown in Figure IV-4 (B), a slight decrease is observed between the normalised fluorescence for the highest toxin concentration when compared to the concentration which provides the highest fluorescence value. The effect which falls outside the bound of the SEM of the various points is seen in PSMs α2 and α4, which show differences of 0.166 and 0.141 units respectively. This decrease was seen in numerous experiments involving both purified toxins and bacterial supernatant. One possible explanation for this phenomenon is peptide amyloid formation. Aggregation of the peptides at higher concentrations has previously been implicated the formation of amyloid like fibrils as structural elements in biofilms [21]. This amyloid formation was induced experimentally using a mixture of synthetic forms of all the PSMs at concentrations of 0.05mg/ml per peptide, which equates to a range from 23µmol dm⁻³ to 11.1µmol dm⁻³ depending on the peptide. Attempts were made to detect amyloid formation in PSM α2, as one of the peptides which showed obvious drop off at high concentrations. A Congo red spectral shift assay and a thioflavin T fluorescence assay were both used to determine if any amyloid formation was taking place [22, 23]. No significant fluorescence change was seen in the thioflavin-T assay with a concentration range from 0.1µmol dm⁻³ to 90µmol
A spectral shift however, was seen over the same concentration range using 7µmol dm⁻³ Congo red (Figure IV-6).

Figure IV-6 Congo red spectral shift assay using PSMα2 in a 0.1 to 90µmol dm⁻³ concentration range and a 7µmol dm⁻³ Congo red. (A) spectra of all Congo red PSM samples: a spectral red shift can be seen with increasing PSM α2 concentration from a lowest λ_max of 490nm to a highest λ_max of 497 nm. (B) plot of λ_max of each PSM α2 concentration demonstrating a general trend towards higher wavelength λ_max. (C) The visible difference between Congo red samples with 90µmol dm⁻³ and 10µmol dm⁻³ of PSM α2; a change in colour from brick red to ruby red is seen with increasing PSM concentration, as is the formation of discrete structures within the microtitre well.

A comparison of averaged spectra in Figure IV-6 (A) demonstrates a gradual red shifting of the Congo red absorbance maxima by around 10nm. A shift of around 50nm to a maxima of 540nm has been demonstrated as indicative of true amyloid formation using other proteins such as lysozyme[24], however shifting has also been shown to be concentration dependant with amyloid β-peptide[25]. A concatenated plot of the absorbance maxima (Figure IV-6 (B)), demonstrates how the λ_max changes are dependent on PSMα2 concentration. Whilst regression analysis of the data has identified a
concentration dependent effect on $\lambda_{\text{max}}$, it appears that this relationship may only manifest above 10$\mu$mol dm$^{-3}$; concentrations below this value show such a degree of cross over in their SEM as to be linear. A visual comparison of the 10 and 90$\mu$mol dm$^{-3}$ samples (Figure IV-6 (C)) demonstrates the characteristic brick red to ruby red transition seen on binding to the $\beta$-sheet structure of amyloid fibrils [22] as well the presence of insoluble particles in the 90$\mu$mol dm$^{-3}$ sample not seen in the 10$\mu$mol dm$^{-3}$ sample.

As evidence for the spectral shift is weaker in the 10$\mu$mol dm$^{-3}$ and lower region (which is the target concentration range used in vesicle testing), a further investigation was made into the ability of peptide aggregates to remove Congo red from solution on centrifugation (Figure IV-7).

As in the previous experiment, 7$\mu$mol dm$^{-3}$ Congo red was mixed with concentrations of PSM$\alpha$2 ranging from 90 to 0.1$\mu$mol dm$^{-3}$. After 10 minute incubation, samples were

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**Figure IV-7 Sequestration of Congo red by PSM$\alpha$2.** (A) Absorbance standard of a Congo red dilution series from 1$\mu$mol dm$^{-3}$ to 50$\mu$mol dm$^{-3}$; the line equation of the fit and the limits of detection and quantification are all shown. (B) Concentrations of Congo red remaining in solution after centrifugation of Congo red PSM $\alpha$2 samples for 10 minutes at 10,000G.; concentrations were determined through reference to the absorbance standard (A). (C) shows the resultant pellets after centrifugation of mixed Congo red PSM $\alpha$2 samples.
centrifuged for 10 minutes at 10,000G. The resulting supernatants were harvested and their absorbance was measured. All supernatants showed a $\lambda_{\text{max}}$ of 490nm, indicative of unbound Congo red. Averaged absorbance values were converted to concentrations of Congo red based on a series of standards (Figure IV-7 (A) and (B)). As can be seen, the concentration of Congo red retained in the supernatant correlates inversely with the concentration of PSMα2 at concentrations above $1\mu\text{mol dm}^{-3}$. The full extent of Congo red sequestration can be seen in Figure IV-7 (C) from the degree of pelleting. Concentrations of PSMα2 above $10\mu\text{mol dm}^{-3}$ gave absorbance values below the limit of quantification.

The linear relation between Congo red sequestration and toxin concentration suggests that, even at very low peptide concentrations, some form of peptide aggregate is being formed. Due to the lack of spectral shifting at these concentrations it seems unlikely that these aggregates are amyloid fibrils in the truest sense, however they may represent smaller aggregates. Experiments by Thiaudiere et al. have previously demonstrated that delta toxin forms aggregates in solution, which form aggregates in an isodesmic fashion above concentrations of $2\mu\text{mol dm}^{-3}$ [17]. At a concentration of $10\mu\text{mol dm}^{-3}$, an aggregate of around 38 monomers would be present. Experiments using mammalian alpha helical membrane disrupting peptides (Islet amyloid polypeptides) have demonstrated an inability of the peptides to lyse DOPG vesicles when in their fibrilar state [26, 27]. It could be hypothesised that the aggregates formed at $10\mu\text{mol dm}^{-3}$ have begun to lose some lytic potential. Indeed some evidence towards this hypothesis has been found in circular dichroism observations of the $\alpha$, $\beta$, and quaternary structure (quaternary structure being the formation of tetramers), of delta haemolysin. These experiments found that at concentrations above $10\mu\text{mol dm}^{-3}$, 56% of the lipid bound toxin was in its $\alpha$ form and 40% was in its quaternary from [17]. What is not known is if these quaternary aggregates limit lytic potential or whether they disassemble on contact with the bilayer. A study examining the activity of delta haemolysin and various synthetic analogues has shown that removal of residues from the wild type toxin can cause both an increase in the haemolytic activity and a decrease in the level of self-aggregation [28]. At this point however, evidence is not direct, and is merely conjecture. The response of the vesicles to these higher peptide concentrations could potentially be important in gauging differences between toxins, and the importance of changes in activity observed when vesicle composition is varied.
(iv) Variations in kinetics of toxin vesicle interactions

The temporal response of vesicles to toxins may also be worthy of consideration; again in relation to the clinical application of such a sensor. In an ideal situation there would be no delay between release of a stimulatory signal and generation of the corresponding response by a receptor. In a realistic situation, there will likely be a diffusional delay between these two events, which will be difficult to compensate for; especially if the sensor is to be kept in a discrete location such as a dressing. However the time taken for the interaction between sensor and toxin should be as rapid as possible. Such rapidity of sensing is necessary to prevent delay of treatment. Such changes in the speed of detection have been seen in the PSM’s, and seem to be related to and intrinsic property of the toxins, rather than the concentration used/normalised fluorescence response attained.

Figure IV-8 Temporal response of vesicles to PSMs at high concentrations. (A) Temporal response of vesicles to 10µmol dm$^{-3}$ of all 4 α-PSMs and delta haemolysin. (B) Temporal response of vesicles to 50µmol dm$^{-3}$ of PSMs β1 and β2.
Fluorescence response over time for the DPPC53 vesicles challenged, with highest concentrations of each of the PSMs is shown in Figure IV-8. Whilst this data is slightly problematic in that fluorescence measurements are not made immediately after the addition of vesicles to the PSM samples (due to the microtitre plate set up, measurements began on average 1.5 minutes after the addition of the first batch of vesicles and no later than 2 minutes after addition), differences between the PSMs are still apparent. From Figure IV-8 (A) differences can be seen between δ haemolysin, and α4 which attains a stable maximum after around 40 minutes, and the remaining α-PSMs which plateaued before the experiment was initiated. Slower fluorescence responses are also seen from the β-PSMs (Figure IV-8 (B)) which reach a stable maximum after around 30 minutes.
Figure IV-9 Comparison of concentration dependant response over time of PSM α3 and PSM β1. (A) The normalised fluorescence response of vesicles over time to individual concentrations of PSM α3 in the range 0.1µmol dm\(^{-3}\) to 10µmol dm\(^{-3}\). (B) Similarly shows normalised fluorescence response over time, except in this case to PSM β1 over a concentration range of 0.1µmol dm\(^{-3}\) to 50µmol dm\(^{-3}\).

Observations of the vesicle responses over the entire range of concentrations tested demonstrates that the kinetics of lysis are roughly maintained, regardless of concentration Figure IV-9. Concentrations of 1µmol dm\(^{-3}\) of PSM α3 and 20µmol dm\(^{-3}\) of PSMβ1 reach roughly equivalent normalised fluorescence values of 0.67 ±0.03 and 0.68 ±0.04 after 60 minutes. The curve characteristics in each case however are markedly different, with α3 showing a gradual incline from a 0 time starting value of 0.41, and β1 showing a much steeper increase from a starting value of 0.10. Whilst these temporal responses, which in the context amount essentially to binding kinetics, do indeed show some relation to concentration in both cases (i.e. velocity of the transition from non-fluorescent to fluorescent is not constant with concentration), inter-peptide differences are also apparent. This may be suggestive of bilayer concentration, or bilayer aggregation, of the peptide as
the rate limiting factors in vesicle lysis for some toxins such as α3. In other cases, lysis is potentially dependant on some intrinsic property of the toxin, such as its bilayer integration efficiency, which may be the case for PSM β1.

(c) Activity of PSMs against eukaryotic cells
In order for a pathogen biosensor system to be useful, it has to at least be capable of signal detection at toxin concentrations equivalent to those which would begin to damage bacterial cells, and ideally should be capable of detection at much lower concentrations to provide the best clinical outcome. Correlating the concentration dependant effect of cell damage with vesicle lysis would be highly convoluted. As such, a decision was made to use a simple assay that would look at T-cell survival, in regards to peptide concentration of the various PSM’s (Figure IV-10). Whilst relatively simplistic, this does allow for a basic determination of vesicle Vs cell toxin sensitivity. Experiments were carried out using a cultured T-cell line. Cells were harvested and exposed to various toxin concentrations, after which they were stained with trypan blue, and counts of live Vs. dead were made. T-cell assays were performed by Maisem Laabei (a current PhD student between the laboratories of Dr Toby Jenkins and Dr Ruth Massey).
Figure IV-10 Response of T-cells to PSMs. The chart shown illustrates the lytic activity of the various PSMs and delta haemolysin against cultures human T-cells. T-cells were treated with peptides for a period of 30 minutes prior to staining with trypan blue. Live and dead cells were then enumerated by eye using a microscope and haemocytometer. Counts are shown as the percentage of cells which survived the treatment. The control cells showed 97.5% survival, which is indicated in the line at the top of the chart.

The range of concentrations used in the T-cell experiments were higher than those used in the vesicle studies due to poor or nominal killing seen over the 0.1-10µmol dm\(^{-3}\) concentration range for the majority of toxins. The general pattern of lysis in terms of the most, and least, lytic species of PSMs correlates well with the pattern seen for the vesicles. In both cases, PSM α2 shows the highest levels of lysis/cell killing, and is closely followed by PSM α3. The EC50 for PSM α1 and delta haemolysin are equivalent in the vesicle assay, but α1 shows significantly greater killing in the T-cell assay. Other than this variation, α4 is again seen to be less “toxic” than delta haemolysin or the rest of the α-PSMs. As with the vesicle assay, the β-PSMs are significantly less lytic than the α types or delta, however the difference between β1 and β2 observed in the vesicle assay are not seen. The pattern of PSM toxicity shows correlation with other studies investigating their effect on other cell types. A study into the effect of the PSMs against erythrocytes demonstrates a similar pattern of toxicity, with the exception that PSMβ1 showed greater haemolytic activity than β2 which is not seen in the T-cell study presented here [29]. Moreover, the β1 to β2 relationship in the vesicle assay is reversed, with β1 showing lower lytic potential.
Another study utilised neutrophils and looked at the effect of a single concentration (10µg/ml equivalent to between 4.6 and 2.2µmol dm$^{-3}$ depending on the peptide) of all the PSMs [2]. Lactate dehydrogenase release was measured as a marker of cell death. At these concentrations, neutrophil lysis was below 10% of the total population when PSMs α1, α2 and delta haemolysin were used. Lysis for PSM α3 was shown to be much higher around 60%. No lysis was seen for the remaining PSMs.

The difference between the vesicle and T-cell studies shown here (which is most relevant to the clinical applications of the sensor), is that at levels of vesicle lysis which would be equivalent to sensor switch-on for all α-PSMs and delta haemolysin concentrations, T-cell lysis is low. Additionally at the highest β-PSM concentrations tested against vesicles, switch-on is seen, but these concentrations show nominal activity against T-cells. In all cases, vesicle switch-on would be expected at toxin concentrations that would generate relatively low levels of damage to T-cells.

**d) The lytic capacity of Staphylococcal supernatant**

Whilst information on individual toxins is useful in highlighting both the relative sensitivity of the vesicles over T-cells and the bio-mimicry which the vesicles present, it is not representative of the combined effect of *S.aureus*’ toxin armoury. In order to be effective, vesicles must be shown to be at the very least, as sensitive to the PSMs as T-cells are to *S.aureus*’ complete array of toxins. As such, a comparison was made of the lytic capacity of *S.aureus* LAC 18 hour supernatant against both T-cells and DPPC53 vesicles Figure IV-11.
Figure IV-11 The lytic capacity of *S. aureus* Lac 18 hour supernatant against T-cells and DPPC53 vesicles. The left Y-axis gives % survival of T-cells and is related to the blue curve. The right Y-axis gives values of normalised fluorescence and is associated with the red curve. Both curves were fitted to a dose response equation using OriginLab 9. Dotted lines indicate the intersect of the fluorescence signal on/off cut-off with the vesicle lysis fit.

At a supernatant concentration of 100%, fluorescence release and T-cell survival are approximately 100% and 0% respectively. On decrease of supernatant concentration however the onset of increases in T-cell survival occurs more rapidly than the onset of fluorescence decrease. This can be observed from the relatively large plateau in normalised fluorescence values above supernatant concentrations of 10%. As a normalised fluorescence response of 0.5 was previously determined as the cut-off for a detectable response (Figure IV-5), an intersect was generated between the 0.5 normalised fluorescence value and the dose response fit to the vesicle lysis data. This value gave a % supernatant concentration of 3% and a T-cell survival of ~80%. These data together suggest that at the vesicle detection limit, cell damage by the full complement of *S. aureus* toxins will be relatively low.

The data shown above demonstrate the bio-mimicry which the DPPC53 vesicles achieve in relation to eukaryotic cell types, as well as their relative sensitivity to the staphylococcal PSM’s. Variations in response to PSMs (especially the low level response to the βPSMs) and the possible aggregation mediated response reduction (seen at high concentrations of
some of the α-PSMs), highlights the potential for sensitivity tuning. This may be especially important in relation to the effect that low concentrations of the PSMs can have on immune response. While it has been demonstrated here and elsewhere that at PSM concentrations which generate vesicle lysis, cell damage is low, the PSMs also have a chemotactic and inflammatory effect in host systems through stimulation of the human formyl peptide receptor 2 which occurs at nanomolar concentrations [30].

(e) *P. aeruginosa* rhamnolipids

*P. aeruginosa* has previously demonstrated the capacity to produce multiple forms of rhamnolipids which were identified in Chapter IV as the primary components in vesicle lysis. As well as simply mono- and di-rhamnolipids which vary in either having one or two rhamnose headgroups, the organism has been shown to produce around 28 different RL congeners [31]. Congeners were shown to vary mainly in the length of one of their two acyl chains (Figure IV-12). The study also identified that congeners were produced simultaneously (many in trace amounts), and that the ratio of the different congeners did not vary over time. The two most abundant rhamnolipid species produced were: L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-Rha-C$_{10}$-C$_{10}$), and it’s mono-rhamnolipid counterpart: L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-C$_{10}$-C$_{10}$) [32]. The majority of studies of rhamnolipids thus far undertaken have focused on their potential as biosurfactants, rather than their lytic capacity.
A fairly thorough investigation has been made into the interaction of the di-RL, Rha-Rha-C_{10}C_{10}, with artificial bilayers which highlights some of the substances biological activities [34]. The relatively large headgroup, and short acyl-chain region of the di-Rl molecule, will confer positive curvature to bilayers (ChapterII-Eq1). However, evidence from the study demonstrated preferential integration into bilayers containing POPE which due to its small head group and large acyl chain volume (a result of an unsaturated C=C bond), preferentially generates negative membrane curvature. This effect was suggested to be due to complementary molecular geometry of the two molecules. A decreased partition coefficient was also seen when cholesterol was included in the membrane (at a 1:1 ratio with POPC); this decrease was suggested to be due to packing inefficiencies between the large headgroups of the two molecules. The geometry of the molecule is also thought to be involved in generation of greater acyl chain disorder within the bilayer, and dehydration of the water bilayer interface, effects which will both lead to decreased membrane stability. These properties have been demonstrated in similar biosurfactants such as the peptide surfactin [35].

Due to the highly complex nature of Pseudomonal rhamnolipid production, the commercially available rhamnolipid preparation R-95 was used as the lytic species in this study. While a graph of DPPC53 vesicles Vs R-95 has been shown previously (Figure IV-24), it is replicated here in order to illustrate key points (Figure IV-13).
Figure IV-13 The normalised fluorescence response of DPPC53 vesicles to a concentration range of R-95 rhamnolipid in PBS. EC50 was determined from fitting to a dose response equation using OriginLab 9 software.

The response to the R-95 concentration range is again fit to a dose response model, which demonstrates an EC50 value of around 21µg/ml. Fluorescence increase is most rapid between 20 and 40µg/ml, after which the fluorescence plateaus at around 1µg/ml. Although data points for the highest three concentrations used are within one another’s SEM, there does appear to be some tailing off of fluorescence values at high concentrations. The critical micellar concentration of the di-RL has previously been demonstrated at 0.110mmol dm$^{-3}$ [36]. Around this concentration, di-RL was demonstrated to have relatively weak detergent properties against POPC bilayers, but which would likely still allow for partitioning to membranes over micelle formation [34]. This lowering of the partition constant at around the CMC may be a reason for the drop off seen in fluorescence at high R-95 concentrations.

The EC50 value shown in Figure IV-13 has also been compared to numerous clinical P.aeruginosa isolates in Chapter IV; however quantification of RL production by this method proved difficult. At the time of writing, an investigation into the effect of R-95 against T-cells had not yet been made, however the effect of supernatant has been
investigated. In this study the well characterised *P. aeruginosa* strain PA01 was used in order to compare supernatant mediated vesicle lysis with supernatant mediated cell death (Figure IV-14.)

![Figure IV-14](image)

**Figure IV-14** Effect of *P. aeruginosa* PA01 concentration on both T-cell survival and vesicle lysis. As with the previous *S. aureus* graph, the left Y-axis gives % survival of T-cells and is related to the blue curve. The right Y-axis gives values of normalised fluorescence and is associated with the red curve. Both curves were fitted to a dose response equation using OriginLab 9 software.

In the case of vesicle lysis, due to the rapidity of increase over supernatant concentrations of 20%, data could not be fitted to a dose response curve as has previously been shown for *S. aureus*. From simple observations of the graph, the intersection between vesicle lysis and T-cell survival occurs low on the y-axis. This suggests that while RL detection in neat supernatant is good (yielding high normalised fluorescence), the loss of a fluorescence signal at the arbitrarily defined 0.5 value occurs at supernatant concentrations somewhere between 20% and 30%. At these sorts of concentrations, supernatant induced T-cell death is still very high at around 70% to 80%. If we take the EC50 value determined in Figure IV-13 to be true (as was done in Chapter IV), then this correlates with data on RL induced...
death of human monocytes, in which complete loss of viability was seen at RL concentrations of 15-26µg/ml [37, 38].

Data shown here is limited to some degree by the lack of differentiation between RL congeners and the potential for variance in production between strains. This is clearly exemplified by the variation in vesicle lysis between the supernatant shown in Figure IV-14 and those shown in Figure IV-22 &23. Although the lytic response of vesicles to RL and P.aeruginosa strains is promising, poor understanding of the variability of RL’s, and the comparatively low sensitivity of the DPPC53 vesicles in relation to T-cell sensitivity, ensures that the remainder of this chapter will focus on altering vesicle sensitivity to S.aureus toxins.

Section 4.04 The effect of varying lipid composition on toxin bilayer interactions

It is well understood that altering the lipid components of a bilayer will affect that bilayers properties in situ as well as interactions that bilayer may have with other amphipathic molecules. The generation of a vesicle system as opposed to a planar bilayer is a result of a balance between different stresses an aggregated lipid structure will endure and the systems free energy. The inherent stability of the vesicle structure will have an effect on the structures lifetime and the effect that other amphiphiles may have upon it. In a planar bilayer the mean spacing between lipid molecules will minimize their free energy. Bending the bilayer to form a closed vesicle will change the molecular spacing in both bilayer sheets, a state that will necessarily require some energy to maintain. The bilayer sheet however being very thin, thus offering little bending resistance, will be susceptible to thermal undulations which increase the structures free energy. Depending on the nature of the composite lipid, the edges of the sheet will either be capped or open to solvent which again will increase free energy, either through solvent hydrophobic interactions or through bending energy imposed on the sheet edge (Figure IV-15).
Considering the tendency to move towards the lowest energy state, vesicle formation can therefore be interpreted as an energy pay off between the cost of bilayer bending, and the cost of thermal undulations and line tension energy [39]. As such, in a thermally stable system, perturbations of a vesicle bilayer which result in the vesicles destabilisation will occur when either the bilayer bending energy is raised or the line tension energy is lowered sufficiently. In the case of δ haemolysin it seems that an increase in bilayer stress (through introduction of positive curvature), and a lowering of line tension energy (through the stabilisation of the hydrophobic portion of the bilayer), are introduced simultaneously. Given what is known of the structure of the phenol soluble modulins and similar amphipathic peptides it can be assumed that these toxins have a similar mode of action.

In order to alter the susceptibility of such a vesicle system to this type of toxin induced damage variations can be made to two key parameters in the destabilisation process. Firstly the capacity of the PSMs to interact with the vesicle bilayer can be altered by varying the bilayer properties. Secondly alterations can be made which will either increase the line tension energy experienced by lipids in a planar bilayer or decrease the bending energy.
imposed on the same lipids in a vesicular formation. In order to vary these parameters an understanding of how they are influenced by their constituent lipids is required.

(a) Altering bilayer fluidity

The molecules that make up a lipid bilayer and the temperature of the system are the primary factors governing membrane fluidity. This fluidity is responsible in part for a bilayers tendency to behave as an elastic sheet under compressive or tensile forces. As such the membrane fluidity has an effect on the bending energy that a membrane can withstand before rupture.

The two most common bilayer phases for pure lipids under physiological conditions are the liquid ordered (L\textsubscript{o}) and liquid disordered phase (L\textsubscript{d}) [40]. The L\textsubscript{d} phase is characterised by lower viscosity and higher diffusion coefficients due to partial melting of acyl chains of individual molecules thus increasing the disorder of the system as a whole. Conversely lipids in the L\textsubscript{o} state show an increase in overall order and an increased viscosity [41]. In addition membranes in the L\textsubscript{d} state have an increased self-healing ability due to the propensity of the bilayer to rearrange. The phase of a lipid system is defined by its chemical make-up. In a single lipid system, factors such as chain length and degree of intermolecular forces such as van der Waals interactions dominate in defining phase behaviour at specific temperatures [42, 43]. As a general rule, the temperature at which transition from the L\textsubscript{o} to L\textsubscript{d} state occurs, increases with increasing acyl chain length [42]. Altering the lipid composition of a bilayer not only alters the temperature at which the phase transition might take place but also broadens the shift so that an ordered/disordered mixed phase (L\textsubscript{m}) becomes apparent.

This is highly apparent when adding cholesterol to bilayers. Bilayers can also be generated from combinations of lipids some of which are in the L\textsubscript{o} phase at a given temperature, and some which are in the L\textsubscript{d} phase. Such bilayers can demonstrate aspects of all three phases at once. A 2007 review used data on inclusion of different membrane lipids to generate a phase diagram for the contribution different phase lipids have to the overall phase of a composite bilayer [40]. As well as phase transition temperatures being important to the structural rigidity of a vesicle, they also have an effect on the ability of objects to insert into the bilayer. Studies using atomic force microscopy have recognised that puncturing a vesicle bilayer requires less force when the membrane is in its L\textsubscript{d} phase. Interestingly
however the required force is decreased even further at temperatures close to the transition temperature [44].

Figure IV-16 Illustration of how variation of the lipid components affects the phase composition of a lipid bilayer. A) a phase diagram showing how at a given temperature varying the mol% of fluid lipids (l\textsubscript{f}), solid lipids (l\textsubscript{s}) and cholesterol can effect the phase composition of a bilayer. Two areas of two phase coexistence are apparent (characterised by the appearance of cholesterol monohydrate crystals), one at very high cholesterol concentrations and another at cholesterol concentrations below 50% where the fluid lipid and solid lipid concentrations are at a ratio of ~1:1 (mixed l\textsubscript{f} and l\textsubscript{s}). There is also a region three phase coexistence (l\textsubscript{f}, l\textsubscript{s} and l\textsubscript{β}) also within the ~1:1 fluid:solid region. Illustrations to the right of the phase diagram indicate how the vesicle bilayer might appear given these phase variations. B) C) and D) are diagrams demonstrating the l\textsubscript{f} to l\textsubscript{s} transition which occurs either with increasing mol% of solid lipid or cholesterol, (illustrated by orange moieties). A) is adapted from the 2007 paper “Phase boundaries and biological membranes” [40]

When in their L\textsubscript{d} or to an extent their L\textsubscript{m} state lipid bilayers can be considered as a two dimensional fluid as individual lipid molecules within the membrane are more or less free to diffuse throughout the whole structure. Molecules within a membrane in the liquid disordered state are free to move either by lateral diffusion through a membrane or by molecular rotation about their axis Figure IV-17. Lipid translocation (flip flop), where a lipid molecule flips from one leaflet of the membrane from the other is also allowed, however this carries quite a high energy penalty and tends to occur as an induced response
such as flip flop of lipids through transient pores generated by discrepancies in ion concentration across a membrane [45].

![Figure IV-17 A two dimensional representation of possible movements of lipid molecules within a bilayer. (A) Lateral diffusion whereby lipid molecules exchange planar locations within a single bilayer leaflet. (B) Rotational movement where by a lipid will remain in the same location relative to other molecules whilst rotating around its own axis. (C) Bilayer translocation or lipid flip flop where lipid molecules may exchange locations across both bilayer leaflets. All three modes of translocation may occur simultaneously.]

The bilayer fluidity and phase forming behaviour and subsequent stability therefore is influenced by both the chemical make-up of its individual molecular components and by the mix of components from which it is made. Generally moieties which decrease bilayer fluidity through either high melting temperatures (i.e. long acyl chain lipids), or through steric hindrances (i.e. cholesterol) will increase the bending energy experienced by membranes, potentially making them more susceptible to lysis.

As well as altering the bending energy experienced by a bilayer changes in fluidity can also affect lateral diffusion coefficients which will change the dynamics of phase separation, the nature of membrane binding events by external molecules and the aggregation of such molecules within the bilayer. Studies have been made into the effect that various properties of lipids such as head group size and charge, chain length and chain saturation have on lateral diffusion. A study performed in 1996 looked at POPC, DOPC, DOPE and DOPS, which vary in all factors listed but chain length. The study found that none of the factors had a significant effect on lateral diffusion, which was equal to between $11.1 \times 10^8$ cm$^2$/s [46]. A more recent study looked at the lateral diffusion of proteins and the lipophilic probe DiD in large unilamellar vesicles (LUV’s) constructed from a variety of lipids [47]. Several di-acyl phosphatidyl choline lipids with chain length ranging
from C-14 to C-22 were used as well as two C-18’s with glycerol and ethanolamine head groups respectively. Lateral mobility of the lipid probe and subsequently its diffusion coefficient decreased with increasing acyl chain lengths. Whilst the decrease in lateral diffusion was not great between acyl chains of minimum length difference, (two carbons) an increase of 4 carbons did show meaningful significance. Diffusion coefficients for 14C, 18C and 22C acyl chains were 13, 8.7 and 5.0um^2/s respectively. The study also showed that a membranes consisting of up to 50mol% of the glycerol head group-containing lipid did not affect the diffusion coefficient significantly but that lipids containing an ethanolamine head group showed a significant decrease in diffusion coefficient between 0 and 50mol%. Changes in lateral diffusion based on increased membrane thickness have been putatively attributed to a number of factors. These include increased van der Waals’s interactions with increased chain length lipids [48] and increased interdigitation of the two leaflets of the bilayer [49]

Cholesterol although mentioned already in terms of broadening of transition temperatures is worth mentioning again in light of effects it has on lipid order and diffusion coefficients. A 2000 study demonstrated that cholesterol has quite general effects on membrane order and reduction of diffusion which function independent of head group charge or structure, chain length or saturation [50]. This leads to stabilisation of the bilayer and decreases the efflux of molecules across the membrane, (in this case carboxyfluorescein). The authors did note that cholesterol had an indirect effect on bilayers consisting of lipid’s displaying charge repulsion via their head groups in that it would separate the charged moiety’s thus stabilising the bilayer. A study from 2006 confirmed that the effect of cholesterol on reducing diffusion coefficients was not chain length specific, but that chain lengths did affect diffusion coefficients separately [51]. It has also been noted by numerous groups that when using a lipid with an acyl chain of 14 carbon’s (DMPC) there is a distinct increase in order and a decrease in the diffusion coefficient between mono lipid bilayers and those with around 30mol% cholesterol [52]. Increasing cholesterol further does not seem to have a significant effect on diffusion coefficient.

Two main models have dominated the diffusion of molecules in lipid bilayers. The free area theory [53, 54] and the hydrodynamic continuum theory. The free area model essentially treats lipids in a membrane as cross sectional areas and attempts to define the space into which they can move. This model has been demonstrated to fit both computational and experimental data of the fluidity of lipids within a bilayer system
reasonably well, despite the conceptual problem of it ignoring bilayer depth where lipids could be reasonably expected to move vertically to occupy space [52]. Whilst the free area model has proved useful for small molecules such as lipids it has been less successful when looking at larger molecules such as proteins. The hydrodynamic model is slightly different in that it treats the bilayer as sheet of viscous liquid on an infinite plan separating infinite regions of less viscous liquid (water) [55]. The protein molecule is regarded as a cylinder with its axis perpendicular to the sheet and which moves under the action of Brownian motion. In this model, diffusion is logarithmically dependent on the radius of the diffusing object and inversely proportional to the thickness and viscosity of the bilayer. Crossover between these two models has been identified for molecules of a certain size. Liu et al looked at the effect of lipid analogues of various sizes in a membrane and found that there was cross over in the models with objects of a ~1nm$^2$ cross-sectional area at the bilayer surface.

Lateral diffusion governed by the hydrodynamic model may be important when regarding its effects on membrane active proteins such as toxins. This is especially important if the proteins action is diffusion limited, for example in structures which must form aggregates such as the alpha toxin of S.aureus [56]. Work using short $\alpha$-helical transmembrane peptides, has illustrated the impact that mismatching between the size of the hydrophobic domain on the protein and the thickness of the hydrophobic region of the bilayer can have [57]. Membranes can adapt in a number of ways to hydrophobic mismatch. For example in cases where the hydrophobic area of the peptide is larger than the membrane could normally accommodate the peptide can tilt, the membrane can distort or the peptide can aggregate. Such adaptation applies stress to the membrane and also increases the effective hydrodynamic radius of the peptide, thus slowing lateral diffusion [47].

The effect that lipid composition has on bilayer behaviour and bilayer response to external factors cannot be understated. The effect that changes in fluidity can have on stability of bilayers to external forces, whether they be physical such as temperature or biological such a protein incorporation, must be considered when designing a system to respond to specific stimuli.
(b) Bilayer domains

Lateral mobility in biological membranes can be orders of magnitude slower than in artificial membranes due to a combination of factors including membrane crowding, domain formation and the formation of cytoskeletal boundaries [58, 59]. Membrane structures such as these do not simply mediate diffusion limitation however, they also affect membrane protein or membrane sugar interactions and stability [60]. Although these structures are less apparent in artificial membranes, they still can occur, either naturally or to some extent through artificial generation.

Lipid rafts were originally identified as clusters of glycosphingolipids in the golgi apparatus of mammalian cells. The rafts tended to be highly buoyant and insoluble in triton X-100 at low temperatures [61]. Lipid rafts in artificial membranes can range from nm to µm in scale, with size being primarily controlled by lipid compositional ratios [62, 63]. Interestingly rafts containing high levels of cholesterol appear in a liquid ordered phase, with acyl chains extended similar to the Lβ state, however lipids in rafts do not show the restriction to lateral diffusion normally seen in the gel phase. Rafts have been shown to form in systems as simple as binary lipid mixtures. A study using C16 phosphatidylcholine and cholesterol noted nm scale domain formation at room temperature in mix ratios of 7:3, DPPC:cholesterol [64]. The same study also showed that the type of raft formed was highly dependent on the lipid species. Whilst DPPC/cholesterol mixtures formed nanoscale structures of phase variation, no such structures were seen in DOPC/cholesterol mixtures. For ternary mixtures containing all three lipids domain formation with larger surface areas than for the DPPC/cholesterol mix but less well defined boundaries were observed. Changes in domain formation and the existence of l_d, l_o and l_m in the bilayers such as these demonstrate the subtlety with which bilayer architectural elements can be modified.
The effect of bilayer cholesterol concentration on PSM lytic behaviour

Incorporation of cholesterol into a bilayer can have numerous effects; including the formation of cholesterol rich domains, changes in bilayer fluidity, broadening of phase transition temperatures, and increasing acyl chain order. Based on the PSMs structure, and subsequently their suspected mode of action (based on that of delta haemolysin), it would not be too great a leap to hypothesise that variations in activity are concurrent with variations in cholesterol concentration. Studies looking at the effect of membrane fluidity on the action of delta toxin have identified that delta toxin is most efficacious when lipids are in the fluid phase, at which point lipid perturbations are apparent and fusion of lipid structures such as vesicles can be observed. [65, 66] Conversely, disturbance of lipids in the gel phase by delta has been demonstrated to be minimal. The effects of delta haemolysin in the gel phase are not nil however, merely subtle. Incorporation of relatively high concentrations of delta into bilayers has been shown to obliterate lipid pre-transition (thought to be related to structural changes of lipids which occur concurrently with chain melting); a process which indicates that interaction with bilayers still occurs in the gel state even if lysis does not [67-69].

Whether cholesterol induced changes in bilayer transition temperature and fluidity have similar effects on delta haemolysin and PSM lytic activity, as was seen in the calorimetric and NMR studies cited above, is unknown. Variations on the DPPC53 vesicles were synthesised, where the percentage of the primary lipid DPPC and cholesterol were varied from 0% cholesterol:73%DPPC, to 50% cholesterol:23%DPPC in 10% increments of each constituent. The percentages of the other two components, TCDA and DPPE at 25% and
2% of total constituents respectively, were not altered. The lytic capacity of all PSMs was then determined against all vesicle types Figure IV-19.

Figure IV-19 The response of vesicles of varying cholesterol concentrations to the PSMSs of S.aureus. DPPC vesicles with cholesterol concentrations from 0% to 50%, were exposed to concentrations of α PSM’s, β PSM’s or delta toxin. Concentrations ranged from 0.1-10µM dm$^{-3}$. On exposure the fluorescence of samples was measured ($\lambda_{ext}$ 485nm, $\lambda_{em}$ 520nm), every minute for a period of 2 hours. Values shown are normalised between a minimum value, (F0), determined as the vesicles response to storage buffer (HEPES buffer) over the measurement period and a maximum value, (Fmax), the vesicles response to 0.01% triton X-100 over the same time period. Values were taken from 1 hour into the experiment when the fluorescence/time response had plateaued. Each graph is labelled as to the toxin used. Data were fitted to a dose response curve using OriginLab 9.

Comparing the data sets in Figure IV-19, the general trend of toxin efficacy that was observed against the DPPC53 (20% cholesterol) vesicles is broadly replicated across all cholesterol concentrations tested. The PSMSs α1, α2, and α3 demonstrate vesicle lysis at the lowest toxin concentrations. These three are followed by delta haemolysin and PSM α4. As seen previously, vesicles show the lowest overall sensitivity to the β-PSMs. As
well as the overall sensitivity of each vesicle type to the individual toxins, the clustering of
data in each case may also be important. For example, fits for all vesicle types (apart from
50% cholesterol) display a high degree of clustering when challenged with both PSMα2
and delta haemolysin, showing maximum fit separations of 0.4 and 0.3 fluorescence units
respectively. Conversely, PSMsα1 and α3 show larger maximum separations of 0.53 and
0.56 normalised fluorescence units. The maximum separation between fits, as well as
visual recognition of clustering, likely gives some indication as to the susceptibility of
membrane integration each peptide experiences as a result of cholesterol concentration, or
the subsequent effects on the bilayer physical characteristics the cholesterol generates.
This variation in “across board” sensitivity is broadly interesting in terms of its potential
for generating multiple vesicle types for the detection of multiple toxins. It is interesting,
more specifically however, in that it may provide some insight into how the differences
between PSMs structure and function manifest.

The arrangement of data in respect to one another in Figure IV-19 is also of some interest.
In all cases (apart from potentially α1), 50% cholesterol vesicles are least sensitive to all of
the PSM’s. In the case of delta haemolysin and the two β-PSMs, this is carried through as
a null response, which may be at least partially explained by the lower demarcation angles
between the hydrophobic and hydrophilic planes in these three. For the other cholesterol
concentrations, there are fewer consensuses in toxin sensitivity. The major consensus seen
between the α-PSMs is that 10%, 20%, or 30% cholesterol vesicles show the highest
sensitivity in all cases; 0% and 40% cholesterol vesicles are never shown to be the most
sensitive vesicle type. Although this seems a vague result, it may be indicative of a
cholesterol “goldilocks zone” whereby the PSMs require a certain amount of cholesterol to
function, but too much or too little is counterproductive. This phenomenon may be
indicative of the PSMs requirement for a certain amount of cholesterol to function, but too
much or too little being counterproductive. Observations to this effect have been made
previously using delta toxin and other amphiphiles [9-11, 70-72]. While in this case the
lipid composition of vesicles used was different to that of our own experiments,
observations were made on the basis of cholesterol to phospholipid ratio and the phase
transitions changes in such induce. It was noted in these studies that delta toxin
preferentially binds to the liquid disordered phase and that perturbation of the bilayer in a
two phase system is approximately similar to that of a pure liquid disordered system
provided that the peptide concentration in the liquid disordered phase is the same in both
cases. If the remainder of the PSMs follow delta toxins lead, we would then expect an increase in cholesterol content to decrease the area of membrane to which the toxins will preferentially bind. Additionally however this would also decrease the bilayer area over which the toxins need to generate perturbations in order to cause lysis.

While a linear inverse relationship between activity and bilayer cholesterol concentration may seem logical with what is known of delta toxins’ tendency to interact preferentially with fluid phase bilayers, the variations seen may make more sense in a biological context. As previously demonstrated, PSMs are active against numerous cell types in numerous organisms, which will have varying and dynamic bilayer compositions. For example, neutrophils of adult rats have been demonstrated to have cholesterol to phospholipid ratio of 0.011 [73], while erythrocytes from adult humans give a ratio of 0.59 [74]. The variation in the PSMs capacity to lyse vesicles of varying cholesterol concentration may therefore be an indicator of redundancy in function. An ability to cope with multiple cell types dependent on the membrane composition, particularly the percentage of CHO. The reduced sensitivity may also be related to biological function, in that natural membranes with very high or very low cholesterol content/fluidity are likely to be uncommon, due to negative effects which may be generated affecting their correct functioning. It has been shown previously that PMNs from different mammals have different susceptibilities to PSMs, with mouse neutrophils (BALB/c and C57/BL6) being more resistant to PSMs than rabbit or human neutrophils [75]. *S. aureus* may have evolved to secrete small peptides which have preferences to cell types based purely on membrane composition, adding to the hugely effective arsenal of toxins in which this bacterium possess [76]. This preference for membrane structure, dictated by CHO gains credibility considering there is no known proteinaceous receptor for PSMs. In contrast to the α-PSMs, the β-PSMs show relatively consistent decreases in lytic activity with increasing cholesterol concentration. As with the α-PSMs, neither β shows greatest activity against vesicles completely lacking cholesterol, so in this way the pattern is maintained.

In order to look more closely at the variations in toxin efficacy against the different cholesterol vesicles, EC50 data from the experiments shown in Figure IV-19 has been collated into Figure IV-20 below. EC50s of samples which did not reach the required cut off of 0.5 normalised fluorescence units have been excluded.
EC50 values of PSMs when challenged with phospholipid vesicles of different cholesterol concentrations. EC50 values were determined from dose response fit’s of the normalised fluorescence Vs concentration data. Where fluorescence maxima did not reach the required 0.5 normalised fluorescence units value, they were excluded.

PSMs $\alpha_2$ and $\alpha_3$ show the lowest effective lytic concentrations of all against 10% and 30% cholesterol vesicles respectively, with values of 0.15µmol dm$^{-3}$ and 0.142 µmol dm$^{-3}$. The lowest sensitivity still above the detection threshold is seen in the $\beta$-PSMs. B1 and B2 give values of 25µmol dm$^{-3}$ and 19µmol dm$^{-3}$ respectively for cholesterol concentrations of 20% and 30%. The overall range of sensitivities is very broad; while it has already been stated that generally a sensor needs to detect the lowest levels of toxin possible, there is some wiggle room in that sensitivity truly need to be based on the level of toxin likely to be produced in a wound environment. Taking into account error bars, in the majority of cases the EC50 values represent a trough angled towards a specific cholesterol concentration, where either side of this concentration EC50 values rise. The PSM $\alpha_3$ and delta haemolysin are exceptions to this rule. $\alpha_3$ shows an increase in EC50 out of the SEM range at a concentration of 20% cholesterol, while delta haemolysins shows a general downward curve rather than a trough. Evidence of such troughs in EC50 data may add some credence to the theory of a “goldilocks zone”.
Several interesting results have come to light as a result of this study which may challenge some of the established knowledge on the PSMs. Until this point, PSMα3 has been thought of as especially toxic amongst the PSMs based on experiments using polymorphonuclear neutrophils (PMNs) [2, 77]. In light of this investigation however, the toxicity seen may be due to the cell type, rather than due to specific high toxicity of α3. The response from vesicles of low cholesterol concentration to the β-PSMs is also of interest. Previously the β-PSMs have been shown to have very low activity against PMNs and intermediate activity against erythrocytes [2, 29, 78]. Additionally, a study performed using POPC vesicles demonstrated high lytic activity of both β-PSMs at concentrations of 0.5µmol dm$^{-3}$ [79]. While the EC50s shown in this experiment do not reach values as low as 0.5, the lack of cholesterol included in the vesicles composition may be part of the reason for the difference in lytic activity seen as compared to PMNs and erythrocytes. The final point is simply that the lytic activity of the PSMs is not as set in stone as is thought; rather it is plastic and dependent upon environment.

(d) The lytic capacity of supernatant against vesicles of varying cholesterol content
While understanding of the effect of individual PSMs against phospholipid bilayers is useful, it does not provide a great deal of context as to how such vesicle systems would react in a more realistic environment. As such, the whole range of cholesterol vesicles were tested against $S. aureus$ LAC supernatant.
Figure IV-21  Lytic efficacy of \textit{S.aureus} LAC supernatant against DPPC vesicles of all cholesterol concentrations.  
(A) The efficacy of dilutions of supernatant against all six vesicle types as determined by fluorescence release.  (B) The EC50 values for each vesicle type treated with \textit{S.aureus} LAC supernatant.  EC50 values were determined through fitting of supernatant dilution Vs normalised fluorescence data to dose response curves.  Fitting was performed using OriginLab 9.
As with the individual PSMs, significant variation is seen in the lytic capacity of supernatant against the full range of cholesterol concentrations. While maximum fluorescence values were above the 0.5 cut off in all cases, there was a marked difference between maximums for the 0%, 10%, and 20% cholesterol vesicles which all showed values above 0.9 and the 30%, 40%, and 50% vesicles whose maximums did not reach above 0.65 normalised fluorescence units. Interestingly in correlation with the α-PSMs, 10% cholesterol vesicles did not show the highest supernatant sensitivity; rather it was middle of the pack in terms of sensitivity. EC50 data (Figure IV-21 (B)) demonstrates similar trough behaviour to that seen in Figure IV-20. The EC50 data corresponds to a certain extent with the maximum values observed in (Figure V-17A), however the one major outlier in this case is the 50% cholesterol vesicles to which LAC shows a significant decrease in activity. Overall, the supernatant shows similar organisation of lytic behaviour among vesicle types to those seen using the α-PSMs. Having said this, the data also shows characteristics of the β-PSMs in the spreading of maximums between the high and low cholesterol concentration vesicles. While this data is useful in identifying toxin sensitivity at fairly realistic toxin concentrations (in-vitro cultures not necessarily representing the toxin load generated in a wound), better understanding of the levels of PSMs present in wound environments is required to fully interpret how these bilayer variations might affect bacterial sensing.

Section 4.05 Changes in PSM sensitivity imparted by varying acyl chains length

Like cholesterol content, acyl chain length of bilayer lipids is also known to affect the fluidity and stability of membranes. Generally longer chain lengths are thought to impart greater membrane stability through the effects of intra-chain attractive forces and decreases in bilayer fluidity. Bilayer disruption often requires the transient distortion of the local lipid environment, in order to generate sufficient difference between bending and line energies to cause rupture, pore formation, or other perturbations. For example, previous studies using delta haemolysin have suggested that bilayer damage may in part be caused by an intermediate delta-lipid structure which imparts high positive curvature, and therefore high bending stresses on the bilayer [10]. As changes in the acyl chain length of
bulk lipids will change the elastic properties of a bilayer, it may be expected that they will also change the effect of interactions with toxins.

In order to determine the effect of changes in acyl chain length on sensor efficacy, three different vesicles were used. As well as the standard DPPC53 vesicles which contain a 16 carbon chain length PC (1,2-dipalmitoyl-sn-glycero-phosphatidylcholine), vesicles containing a 14 carbon PC (1,2-dimiristoyl-sn-glycero-phosphatidyl choline) and those containing an 18 carbon PC, (1,2-distearoyl-sn-glycero-phosphatidylcholine) were also synthesised. The headgroups and the chain saturation remained the same amongst the three molecules to ensure as much as possible that differences observed were a result of chain length variations. The other constituents of the vesicles, cholesterol, DPPE and TCDA remained at 20%, 2% and 25% respectively. From this point, the three vesicle types will be referred to as DMPC53 (14:0 PC), DPPC53 (16:0 PC) and DSPC53 (18:0 PC). Due to constraints on the availability of synthetic toxins, data for PSMs α3 and βs 1and 2 have unfortunately not been included.

Figure IV-22 Sensitivity of vesicles of varying acyl chain lengths to select α-PSMs. (A, B, C, D) Show the response measured in normalised fluorescence of all three vesicle types to PSMs α1, α2, α4 and delta haemolysin respectively. All data were fix using a dose response equation using OriginLab9..
As with experiments of varying cholesterol concentration, some general patterns of PSM activity are upheld. Both PSMs α4 and delta haemolysin show reduced lytic capacity across the acyl length ranges. Both also show reduced normalised fluorescence maximums in the case of DSPC53 vesicles with values below the 0.5 unit cut off. In all cases there is an inverse correlation between acyl chain length and fluorescence maximum, where DMPC53 vesicles give the highest normalised fluorescence across the whole PSM concentration range. For all toxins, DMPC vesicles are not the most concentration sensitive. This is interesting to note, as it has been previously demonstrated with delta toxin that there is a positive correlation between bending modulus and membrane perturbations [80]. As the chain length increases, the bending modulus also increases as a general rule; however this is highly dependent on other factors such as temperature, total bilayer components and bilayer type. In this case, the differences may arise from differences in bilayer constituents which were multiple in this study but were singular in the previous study; temperature and pH as peptides in the previous study were dissolved at ~pH 3. Also of note is the fact that lipids of chain length 16:0 PC and higher were used in the previous experiment, but 14:0 PC lipids were not included.

Again to observe variations in sensitivity more closely, the EC50 values of each toxin and vesicle were plotted. Values for delta and PSMα4 Vs DSPC were not included due maximum values not reaching the required threshold values (Figure IV-23).
EC50 values highlight an interesting difference between the toxins, in that the normal pattern of efficacy seen between the toxins (i.e. $\alpha_1/\alpha_2/\alpha_3>\delta>\alpha_4$) do not apply when DMPC53 vesicles are the target. The pattern is disrupted by the transposition of $\alpha_4$ and delta. It is worth again pointing out the surprisingly high EC50 values seen for DMPC53 vesicles across all toxins. A partial answer to this unexpected variation may indeed be gained by looking at previously determined bending moduli of pure lipid, giant unilamellar vesicles. One study generated values of $1.27\times10^{-19}$J at 40°C for DMPC and $0.54\times10^{-19}$J at 42°C for DPPC [81, 82]. Although these values were determined at different temperatures, values were shown to increase with increasing temperature for both DMPC and DPPC at rates of $0.031\times10^{-19}$J ±0.019 and $1.33\times10^{-19}$J ±1.86. As such, a 2°C temperature increase is unlikely to lower the bending modulus of DMPC below that of DPPC. The inclusion of cholesterol and other bilayer constituents will also have an effect on the bending moduli; however the aforementioned relationship between bending modulus and delta toxin induced membrane perturbations should still be considered based on this correlation.
Overall the data demonstrate the higher sensitivity toward the staphylococcal PSMs experienced by DPPC. The relatively lower sensitivity of the other two acyl chain lengths may also be interesting in situations where a PSM insensitive vesicle is required. Further experimentation to see if this pattern carries through to the untested PSMs would also be useful.

(a) Sensitivity to *S. aureus* LAC supernatant generated through varying acyl chain lengths

*S. aureus* Lac supernatant was again used to qualify the effect of more realistic toxin concentrations on the variable acyl chain vesicles. As before, supernatant was harvested from 18 hour cultures and vesicles were treated with various supernatant dilutions (Figure IV-24).

![Graph showing response of phospholipid vesicles of varying acyl chain length to 18 hour supernatant from *S. aureus*.](image)

Response of the different chain length vesicles to LAC supernatant yields some surprising results, given data shown for the PSMs. Whilst maximums across the three vesicle types
correlate with PSM data in that DMPC53>DPPC53>DSPC53, the order of sensitivity is less expected. In none of the PSMs tested was lytic efficacy shown to be greatest against DMPC53 vesicles, as is shown with LAC supernatant. This result seems to suggest that either one of the untested PSMs shows considerable difference to those tested in terms of its lytic profile, or that some synergistic effect is occurring between the PSMs which is not noticed when testing in isolation. EC50s have not been compared directly in this study, mainly due to poor fitting seen in relation to the DSPC53 vesicle type. This may be related to the particularly large decreases in normalised fluorescence seen at high supernatant concentrations for both DSPC53 and DMPC53. While it is possible that such decreases are a result of lowered efficacy of the previously hypothesised aggregates against these vesicle types, a similar result would also have been expected with the individually tested PSMs. While it is possible that one of the untested toxins is causing these effects, they may also be the result of interactions between some other portion of the supernatant and the vesicles.

Section 4.06 Conclusion

While investigations made here are by no means entirely comprehensive or complete, they have nonetheless yielded some interesting results on both the functioning of the PSMs, and the sensitivity of the vesicle types tested. General observations of the phenol soluble modulins with DPPC53 vesicles have identified several factors (EC50, lysis maximum, and fluorescence leakage over time), which are useful in comparing toxin lytic efficacy. These factors have initially highlighted, as the peptide structures suggest, that lytic efficacy varies considerably between toxins. In general, the toxins go from most lytic to least lytic in the order $\alpha_2>\alpha_3>\alpha_1>\delta>\alpha_4>\beta_2>\beta_1$. Whilst the lower sensitivity of $\alpha_4$ has been demonstrated in other studies [2, 29], it has not as of yet been commented on, nor seen with such regularity.

This study has also highlighted that the relationship between toxin concentration and lytic activity may be more complex than a simple dose response model can describe. Investigation into decreases in PSM$\alpha_2$’s efficacy at high toxin concentrations suggests that aggregation of peptides may have a role in reduction of lytic potential. This is especially interesting in light of recent investigations into the PSMs role as structural elements within
In order to investigate this further, analysis of the aggregation behaviour of all peptides would need to be determined. Studies of the lytic potential when aggregation is disallowed, such as at high pH, may also be necessary. The effect, which such peptide aggregation may have on the vesicles as a sensor system, is unknown, as measurements of PSM production in a wound or simulated wound environment have not yet been made. If aggregation does prove to be a factor in peptide transition from a lytic to non-lytic state this could explain how such high concentrations of peptide can be found within biofilms which seem minimally lytic to mammalian cells. It would be interesting then to see how aggregation behaviour may affect lysis.

Comparisons were made of the lytic activity of PSMs and rhamnolipids between vesicles and T-cells which suggested that while vesicles may be a suitable sensor system for the detection of *S.aureus*, this may not be the case for *P.aeruginosa*. In both cases however, further measurements of a number of strains would be required to determine how vesicle susceptibility varies.

Variation in bilayer compositions has demonstrated general trends in the efficacy of the toxins based on fluidity and phase formation. Variations in cholesterol concentration have shown that the PSMs are generally more efficacious when cholesterol is neither too high nor too low. A partial explanation for this can be found in delta haemolysins integration to membranes that contain sphingomyelin-cholesterol rafts [70, 72], specifically within the liquid disordered domains of such bilayers. It has been hypothesised that increases in raft formation within bilayers lead to fewer haemolysin insertion sites. As such any increased bending exerted on the membrane is likely to occur over a much smaller area, leading to membrane rupture at relatively low peptide concentrations; this may then be the case generally with all of the PSMs.

Variations amongst different acyl chain lengths also yielded some interesting results, particularly in the apparent sensitivity of DPPC53 vesicles to the PSMs. Although results gained using *S.aureus* supernatant may seem anathematic to those from the individual peptides, differences in susceptibility of the differing acyl length vesicles are actually very small (around 3% points difference), and additionally may be accounted for by the missing peptides.

Overall this study has identified tuneable factors in the development of a vesicle system, and has presented possibilities for creating both high and low sensitivity vesicles to either...
one or multiple toxins. As well as the desired goal of this project, to generate a sensor which can both identify and distinguish between different pathogens such vesicle systems also demonstrate potential as rapid lab based sensors. As the work in chapter 4 has already illustrated the system could be used to determine the correct functioning of genetic elements. However depending on how finely the sensitivity of such a system can be tuned there is also the potential to develop a sensor system with the capacity to map the membrane active compounds produced by a particular organism. The current setup used for analysis is based on a micro-titre plate loaded with vesicles with toxin concentrations varied. Due to the relatively long shelf life of these vesicles it is easy to envisage instead, a system whereby the types of vesicles are varied. In this way bacterial supernatant could be delivered to the entire plate with each well having the capacity to detect the presence of a particular bacterial toxin. Such a system would then have the potential to detect the toxin production of different strains of a particular organism and also the toxin production based on varied growth conditions. The use of a vesicle bilayer as a sensor in this way presents a powerful tool for the investigation of both the toxome and potentially the genome of a target organism.

Moving forward however, several considerations must be made to enable the practical use of such a system both for its intended clinical use and its potential laboratory usage. First and foremost is the quantification of PSM release in a wound environment or pseudo-wound environment. Whilst lab grown cultures are useful for preliminary study, more in-depth knowledge is required for further investigation. One possibility for such analysis may be in the use of MALDI-TOF which has previously been used for the quantification of PSMs across a single S.aureus colony [83]. Greater clarity must also be achieved in understanding the sensing mechanism of vesicles in order improve on the design. The lack of an all-or-nothing response in numerous cases, and the variation in maximum fluorescence release, suggests that peptides are not simply lysing vesicles. Instead the ability of PSMs to create a saturated response at fluorescence concentrations below the maximum suggests a ligand receptor type model of response that may include vesicle fusion lysis inhibition. All of these questions provide ample material for further study and the basis of a system for the detection of bacterial pathogens.
References


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Chapter V. The efficacy of novel zinc organometallics as antimicrobial surface coatings

Whilst the primary goal of this study was the development of an in-vivo detection system for bacterial pathogens the second aim was the reduction of pathogen counts in the same wound environment. This goal was originally conceived as useful stop-gap between microbial detection by the biosensor element of the system and subsequent treatment of the detected infection. The concept was to repress bacterial growth while the pathogen was both determined and treated, without putting unnecessary selection pressures on the organism. The idea was thought to be prevalent both in terms of the rapid treatment required in burn patients, and the decreased bacterial load required for infection associated with wound dressings [1-4]

Initially, attempts were made to alter the phospholipid vesicles used for microbial detection (Chapters IV and V) to include antimicrobial compounds. The aminoglycoside antibiotic gentamicin and silver nitrate [5, 6] were initially trialled as proofs of concept. As previously stated, the vulnerability of lipid vesicles relies upon surfactant-like toxins, which are produced in response to bacterial quorum signals. As such, microbial growth would be expected up to the point of vesicle lysis, at which time the antimicrobial components would then be released. However, no growth was seen with either antimicrobial. The problem was thought to be due to inappropriate methods of post synthesis vesicle purification. In the production of CF containing vesicles, excess dye is removed by a size exclusion sephadex column, a process which may not be appropriate for the removal of excess antimicrobials.

Due the difficulty with the vesicles, a second concept was generated. Rather than an antimicrobial being released in response to bacteria, a surface component of the dressing would passively provide antimicrobial activity to the dressing surface. Due to the passive nature of the antimicrobial, it would be required to show low incidence of development of resistance. Zinc was chosen as the antimicrobial component of this surface treatment, as it has demonstrated low resistance selective pressures, antimicrobial activity[7, 8], low eukaryotic toxicity and is relatively cheap.
In order to generate a surface coating of zinc, a carrier Schiff base was employed. The use of such a molecular architecture has a number of benefits. The ease of synthesis would make compounds ideal industrial candidates. The two R groups present in these Schiff bases also allow access to a wide variety of chemistry (should it be needed), through their substitution. This is demonstrated in the synthesis schematic Figure V-1.

![Figure V-1 Schematic Zinc Schiff base synthesis. The Schiff base ligand is formed through nucleophilic addition of an aromatic amine and a carbonyl compound. Zinc is then ligated through the reaction of the Schiff base with dimethyl zinc.](image)

The availability of a C=C group in the compound, in the form of cyclohexane ring should be more susceptible to ionisation, or radicalisation. This makes the compound more suited to attachment processes such as plasma deposition and UV grafting. It also provides a degree of control over attachment sites when using these methods. Attachment of the compound to the fabric substrate in this manner should hopefully allow for adherence with minimal changes in the compounds overall structure.
This Schiff base generated was based on 5-methoxysalicaldehyde, to which 2-(1-cyclohexenyl)-ethylamine was attached. Dimethyl-zinc was added to form the zinc Schiff base compound ZSB2 (consisting of 2 ligand molecules and a single zinc centre). The compound was synthesised by Dr Charlotte James, a prior student of the Jenkins (biophysical chemistry) research group and is shown in Figure V-2.

Aims & Objectives

Section 5.01 Aims & Objectives

With the required zinc containing Schiff base developed, several aims had to be realised simultaneously in order to achieve the desired goal of generating an antimicrobial surface coating for use in wound dressings. Initially the antimicrobial activity of the compound against target organisms would have to be ascertained in order to determine how it was affected by any attachment method. This target was achieved simply however through the use of MIC determination (see Figure V-3). Subsequently an appropriate method of surface attachment to non-woven polypropylene would have to be established. Once this goal was reached various parameters of the resultant coating would need to be resolved.
including, antimicrobial efficacy, leaching properties and maintenance of the original compounds structure.

Attempts to generate a contiguous surface coating of the zinc Schiff base compound were made using one of two methods namely; inductively coupled pulsed plasma deposition and photo radical initiated grafting. When attempting compound deposition through the use of the inductively coupled plasma method parameters such as input power, total power, monomer concentration, flow rate and deposition time were all varied. In the case of the photo radical grafting method pre-activation of the polypropylene surface was compared to direct attempts at grafting. Additionally the total energy delivered to the surface and the initial compound concentration was also varied.

To determine how each of these grafting procedures and the variables there in affected the antimicrobial efficacy of the graft compound, a refinement of the Japanese industrial standard assay for the antimicrobial activity of fabrics was performed. This assay involves measuring the relative inhibition of bacterial growth cause by the treated fabrics in comparison to an untreated control. Additionally variations in graft efficiency were investigated using fluorescence spectroscopy to determine the degree of compound structural retention in treated surfaces; confocal microscopy to observe the ratio of live to dead bacteria attached to treated poly propylene fibres; scanning electron microscopy to identify topographical differences in treated and untreated surfaces; and energy dispersive X-ray spectroscopy to investigate the zinc content of treated surfaces. The degree of compound leaching from grafted surfaces was measured using UV/visible light spectroscopy.

Finally attempts were made to determine the mode of action of the un-grafted monomer. Activity of the compound was monitored using a bacterial biosensor sensitive to the presence of free Zn$^{2+}$ ions. This activity was then compared to killing of the biosensor associated with the same compound concentrations. These results were compared in order to elucidate a likely route by which the Schiff bases antimicrobial activity was achieved.
A primary concern was determination of the efficacy of ZSB2. The compound was required to show evidence of surface killing. Release of the compound was not to be allowed as the eukaryotic toxicity of the newly generated molecule was unknown. Initial investigations were based on simple minimum inhibitory concentration (MIC) assays. ZSB2 should also be able to demonstrate antimicrobial activity either equal to, or greater than, the sum of its parts.

(a) Determination of MIC values for ZSB2

MIC values were established for ZSB2 against *S. aureus* MSSA 476, and *P. aeruginosa* PA01, at concentrations of $10^5$ CFU/ml. The MIC curves for both compounds are shown below. MIC curves were established by plotting the area under the curve of absorbance values for treated bacterial cultures after 18 hours. The resulting curve was fit to a dose response model. A concentration range from 900µmol dm$^{-3}$ to 50 µmol dm$^{-3}$ was used. The ligand and the metal ligand complex were both dissolved in 2% DMSO due to very poor water solubility.

<table>
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<tr>
<th>Compound</th>
<th>MIC$_{50}$ (µmol dm$^{-3}$)</th>
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<tbody>
<tr>
<td></td>
<td>MSSA 476</td>
</tr>
<tr>
<td>ZSB2 + 2% DMSO</td>
<td>442</td>
</tr>
<tr>
<td>SB2 ligand +2% DMSO</td>
<td>598</td>
</tr>
<tr>
<td>Zn acetate</td>
<td>755</td>
</tr>
<tr>
<td>Zn acetate + 2% DMSO</td>
<td>700</td>
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MIC values were determined for ZSB2 against both organisms (Table V-1). Integration of absorbance data over an 18-hour period was used in order to avoid problems of discrete variation in absorbance values at specific time points (Figure V-2 (A)). However, variation in total growth was still observed between species, due to differences in maximum OD reached and changes in absorbance seen from *P. aeruginosa* PA01 in stationary phase (Figure V-3 (B)). Growth variations should not however affect overall MIC$_{50}$ values, which are a measure of half maximum of inhibition of growth.

Of the two 2 bacterial species ZSB2 shows greatest efficacy against *P. aeruginosa* PA01. The compound shows efficacy at a concentration 120µmol dm$^{-3}$ (or 28%), lower than that
seen for the *S.aureus* strain. In both cases, the SB2 ligand is a less effective antimicrobial than the ligand metal complex. This difference suggests some synergistic activity between the zinc and the ligand. The difference between the two also varied considerably between species.

While the difference between MICs for ZSB2 and SB2 against MSSA 476 was only 156µmol dm$^{-3}$, the differences in MICs against *P.aeruginosa* was much larger at 3539µmol dm$^{-3}$, close to an order of magnitude difference. A comparison of the MICs of Zn acetate and ZSB2 highlighted similar differences between the two organisms. In this case (using values for Zn acetate + 2% DMSO), MSSA 476 showed a difference of 258µmol dm$^{-3}$, while PA01 showed around a 16 fold decrease in sensitivity against Zn acetate as compared to ZSB2, a 4859µmol dm$^{-3}$ difference.

While the effect of ZSB2 against *P.aeruginosa* may be representative of a true synergistic activity, due to the low efficacy of either the ligand or the metal centre as an antimicrobial, its activity against MSSA476 is not quite so clear-cut. Although an increase in sensitivity is seen to ZSB2, this may be a consequence of the complexation process, as each mole of ZSB2 consists of two moles of ligand to one of zinc. Assuming a linear correlation between ligand concentration and antimicrobial efficacy, we would expect a doubling of the ligand concentration to give the MIC$_{100}$ of the compound. If this held true the MIC$_{100}$ of SB2 would be lower than the MIC$_{50}$ of ZSB2 (a value of 299µmol dm$^{-3}$). The fact that the MIC$_{50}$ of ZSB2 is not as low as for MSSA476 generates a couple of possibilities. Firstly and most likely is that the antimicrobial activity of the Schiff base and its constituent parts are not linked, and that the mechanism of action of ZSB2 is different to than of either SB2 or zinc acetate. Another possibility is that the ligand molecule has multiple antimicrobial “active sites”, and that complexation with zinc is occluding these sites. It seems however that if this second possibility was correct and each molecule contained more than one antimicrobial site, any metal complexation which eliminates a site would do so on both complex molecules. As such, the MIC of the SB2 ligand would be expected to be even lower.

While the activity is difficult to compare to gentamicin, which can be a mixture of slightly different isomers, the activity can be compared to that of silver nitrate, which was determined in preliminary experiments with antimicrobial vesicles, (as mentioned at the beginning of the chapter). Silver nitrate shows MIC$_{50}$ values of 4.2µmol dm$^{-3}$ against
MSSA 476, and 8.8µmol dm$^{-3}$ against PA01. Although these MIC$_{50}$ values are much lower than those seen for ZSB2, the mechanism of action in both cases must be considered. While silver nitrate was to be released from vesicles, the concept of ZSB2 is to generate a surface-active compound. As such, the relative concentration of the compound will likely be higher at the dressing surface. Overall, ZSB2 shows a decent level of antimicrobial activity against both organisms, however further investigation is required; especially in regards to retention of activity upon surface binding.

Section 5.03 Compound grafting and antimicrobial activity

Polypropylene non-woven fabrics were chosen as the material for development of prototype antimicrobial surfaces. Two approaches were attempted in the attachment of ZSB2 to such surfaces: plasma deposition [9], and a UV graft to approach [10]. Each process has its own merits and drawbacks. For example, while plasma deposition is a one-step process whereby additional chemicals are not required for attachment, it relies upon input power for the grafting process, which can be highly temperamental and dependant on numerous system parameters such as the temperature, pressure, and gas composition inside the reaction chamber. UV grafting on the other hand is a wet chemical approach, and requires the tuning of multiple parameters. These include adjustment of the concentration of the UV initiating compound, benzophenone (BP), adjustment of irradiation energy and adjustment of the monomer: initiator ratio rather than simply adjustment of the input energy. Determination of antimicrobial activity was made by assessing the viable bacterial load that could be recovered from treated fabrics after an exposure time of 18hrs.

(a) Plasma processes

Primary investigations were made into plasma deposition as a potential surface modification procedure. As such an understanding of plasma processes was required in order to grasp how the variation of parameters or techniques which might affect the resultant coating.

Briefly plasma is an ionized gas, a mixture of negatively and positively charged particles with an approximately neutral net charge density. The heating of a substance to reach
plasma state raises the kinetic energy of the atoms and molecules to the point at which ionisation occurs. However the type of energy input does not necessarily have to be thermal, it merely has to provide enough energy to the electron in question, to allow it pass the potential barrier of the atom or molecule it is going to leave or join. This is defined as the energy difference between the highest occupied atomic or molecular orbital and the highest possible orbital for that substance. The ionisation required to form a plasma can take also place through the addition of radiative energy.

There are several industrially viable methods of plasma generation including DC and DC pulse discharge, RF and microwave discharge and plasma created using laser or ion beams [11]. Two of the most commonly used types in thin film deposition (the required goal in this case), are capacitively coupled and inductively coupled RF plasmas. In this case an inductively coupled plasma (ICP) setup was utilised. Both of these categories of plasma are “non-equilibrium plasmas” where neutral particle temperatures are much lower than in a number of plasmas at atmospheric pressure. Such low temperature plasma was suitable in this case as a non-destructive method, which would minimally alter the starting monomers molecular structure as was required from this nanofabrication process. Figure V-4 is a schematic representation of the ICP setup in the biophysical chemistry laboratory.
In this diagram the anode and the cathode are represented as separate entities for simplicities sake, and is more akin to a capacitively coupled plasma generator. However in reality the electrodes take the form of a copper coil around the reactor connected to the power supply at one end and grounded at the other.

(i) **Plasma deposition**

The purpose of the plasma treatment of the antimicrobial compound is to attach the compound to a substrate surface for use in a medical setting. Deposition of organometallics is usually performed through plasma enhanced chemical vapour deposition, (PECVD). In PECVD the compound to be deposited either forms a vapour at the low pressures generated in the reaction vessel, or is heated before being released into
the reaction chamber to raise its volatility. The problem with using this method is that the high degree of heating has the potential to damage either the monomer or the substrate.

Vapour deposition can also be used within plasma processes, however due to low volatility of the starting monomer plasma deposition was not performed in the gas phase. Instead, polypropylene fabric is pre-coated with a solvated form of the compound. This changes the deposition process occurring at the surface due to the development of a plasma sheath at the substrate surface. This sheath is a result of Debye shielding, a dielectric phenomenon involving the redistribution of space charge by the polarization of the plasma medium (in this case the charge carriers resulting from the excited gas), to decrease the magnitude of internal electric fields. A simple example to consider is a point charge introduced into a plasma. For simplicity’s sake we will assume that the plasma consists of only free electrons, with a charge $1^{-ve}$, and ions which have lost only one electron thus having a resulting charge $1^{+ve}$. The plasma is also homogenous in terms of temperature and charge distribution. The point charge will attract electrons and repel ions creating a dampening field of electrons around it, thus shielding its electric field from the rest of the medium. However electrons cannot simply collapse onto the point charge as in their plasma state they are still experiencing an abundance of kinetic energy. At the point in time where coulombs attraction between the point charge and the electrons, and the kinetic energy of the electrons has come to equilibrium we will observe Debye shielding. This shielding effect acts over a specific length, which is dependent on exactly the two forces previously mentioned, the kinetic energy of the particles in the plasma and the magnitude of the coulombs attraction [12]. Debye length of our system can be determined by using the equation:

$$\lambda_D^2 = \frac{\varepsilon_0 k T_c}{n_0 e^2}$$  \hspace{1cm} (V-1)

Where $\lambda_D$ is the Debye length, $\varepsilon_0$ is the permittivity of free space, $T_c$ is the temperature of the system and $n_0$ is the number density of ions in the system. A schematic of this concept is presented in Figure V-5.
A schematic representation of the Debye shielding concept. A) The hypothetical reaction chamber containing neutral un-charged polypropylene fabric (grey mesh) and an even number of perfectly evenly distributed high velocity, positive (blue) and negative (red), charges. B) A positive charge is introduced to the polypropylene causing the net movement of negative charges towards and positive charges away from the fabric. C) Accumulation of a net negative charge at the fabric surface due to the rapid translocation of electrons within the space generates a shielding effect at the fabric surface.

Due to the higher velocities of electrons in the plasma relative to ions, the surface will build up a negative potential reflecting further electrons away from the surface. This negative potential will be balanced by an influx of positive ions and neutral particles, creating a sheath at one of the substrate surfaces [13], which is dependent on the orientation of the electromagnetic field. Due to the fluctuating potential of the applied electric field, the plasma sheath should fluctuate correspondingly around any object immersed in the plasma [14]. This leads to the substrate surface being periodically being bombarded with neutral but reactive free radicals and positive ions followed by immersion in the net neutral plasma bulk, thus leading to deposition on all sides of an object suspended in the reactor.

The point of molecular weakness (in the form of a cyclohexane ring) designed into ZSB2’s structure should allow for the occurrence of a number of reactions potentially generating the following free radicals which could propagate the surface reactivity [15]:

\[ AB + e \rightarrow AB^- \]

\[ AB + e \rightarrow AB^+ + 2e \]

\[ AB + e \rightarrow A^+ + B + 2e \]

\[ AB + e \rightarrow A + B + e \]

\[ AB + e \rightarrow A + B^- \]
Because of their unsatisfied bonds, these free radicals are usually the primary deposition reactants reacting with both one another and the substrate surface. An image of the plasma reactor in use is seen in Figure V-6.

![Plasma reactor](image)

**Figure V-6** Plasma reactor during a continuous wave duty cycle at 100W. The blue photons being generated are a characteristic of the gas in the chamber in this case oxygen.

**(b) Plasma parameters**

Several conditions can be altered in order to generate plasmas with properties which will affect the deposition process:

1. By altering the input RF, ion energy distribution in the plasma is altered, with a greater spread of ion energies at lower RF’s [16].
2. Changes in pressure will alter the interactions of particles with the surface as ion-neutral collisions in the plasma sheath become more prevalent. An increase in pressure leads to a wider distribution of ion energies and an overall decrease in mean ion energy [16].
3. Duty cycle is the length of time power is supplied to the reactor for. Commonly the duty cycle will be continuous wave where the power is supplied constantly. Other duty cycles would consist of power being supplied to the reactor for fractions of a second and then being switched off for fractions of a second at a specific ratio.
Control of the duty cycle is maintained through the use of an oscilloscope, (as per Figure V-4) which modulates the “on/off” time of the frequency generator. In an example from the following experiments, plasma was pulsed at a duty cycle of 1ms on 40ms off. Pulsing plasma changes the time averaged ion flux to the substrate surface [17]. Ion flux is generally increased with higher pulse rates.

4. Input power can be increased or decreased. Increasing the input wattage will increase the overall energy of the system generally increasing the number of reactions by increasing the electron and ion temperatures.

These last two points are important as whilst higher input energies generally create more reactive species, they also tend to generate more damage to the sample surface. By changing the duty cycle high energy plasmas containing highly reactive species can still be generated but the shorter “on” time means that total energy which the surface is exposed to is lower thus limiting damage to the surface [18]. This relationship can be described through the following:

\[
P_{eq} = P_{peak} \left( \frac{t_{on}}{t_{on} + t_{off}} \right)
\]

(23)

Where \(P_{eq}\) is the equivalent power, \(P_{peak}\) is the maximum input power and \(t_{on}\) and \(t_{off}\) are the on and off times of the RF generator. Controlling of such variables allows for a degree of tuning of properties of the deposited film.

(c) ICP deposition of ZSB2

Degree of ionisation and radical generation from plasma deposition processes were varied initially through the input power supplied to the reactor. This variation took the form of changes to both input wattage and duty cycle. Fabric sheets with a surface area of 200cm\(^2\) were dip coated in a 10mM solution of ZSB2 dissolved in methanol, prior to entering the reactor. Reaction times were 30 minutes regardless of input power. Four input duty cycles were initially tested: continuous, 40ms on 40ms off, 10ms on 40ms off, and 1ms on 40ms off, each at a power of 50 watts. In order to determine the antimicrobial activity of treated samples, a modified version of the Japanese industrial standard (JIS) assay for the antimicrobial activity of fabrics (JIS Z 2801) [19] was used (Chapter II). Circular
swatches, with a diameter of 3cm of treated fabrics, were inoculated with 1ml of bacterial culture of an OD600 of 0.001; equivalent to a concentration of $10^6$ CFU/ml. Samples were incubated at 37°C for 18 hours before quantification of attached cells was made.

![CFU counts of MSSA 476 and PA01 recovered from fabric plasma treated at 50 watts with 10mM of ZSB2 under varying duty cycles. Swatches were coated with ZSB2 and subsequently treated under 1 of 4 duty (X-axis legend gives on and off times for duty cycles). The counts represent bacterial colonies recovered from the fabric after an 18 hour period.](image)

CFU/ml counts of bacteria recovered from control untreated fabrics (no coating or plasma treatment) were less than a log greater than CFU/ml recovered from any of the treated fabrics from either organism. The greatest difference between control and treated samples was at a duty cycle of 1ms/40ms for both MSSA476 and PA01; differences were $1.22 \times 10^7$ and $9.79 \times 10^6$. While it at first may appear that treated fabrics demonstrated higher killing activity against *P. aeruginosa*, it must be noted that the difference between the two
organisms is likely negated by the differences in the controls. Both organisms show significantly lower CFU counts under the 1ms/40ms duty cycle when compared to any other duty cycle. This decrease in killing activity may be indicative of the lower energy cycle generating lower damage to the ZSB2 compound [20, 21].

The high initial concentration of ZSB2 on the fabric surface should have been sufficient to kill all organisms, considering the MIC of the compounds was determined using similar starting concentrations of cells. The partial loss of the antimicrobial activity of the compound presents several possibilities. Primarily, the plasma has the potential to disrupt the activity of compounds by destroying parts of the molecule. While the molecules were designed with a cyclohexene ring in place as a potential attachment/polymerisation site, too high an input energy may disrupt other parts of the molecule. It is also possible that the cyclohexene group is key to the antimicrobial functioning of the molecule and thus removal of this group through grafting lessens its activity. A third possibility is that the coating is not complete thus leaving surface area for organisms to colonise. A related concept would be that, cells initially killed at the fabric surface could create an insulating barrier against the compound’s effects.

In an attempt to rule out some of these possibilities, the experiment was repeated using a lower plasma duty cycle of 20 watts (Figure V-8).
Overall, a general decrease in colony counts can be seen when comparing the 50 and the 20 watt duty cycle. While the S.aureus control shows an increase, the P.aeruginosa control has shown a decrease in CFU in comparison the 50 watt experiment. Average differences between the controls and samples in the two experiments (shown in inset of Figure V-8) suggest that decreases in samples are greater for both organisms in the 20-watt sample, as compared to the control. The pattern seen whereby the 1ms/40ms sample demonstrated highest killing in the 50 watt experiment is absent from the 20 watt experiment. This may suggest that the previous result was an experimental artefact, or potentially that the decreased destruction of ZSB2 by the lower total power has been negated; in this case by a lower input power.
While levels of antimicrobial efficacy do seem to have increased as a result of decreased input power, the reduction is still not bacteriostatic; growth has been reduced, but not sufficiently so. This finding supports the possibility that power being supplied to the surface may be damaging the compound. Indeed, when individual circular swatches are treated under various conditions some damage in the form of changes in colouration is seen which appears to be dependent on input power (Figure V-9).

![Control](image)

![20 watts](image)

![50 watts](image)

![100 watts](image)

Figure V-9 Example of the visible appearance of ZSB2 treated samples after removal from the plasma reactor under different input powers. Rows of samples from top to bottom are an untreated control, samples treated at 20 watts, samples treated at 50 watts and samples treated at 100 watts. All plasma treatments were performed in triplicate under a continuous wave duty cycle for 30 minutes.

As such, it was decided that a different method of surface grafting would be attempted in an attempt to create a prototype with greater antimicrobial efficacy.

(d) Photo-initiated grafting

Surface grafting of the antimicrobial was next attempted through the use of photoinitiated radical grafting (PRG). In this process a photoinitiator is exposed to UV or visible light
and either yields free radicals through bond cleavage (type I photoinitiator), or is promoted to an excited state whereby it is free to react with another molecule, (a co-initiator) to generate the free radicals necessary to drive the reaction forward [22]. These reactive species can be part of either the compound to be grafted (graft to approach), or on the surface itself, (graft from approach). The generated species is then free to react with either the surface or the compound respectively forming covalent attachments.

Benzophenone was the chosen photoinitiator for the following experiments. As a type II photoinitiator the proposed mechanisms of graft initiation is through abstraction of hydrogen from polypropylene (the surface chosen as a dressing prototype). The resulting radical allows for the graft reaction to proceed [23] Figure V-10.

Figure V-10 Schematic of ketyl radical formation prior to grafting. Benzophenone is excited into its triplet state by photons at 254nm. The excited state benzophenone then abstracts hydrogen from the polypropylene surface to create a ketyl radical.

The absorbance spectrum of the photoinitiator is important when considering a graft reaction. Choosing a compound which absorbs more strongly in the UV region is useful in terms of limiting the graft reaction. The limited UV content of ambient light ensures that the reaction is unlikely to proceed outside of the confines of the experiment and so can be limited with relative ease. It is also important that there is limited cross over between the absorbance spectra of the photoinitiator and the graft compound or other components of the reaction mixture to ensure minimum competition which could unwoundly limit the reaction. The absorbance spectra of benzophenone are show in Figure V-11.
The $\lambda_{\text{max}}$ of benzophenone is 254nm in water the $\lambda_{\text{max}}$ of ZSB2 however is 362nm. This provides a good degree of separation (108nm) especially considering the major component of the UV flood lamp used is at 254nm.

The solvent in which grafting experiments are performed should also be considered due to slovachromatic effects. Increases in solvent polarity have a tendency to red shift the $\lambda_{\text{max}}$ of benzophenones $\pi$ to $\pi^*$ transition thus allowing for hydrogen abstraction at longer wavelengths closer to visible portion of the UV spectrum. This allows for both tuning of which part of the spectrum grafting is performed in, but also (if using a fixed light source with a consistent spectral output), control over reaction rate by solvent mixture modification [24]. Like other experiments performed, process involved in UV grafting are complex, often more so than they at first appear and proper consideration of these processes is often required to fully comprehend experimental outputs.
Prior to UV exposure fabrics were coated with a mixture of the compound monomer (ZSB2), and a grafting initiator (BP), dissolved in methanol at concentrations of 10mM and 100µM respectively. Grafting was performed using a 400 watt UV flood lamp over a 5 minute period, with a total energy reaching the surface of ~7J/cm². All measurements were performed in a positive pressure nitrogen flood tank. JIS measurements were performed as previously described.

Initial experiments demonstrated complete killing of both *P. aeruginosa* and *S. aureus*, with no cells recovered after a single JIS experiment. To ensure that the level of antimicrobial activity found was not a result of the benzophenone, rather than the ZSB2, a control sample of fabric which had been treated with BP but without ZSB2 was also included; growth comparable with the control was observed.

Such a result seems to indicate that the plasma treatment was indeed too harsh, and as such, was damaging the compound. As bacterial killing was so complete, attempts were made to remove unbound/ungrafted compound from the fabric surface. Due to the high initial concentration of ZSB2 used, it was likely that some of the compound was physadsorbed, rather than bound to the surface.

One of two wash treatments was used to remove unbound compound. The more gentle wash cycle consisted of soaking fabric swatches in sterile PBS containing 2% DMSO for 10 minutes before being rinsed in fresh PBS and dried. The harsher wash cycle involved sonicating coated swatches in methanol for 10 minutes and subsequently rinsing in fresh methanol and then rinsing in PBS 2% DMSO before drying. In an attempt to distinguish between physadsorption and UV grafting with more clarity, swatches which had been dip coated in the ZSB2/BP solution, but had not undergone any UV treatment, were also involved in the assay.
Absence of colony forming units recovered from the fabric after gentle wash cycles demonstrates that 2%DMSO included in the PBS wash is not sufficient to re-dissolve the physisorbed compound from the fabric surface (red crosses Figure V-12). By comparison, sonication in methanol seems to remove almost all antimicrobial activity. In the case of the sample that had not been UV treated, bacterial counts return to the level associated with the control sample, as expected. The UV treated samples lose much of their antimicrobial activity in both cases; however in each case bacterial counts fall outside of the SEM ranges of their control sample counterparts. This result was not entirely expected, as while a certain amount of the surface coating was seen to re-solubilise from the fabric surface upon methanol washing, fabrics still retained the characteristic yellow colour of the ZSB2 unbound monomer (Figure V-13).
There is an obvious possibility that yellowing of the fabric in this manner is not due to bound ZSB2 but rather a result of photo-degradation and yellowing of the polypropylene surface [25]. In an attempt to determine if the decreased antibacterial efficacy of the washed fabrics was caused by loss of compound above the MIC, or other factors such as damage to the compound through grafting, several analytical methods were employed to examine the UV treated washed surfaces.

Section 5.04  Determination of ZSB2 attachment

Several surface analytical methods were employed in an effort to both detect and quantify the surface bound compound in some respect. These included Attenuated Total Internal Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy, and fluorescence spectroscopy.

(a)  ATR-FTIR examination of ZSB2 treated fabrics
Swatches of fabric from all UV treatments (UV treated and un-treated, and both washes), were analysed by FT-IR in order to determine the treatments effect on the chemical structure of either the fabric or the compound. The 3 plots in Figure VI-8 show the FT-IR spectra of the non-woven polypropylene fabric, the ZSB compound, and polypropylene fabric coated with ZSB and treated under a 1/40 duty cycle at 100W.
Figure V-14 FT-IR spectra of UV-grafted and non-grafted samples of ZSB2 treated polypropylene (A) FT-IR spectra of Non-Woven polypropylene fabric, (B) FT-IR of polypropylene fabric dip coated in ZSB and subsequently UV treated for 5 minutes with 10mmol dm$^{-3}$ ZSB2 and 100µmol dm$^{-3}$ BP , (C) FT-IR of UV treated sample (B), after the harsh wash step of methanol sonication.

Figure V-14 (A) shows the spectra of untreated polypropylene fabric. The two peaks at 2918 cm$^{-1}$ and 2852 cm$^{-1}$ fall into the alkane C-H stretch region (2950-2850 cm$^{-1}$), and are likely associated with the methyl and propyl C-H bonds on the polypropylene chain. The peak at 1463 cm$^{-1}$ falls neatly into the C-H bend/scissor region, (1470-1450 cm$^{-1}$). The peak at 1377 cm$^{-1}$ falls slightly outside of the expected C-H rock region expected of methyl groups (1370-1350 cm$^{-1}$); however this is not likely to be significant especially as the peak is sharp. The final peak at 720 cm$^{-1}$ can be associated with the C-H methyl rock region associated with long chain alkenes (725-720).

Figure V-14 (B) is the spectra from ZSB2; UV treated polypropylene prior to methanol washing. The compound shows a complex fingerprint region, however bands corresponding to several key functional groups have been highlighted. A peak at 1622 cm$^{-1}$ corresponds well to the aromatic C=C stretch at around 1625 cm$^{-1}$. The peak at 1599 lies within a C=N stretch ranging from around 1600-1700 cm$^{-1}$, while the peak at 1291 cm$^{-1}$ falls into the C-O stretch between 1320-1210 cm$^{-1}$. The peak seen at 766 cm$^{-1}$ falls into the
band recognised for C-H bending in disubstituted 1,2 (ortho), aromatic rings, (770-735 cm⁻¹). Unfortunately when viewing UV treated Vs non-UV treated samples coated with ZSB2, no difference can be observed in the 1625 cm⁻¹ C=C stretch, likely due to multiple bonds of this type existing within the compound. On a more positive note, no evidence of the hydroperoxide groups (POOH) was found, nor density increases in the small 997 cm⁻¹ and 992 cm⁻¹ peaks, which are commonly associated with UV induced damage are seen [25].

Figure V-14 (C) shows the spectra for the same sample as (B), but after the methanol wash step. All of the C-H regions observed in the untreated polypropylene can be recognised, however no additional bands are present. This would suggest that either there is no coating remaining on the surface after the UV treatment, or that the coating is present at a level that cannot be detected by FT-IR. As already established, the latent low-level antimicrobial activity seen after such wash steps, and the intrinsic colour difference between the treated and untreated samples, suggests that compound does still remain on the surface. The lack of detection may be due to masking by the abundantly strong polypropylene signals. Alternatively, it could be due to the technique probing too far into the sample [26].

(b) Fluorescence of UV grafted ZSB
Examination of ZSB2 coated materials under UV light demonstrated that the compound exhibits blue green luminescence; an effect which likely relates to high levels of conjugation throughout the compound structure. Interestingly, the ligand does not demonstrate the luminescent effect on its own; luminescence is only seen upon interaction with the metal. Similar ligand metal specific luminescence effects have been observed in other compounds, and are proposed to be generated by structural rearrangements of the molecule upon metal binding [27].
Figure V-15 Luminescent properties of ZSB2. (A) 10µmol dm$^{-3}$ compound under normal lab lighting (B) 10µl compound under UV lamp with major component at 354nm.

It was postulated that level of luminescence displayed by the deposited compound would allow for a semi-quantitative determination of how UV grafting might affect the compound structure (given that the ligand on its own shows no luminescent behaviour). It would therefore also provide the potential to determine differences between UV treatment types. The fluorescence profile of ZSB2 was measured and is shown below.
Figure V-16 The excitation and emission spectra of ZSB2. Spectra were determined using a Perkin Elmer LS50 B Luminescence Spectrometer. A solvated form of the compound, (in methanol), was scanned across the visible spectrum first for excitation peaks. A peak at 387nm was chosen and the compound was subsequently scanned again revealing the emission peak at 487nm.

The compound showed an excitation peak at 387nm and a corresponding emission peak at 487nm. The large stoke shift seen (100nm), may be indicative of phosphorescence rather than fluorescence. For this study the large shift is useful in the semi-quantitative efforts undertaken.

4 sheets of coated fabric were treated using 5 different UV treatment cycles, based on BP pre-treatments to activate the fabric. All cycles were five minutes in length but varied as to when the 10mM ZSB2 solution was added. Pre-treatments with BP 100µmol dm$^{-3}$ in methanol were made for 0, 1, 2, 3, and 4 minutes, before the addition of the ZSB2 solution. After treatment, all samples were left under nitrogen for half an hour before undergoing methanol washing. 3 circular swatches were cut from each UV treated sheet. Each swatch was placed in a 6 well plate and subsequently scanned in a 6×6 matrix. The gain for the well scanning assay was auto set at 1675.
Figure V-17: Example of relative fluorescence of swatches of ZSB2 coated treated fabric after different UV treatment times. Fabrics were either not pre-treated (A) pre-treated for 1 minute (B), 2 minutes (C), three minutes (D) of four minutes after which ZSB2 was added and treatment continued for a full five minute period. Fluorescence was measured using a BMG LABTECH FLUOstar Omega plate reader. High levels of fluorescence are indicated by colours close to the red end of the scale whilst colours close to the purple end indicate low levels of fluorescence.

Variation in the fluorescence intensity can be seen both between the swatches with different pre-treatment conditions, and within individual swatches (Figure V-17). Interestingly, the maximum and minimum emissions seen within a particular pre-treatment group are not confined to individual wells; the peaks and troughs of emission can be seen in more than one well for each duty cycle. This would suggest localized changes in grafting conditions, rather than changes over the whole sheet. These localized changes may be due to small-scale difference in UV exposure, or due to changes in the fabric’s density in such areas. Changes in density can be clearly seen when coated fabric is observed under a U.V. source (Figure VI-12).
Figure V-18 Non-woven polypropylene fabric coated with a 10mM solution of ZSB2 dissolved in methanol. The fabric sheet has been exposed to U.V. light at 354nm. The dissolved compound was allowed to dry to the fabric before the image was taken.

The fluorescence of the compound coated on to the fabric demonstrates clear striations down the fabric length. These physical anomalies are likely due to density variations with in the fabric weave caused by changes in pressure of the rolling mechanism used in manufacture. Significant differences in fluorescence intensities were also seen between the duty cycles when measurements by the dot matrix process were made.
Average fluorescence intensities generated from fabric samples treated with BP and ZSB2 after different graft cycles. Grafting with BP was performed for either; 0, 1, 2, 3, or 4 minutes, before the addition of a 10mmol dm$^{-3}$ ZSB2 solution. Intensities are representative of averages of well scans of three replicates after methanol washing. Gain was set to 1675. The control shown is fabric treated with BP for 5 minutes without the addition of ZSB2.

A decent correlation was observed in this study between pre-treatment time and retention of fluorescence (Figure V-19). The highest levels are seen in swatches treated for three minutes prior to adding the ZSB2 methanol component. A 2-minute pre-treatment gives the next highest value (around 50000 AU lower), while 0, 1, and 4 minutes pre-treatment give approximately similar values of around 100000. The control samples (non-UV treated fabric) show fluorescence around 50000. The peak and then decline in fluorescence values after 3 minutes may suggest that activation of the polypropylene surface (presumably by hydrogen abstraction) is required for grafting to occur. Alternatively, the time constraint on grafting may be a result of BP damaging the compound, if allowed to react over the whole five-minute period. While this second theory is marginally backed up by the significant difference in SEMs between the 0 and 4 minute
pre-treatments, it seems unlikely that the 4 minute pre-treatment (likely low due to insufficient grafting time) would show the same level of fluorescence as a damaged sample. A further possibility is that high levels of fluorescence may indicate high levels of structure retention, with lower levels indicating highly UV modified ZSB2, and therefore greater surface grafting and lower retention of original structure. This again seems unlikely mainly due to the fairly substantial wash cycles used before measurements were made, and the lack of fluorescence retention in the control.

In order to determine if alterations in pre-treatment time and increased fluorescence result in greater antimicrobial efficacy, a further JIS was performed using three-minute pre-treatment ZSB2 grafted samples. These were then either washed in methanol prior to use, or washed in Saline 2% DMSO.

Figure V-20 JIS of fabric, UV grafted with ZSB2 under optimised conditions. Optimisation consisted of a three minute pre-treatment with 100 µmol dm$^{-3}$ BP methanol solution, followed by two minutes in the presence of 10mM ZSB2 also in methanol.
Similar results were seen as to un-optimised conditions (Figure V-20). While the gentle wash cycle again showed complete killing, no reduction was seen in this occasion between the harsh methanol washed sample and the control. This leads to two possibilities; either the fluorescence and the colour retained on the fabric is a result of relatively low levels of the ZSB2 compound in comparison to the MIC for either organisms, or, the bound compound shows low or no antimicrobial efficacy and the antimicrobial action seen is a result of total or partial solvation of the unbound coating.

(c) SEM and EDX examination of UV treated polypropylene
Further investigations were made of the homogeneity of the coating on the surface using SEM to visualise surface changes, and EDX to probe the fabrics for zinc. SEM investigations into changes in the fabric were performed on samples after UV, no UV, both gentle and harsh wash cycles, and also on control fabrics (Figure V-21).
Observations of the control polypropylene fibres (Figure V-21 (A)) show a smooth relatively featureless surface that has small imperfections likely generated during manufacture. The ZSB2 UV grafted but unwashed polypropylene (Figure V-21 (B)) shows a much more highly textured surface. The coating can clearly be seen in the lower left hand portion of the image; it is interesting to note that the coating seen in this image is highly heterogeneous, and appears to be much thicker in some locations than in others. ZSB2 UV treated and methanol washed polypropylene (Figure V-21 (C)), shows inconsistencies and pitting on the fibre surface in some (but not all) locations. It is possible that the dark “pits” are actually localised areas of ZSB2 grafting. The non-UV treated methanol washed fabric shows a virtual return to the level of smoothness shown from the control fibre. Some surface variations do now appear to be present and may be due to UV damage generated during the grafting process.
EDX measurements were also performed using the microscope to probe the surface for its zinc content.

Figure V-22 EDX spectra of ZSB2 treated samples from select locations along sample fibres. (A) ZSB2 coated (no UV treatment) and methanol washed polypropylene sample. (B) ZSB2 coated and UV treated sample after methanol washing. Data was obtained using a JEOL SEM6480LV scanning electron microscope paired with an Oxford INCA X-ray analyser.

Data from EDX spectra of non-UV treated methanol washed fabrics (Figure V-22 (A)) consistently demonstrated a single large carbon peak; indicative of the composition of the polypropylene itself. Occasional chlorine and calcium peaks were also observed; however these were attributed to remnant constituents of PBS. In the UV treated samples, the “pit” like structures observed in SEM images (Figure V-21(C)), were interrogated for the presence of ZSB. Figure V-22 (B) is an example of the spectra found from the examination of one of these pits. Peaks for zinc, and for oxygen, can both be seen. The presence of zinc is almost undoubtedly due to the ZSB2, the oxygen peaks however may
either be due to the compound, remnants of BP or possibly as a result of the UV treatment itself. It should be noted that the appearance of zinc peaks was not ubiquitous under analysis of these pits; peaks were only seen in 3 of 10 examined. Zinc was however also found elsewhere on the fibre surface un-associated with any visible physical structures.

This data along with the fluorescence data suggests the possibility of a very thin layer of ZSB2 associated with the polypropylene surface. The compound could potentially be chemically bonded, or physadsorbed, to the surface. If the coating is very thin, this may suggest why it could not be picked up by ATR-FTIR, which can probe much further into the sample surface than EDX tends to [28, 29]. All in all, the data presented suggests a diffuse thin coating of ZSB2 after UV grafting.

Section 5.05 Close examination of UV grafted ZSB2 antimicrobial efficacy

Data shown up to this point does not definitively establish whether the bound compound is too low in quantity to generate the desired antimicrobial efficacy, or whether the bound compound is simply ineffective as an antimicrobial. It also suggests that antimicrobial efficacy of unwashed samples is likely due to the unbound bulk of ZSB2 on the polymer surface. Due to the low water solubility of the compound, several studies were set up to determine if the antimicrobial efficacy of the compound seen in Figure V-12 and Figure V-20 was due to surface killing of organisms or partially solubilised monomer.

(a) Live/Dead Staining of ZSB2 treated and untreated fabrics

Live/Dead staining was used to determine the effects of treated and untreated fabrics on bacterial adhesion and killing. Fabrics which had been UV grafted with ZSB2 were exposed to either the gentle or harsh wash cycle before inoculation, using bacterial isolates. A positive and negative control was also included. The positive control was a sterile sample of PBS washed polypropylene fabric, and the negative control consisted of a similar sample that was dipped in 70% ethanol two hours after inoculation. All samples were inoculated with a bacterial culture (either MSSA or PAO1) of $10^6$ CFU/ml, as per the JIS studies. The inoculated swatches were incubated at 37°C for 4 hours before being washed, to remove interstitial culture, and stained with Invitrogen’s LIVE/DEAD BacLight.
L13152 stain. Stained fabrics were left for an hour before a further wash step and subsequent visualisation by confocal epifluorescence microscopy. Micrographs are show in Figure V-23 and Figure V-24 and are representative of multiple images from three separate samples. Green fluorescence represents live cells whereas red fluorescence represents dead cells.

Figure V-23 Confocal fluorescence micrographs of live/dead staining of fabric swatches inoculated with *S.aureus* MSSA 476 (A) Uncoated fabric (positive control) (B) uncoated fabric treated with 70% ethanol 1 hour post incubation (negative control, (C) ZSB2 coated fabric UV treated under 3 minute pre-treatment cycle washed in saline 2%DMSO D) ZSB2 coated fabric UV treated under 3 minute pre-treatment cycle washed in methanol and PBS prior to inoculation. All swatches were incubated for 5hrs before staining. Images were taken using a Nikon eclipse 90i confocal epifluorescence microscope. All micrographs were taken at 200x magnification.
Fabric in panel (A), (Figure V-23), shows uncoated fabric inoculated with MSSA. The small green spots seen on the fabric fibres are likely to be clusters of bacterial cells or micro-colonies. As we are attempting to test if treated fabric shows killing efficacy on the fabric surface, the ability of cells to grow on this control is important. The cell density on the untreated swatch can also be used comparatively with other samples. The fabric in panel (B) was treated with 70% ethanol two hours post inoculation. The small red dots are clusters of dead cells attached to the surface of the fibres. The number of colonies seen on the fibre is relatively few, and clusters of colonies do not appear. This most likely due to the short colonisation and attachment time allowed by the 70% ethanol treatment. Panel C shows a fabric swatch coated with ZSB2 and treated by UV grafting. Very little fluorescence can be seen on the fibres. Any fluorescence that is found seems to represent small isolated colonies or clusters. This may be indicative of bacterial binding to occluded sites within the fibres and subsequent growth protected from the antimicrobial ZSB2. The distinct lack of dead cell staining on these fibres is also interesting, as it suggests very limited binding of bacteria to the fabric in any form. This may be in keeping with results from the JIS where swatches that had undergone gentle wash cycles retained antimicrobial activity. The lack of bound bacteria on the surface then is suggestive of killing in solution, which would remove the opportunity to attach to fabric. Panel D shows ZSB2 treated fabric which was methanol washed prior to inoculation. Numerous clusters of live cells on the surface suggest the majority of the fabric’s antimicrobial efficacy has been lost, despite the latent fluorescence exhibited in well scanning experiments.
Figure V-24 Confocal fluorescence micrographs of live/dead staining of fabric swatches inoculated with *P. aeruginosa* PA01 (A) Uncoated fabric (positive control) (B) uncoated fabric treated with 70% ethanol 1 hour post incubation (negative control. (C) ZSB2 coated fabric UV treated under 3 minute pre-treatment cycle washed in saline 2%DMSO D) ZSB2 coated fabric UV treated under 3 minute pre-treatment cycle washed in methanol and PBS prior to inoculation. All swatches were incubated for 5hrs before staining. Images were taken using a Nikon eclipse 90i confocal epifluorescence microscope. All micrographs were taken at 200x magnification.

Figure V-24 shows treated and untreated fabric swatches that have been inoculated with *P. aeruginosa* PA01. Like Figure V-23, panels A and B in Figure V-24 again show fabric swatches, which are uncoated and uncoated 70% ethanol, treated respectively. As expected, high levels of growth are seen on the untreated fabric, and a number of smaller dead colonies are seen on the swatch treated with ethanol prior to staining. There does seem to be a higher level of background fluorescence on both of the swatches in comparison to the fluorescence seen in panels A and B in Figure V-23, despite the same gain being used. This is may be due to PAO1’s strong biofilm forming behaviour and
often mucoidy phenotypes [30]. Panel C (ZSB2 coated fabric UV grafted on the 3 minute pre-treatment cycle) again appears similar to its respective counterpart in Figure V-23, demonstrating low growth and limited overall attachment. Fibres in panel D, which were treated in the same manner as the swatch in panel C (aside from the methanol treatment prior to inoculation), show similar growth to their *S.aureus* counterpart in Figure V-23.

The micrographs collectively demonstrate very limited attachment of organisms to treated but gently washed fibres, suggesting that the antimicrobial action of the ZSB2 compound may be occurring away from the treated surface. The lack of difference between the positive control swatches and treated swatches which underwent methanol washing suggests that any compound bound to the fabric has lost antimicrobial activity. While it is also possible that the remaining bound compound is merely too low to generate large scale reductions in the number of *S.aureus* or *P.aeruginosa* cells present, some reduction in live bacterial numbers or an increase in dead cells would still be expected close to the fabric surface. As such the lack of difference is likely indicative of the former.

As ZSB2 presents low water solubility, it was decided to investigate leaching of the compound from UV grafted ZSB2 fabrics which had been exposed only to the gentle wash step.

**Section 5.06 ZSB2 leaching from UV grafted surfaces**

In order to determine the loss of ZSB2 from the fabric in a physiological environment, a method of determining leaching of compound from the fabric was devised. Swatches of fabric UV treated under four of the five pre-treatment conditions (0 minutes pre-treatment was excluded), were immersed in 10ml of PBS, and placed in a shaking incubator for 5 days. Samples of the saline solution were removed at 24hr intervals, and their absorbance was measured. In order to determine the concentration of ZSB in the saline solutions, a standard had to first be determined as shown in Figure V-25.
The absorption profile of ZSB2 concentrations in 0.9% saline containing 2% DMSO, absorption spectra was blanked against a saline DMSO solution. Values below the limits of detection and quantification were included to demonstrate the cut off level of ZSB2 detection.

$\lambda_{\text{max}}$ of ZSB2 of 387nm was used to generate the absorption profile of the compound. A line of best fit was introduced with a good $R^2$ value. The gradient of the line was determined as was the y intercept. These two along with the value of y (OD$_{380}$nm), were used to determine the unknown concentration of the solutions in which the swatches were soaked.

Fabric from each UV treatment cycle was measured in triplicate for two separate depositions; 6 swatches in total for each duty cycle. The average change in absorbance, over the 5 day period for all duty cycles, is shown in Figure VI-20.
The change in concentration of ZSB2 present in soaking medium of swatches over a 5 day period. UV grafted ZSB2 fabric swatches and a control swatch were washed under gentle cycle conditions post grafting. Swatches were then transferred to falcon tubes containing 10ml of PBS. Absorbance measurement of 200µl of soaking medium was made at 387nm each day of a five day period. Absorbance values were used to calculate concentration of ZSB2 in the medium using the equation from Figure V-25.

All samples showed high levels of cross over on all days suggesting no dependence in the levels of leaching on UV treatment cycle. The majority of the samples are above the LOD but may stray slightly under the LOQ, suggesting that values may not be particularly accurate. The lack of differences over the total time period in any of the samples indicates that any leaching which occurs does so rapidly within the first day. The low overall concentrations of the samples (≈55µmol dm\(^{-3}\) to ≈70µmol dm\(^{-3}\)) are much lower than the MIC values for either organism. However, when values are adjusted for dilution between the JIS volume (1ml) and the 10ml used in this experiment, concentrations would be in the range of ≈550µmol dm\(^{-3}\) to ≈700µmol dm\(^{-3}\) which are above the MIC values for both \textit{P.aeruginosa} PA01 and \textit{S.aureus} MSSA476.

Whilst the majority of the evidence now heavily leans towards bound ZSB2 not having the same (if any) antimicrobial activity as its monomer form counterpart, the reason behind the loss of activity is still not understood. Changes in ZSB2s activity could be put down to
structural changes to the compound on grafting and subsequent destruction of the compounds “active site”; destruction of the compound on grafting to the surface, or that surface binding occludes the antimicrobial activity of the molecule in some way. Complete destruction of the molecule can almost certainly be ruled out due to retention of fluorescence on surface grafting. Changes in the unbound monomer after a grafting treatment can be discounted with some confidence due to no spectral shift being seen between unbound monomer from UV treated and non-UV treated leachate (data not shown). This suggests that surface attachment is occluding the antimicrobials molecular mechanism in some manner.

Section 5.07 Detection of mode of action of ZSB2 in solution using a zinc biosensor

It was postulated that antimicrobial activity of ZSB2 in solution might be caused by transmetallation of the zinc centre, either intra or extracellularly, with zinc being exchanged for other protein bound metals vital to cellular function and thus disrupting their activity. A “lights on” zinc biosensor consisting of an E.coli MC1061 strain containing a zinc reporter plasmid (pSLzntR/pDNPzntAlux) was used to determine if exposure to ZSB2 resulted in increased levels of free intracellular zinc [31]. By comparing luminescence values gained from exposing the zinc sensor to free zinc, ZSB2, and its ligand, it should be possible to determine if zinc is being released from the Schiff base complex. Preliminary work was performed to determine the MIC of ZSB2, the SB2 ligand, and zinc acetate against the E.coli strain used.

Table V-2 MIC\textsubscript{50} values for zinc acetate, ZSB2 and SB2 Schiff base ligand when challenged against E.coli MC1061. Values were obtained by plotting the area under the growth curve (as measured by optical density at 600nm), against treatment concentration of the compound in question.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC\textsubscript{50} µmol dm\textsuperscript{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZSB2</td>
<td>325</td>
</tr>
<tr>
<td>SB2 ligand</td>
<td>447</td>
</tr>
<tr>
<td>Zinc acetate</td>
<td>860</td>
</tr>
</tbody>
</table>
As can be seen, the MIC$_{50}$ for ZSB2 and its ligand are considerably lower than the value for zinc acetate. As such a concentration range was chosen from 50-500µmol dm$^{-3}$ of all three compounds in order to demonstrate zinc response without the associated antimicrobial effect. Zinc free cultures of both organisms were exposed to the compounds at 37°C for four hours before luminescence measurements were taken (Figure V-27).
Figure V-27 Response of zinc biosensor and luminescence control to zinc acetate, ZSB2 and SB ligand. (A) Luminescence response of zinc biosensor E.coli MC1061 (pSLzntR/pDNPzntAlux) to zinc acetate, zinc Schiff compound and Schiff base ligand. (B) luminescence response of control organism E.coli MC1061 (pSLux) to zinc acetate, zinc Schiff compound and Schiff base ligand.

Exposure of the zinc biosensor (E.coli MC1061 (pSLzntR/pDNPzntAlux) to zinc acetate (Figure V-27), shows an increase in luminescence over the entire 50-500 µmol dm\(^{-3}\).
concentration range. A similar increase is seen, with error overlap at multiple points, within the same concentration range of ZSB2. The SB2 ligand shows lower level luminescence with no increase over the concentration range. The mirroring of luminescence increase between zinc acetate and ZSB2 suggest that in both cases the organism is exposed to similar concentrations of zinc. This suggests that zinc is released from the ZSB2 compound intra-cellularly as hypothesised. The lack of luminescence response of the biosensor to the SB2 ligand indicates that zinc is indeed the cause of the increase and not any extraneous factor.

Turning to the control organism which constitutively expresses the lux operon (expression of the luxCDABE genes controlled under the lac promoter), we see a general decrease in luminescence with increasing compound concentration across all three compounds. Both ZSB2 and SB2 show lower level luminescence across all concentrations. At the highest concentration of all three compounds, luminescence is highest in the zinc acetate treated sample, and lowest in the ZSB2 treated sample. These factors suggest that the cross board decrease in luminescence of the control is likely due to antimicrobial activity of the three compounds on the control organism.

Only a slight decrease in luminescence is seen between zinc acetate and ZSB2 at the highest concentration of both compounds when used to challenge the zinc biosensor. The lack of overall decrease in luminescence, equivalent to that seen from the control organism, is likely caused by the antimicrobial effects the compounds may be have being outweighed by the much larger response to zinc.

Section 5.08 Conclusion

The work highlighted in this chapter shows the development of a novel organometallic compound with antimicrobial activity as a surface coating agent for polypropylene fabrics. Both plasma deposition and UV grafting were investigated as methods to attach the compound to the surface. Work with plasma deposition suggests that the high energies involved in the process may be damaging to the coating. While UV grafting at first seemed more successful, it was determined that the active compound was not chemically bonded to the polypropylene surface. Subsequent investigations have revealed that while
some of the compound may remain bound to the surface after UV grafting surface attachment, and antimicrobial efficacy in this case are likely mutually exclusive. Investigation into the compounds mode of action using a zinc biosensor suggests that zinc may be released from the compound upon entering a bacterial cell. The compound therefore may be acting as a carrier molecule.

While the goal of developing an antimicrobial surface was not achieved in this study, the individual goals of generating an antimicrobial and surface attachment, to some extent were met. As such, this study is not a complete loss, and may allow for the development of other compounds with varying modes of action. Additionally such methods of surface grafting may hold promise with the use of different substrate materials especially considering polypropylenes relatively unreactive nature.

Section 5.09 References

27. Kim KB, Kim H, Song EJ, Kim S, Noh I, Kim C: A cap-type Schiff base acting as a fluorescence sensor for zinc(ii) and a colorimetric sensor for iron(ii), copper(ii), and zinc(ii) in aqueous media. Dalton Transactions 2013.


31. Ivask A, Rolova T, Kahru A: **A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing.** *BMC biotechnology* 2009, **9**:41.
Chapter VI. Conclusion and future directions

Section 6.01 Summary

The initial aims of this body of work were to generate methods of detecting and to some extent treating a bacterial infection within a paediatric burn wound. The potential for the first part of this goal has been realised in the modification and close examination of a dye encapsulating lipid vesicle system, which has previously been used to investigate lipid protein interactions.

In investigating the susceptibility of vesicles to various pathogenic bacteria two main organisms were identified to which this sensor demonstrates broad applicability. The first of these was *Staphylococcus aureus* to which the bilayer based sensor demonstrates sensitivity against a broad range of clinical isolates, >80% of those investigated. The molecular mechanism of sensing in *S. aureus* was identified as the quorum response associated PSM’s and delta haemolysin. This is important both in terms of the highly conserved nature of *S. aureus* AGR quorum sensing system [1] but also in the redundancy it provides. While the PSMs and delta haemolysin are both regulated by the AGR component AgrA, they are not regulated under the same operon [2].

Investigation into activity of individual PSMs showed high levels of sensitivity across the board when compared to the lytic potential of the toxins against T-cells. Vesicles were also demonstrated to show sensitivity between the PSMs. This may provide the potential for tuning to sense individual toxins or toxin groups. The possibility of sensor tuning was expanded on further by examining the effect of both variable cholesterol concentrations and variable lipid acyl chain lengths on sensor activity. Varying bilayer cholesterol demonstrated a requirement across both the αPSMs and delta haemolysin for bilayer cholesterol concentrations that are neither very high (>40%), nor very low (<10%). These experiments also provided some insight into the structure function relationship between the molecules. This is based on the much larger and less hydrophobic βPSMs preference for low cholesterol bilayers. The βPSMs have often been thought to show less lytic activity than the αPSMs[3] mainly holding a role in inciting immune responses through their activity on the formyl peptide receptor [4]. This evidence coupled with other recent reports of the βPSMs activity at low bilayer cholesterol concentrations [5] may suggest a
role for βPSMs against cell types showing cholesterol depletion. These molecules also showed interesting variation and patterns of activity against varying acyl chains. Reduced activity against shorter acyl chain lipids, which are typically thought of as less stable provides a further possibility for tuning to exclude one toxin type while potentially increasing sensitivity to another.

The demonstration of vesicle sensitivity to rhamnolipids produced by \textit{P.aeruginosa} also provides some interesting possibilities not least of which is the pattern of rhamnolipid production seen between chronic and acute wound isolates. In this regard the vesicle system may have some role to play in the discrimination of infection type within an organism. Whilst the relatively low sensitivity of the vesicle system to rhamnolipid in comparison to T-cells is disappointing variation in bilayer formation may prove to be a solution to this problem. Some initial work has been performed to this extent as can be seen from below.

As well as the current clinical aims of this project, a vesicle based sensor system such as this may fill other types of diagnostic role. For example the vesicles have already shown potential in discriminating between AGR positive and negative strains and may therefore provide as a rapid screening tool for AGR dysfunction \cite{6}. In the case of rhamnolipid the vesicle system may prove useful as semi quantitative tool for the comparison of biosurfactant production between strains as well as a means to distinguish between organisms more likely to cause chronic as compared to acute infections.

The similarity in mechanism of action of the two toxins from the two separate organisms may also be useful. The fact that in both cases it was small amphipathic molecules that demonstrated lytic activity against the vesicles provides a marker to look for when screening other organisms. Such a sensor system could also potentially have an entirely lab-based function in assessing the lipid active compounds produced by various bacterial species. Such a system could be envisioned to work through the exposure of vesicles of various selective bilayer compositions to bacterial supernatant.

Work on the ZSB2 compound also provided some interesting if not slightly disappointing results. The fact that the compound could be attached to the fabric surface as predicted suggests a starting point for other potential ligand systems. This coupled with the fluorescence switch on seen upon zinc binding may provide a rather esoteric role for a molecule which failed as a surface bound antimicrobial as a surface bound zinc sensor.
The seeming carrier function of the Schiff base ligand also has some potential as an agent to bypass bacterial defences against attack by metal ions.

Section 6.02 Future directions

Through the work performed thus far, several key lines of investigation have been identified which will need to be considered should the work presented here be taken any further.

(a) Vesicles as sensors for Rhamnolipid

In regards to the vesicle systems sensitivity to the biosurfactant rhamnolipid produced by *P. aeruginosa*, data needs to be accumulated on the varying sensitivity of differing bilayers to these molecules. Some preliminary experiments were carried out looking to determine how vesicle sensitivity to commercially available rhamnolipid varies dependant on cholesterol concentration. However while this data could potentially be useful it should be noted that the rhamnolipid used is isolated from *P. aeruginosa* PA01 and is likely a mix of different RL congeners. Good understanding of the different congeners produced by different bacterial strains, how prevalent each congener is and knowledge of how bilayer active different congeners are would allow for further progress in the use of this vesicle system as a sensor for the detection of *P. aeruginosa*.

(b) Application of vesicles as a staphylococcal sensor in clinical settings.

While the studies described have demonstrated that the developed vesicle system is highly applicable to detection of *Staphylococcus aureus* in a lab setting, the ability to identify it in a clinical setting has not been established.

Several questions about the systems usefulness in a medical environment are key. Firstly is the stability of the system in such an environment. As a preliminary test vesicles have been exposed to pooled human serum to determine if they are stable. The test was performed over a 14 day period as this was established to be a likely time period for a dressing to be in place in the case of paediatric burns.
Results of this test demonstrated that while vesicles were stable in serum over this time period they showed reduced sensitivity to both Staphylococcal PSMs and the positive lysis control triton X-100. While this data is useful as a starting point exposure of the vesicles to complement activated human serum may be a better test of the systems applicability. At a future date prototypes of a dressing system containing this technology will also need to be assessed as to their proper functioning and how well the system survives normal wear and tear. Such testing of prototypes however may be beyond the remit of this study.

As well as how well the system survives contact with human serum, plasma or wound exudate, an assessment of correct functioning when under these conditions must be made. Such an initial investigation have been made by exposing the vesicle system to bacterial supernatant in the presence of wound exudate/serum collected from intact blisters of burn patients at the children’s burn unit, Frenchay hospital in Bristol (Figure VI-1).

![Figure VI-1 The response of DPPC53 vesicles to bacterial supernatant in the presence of varying concentrations of human serum. The plot shows the fluorescence response of vesicles exposed to 100µl of supernatant from 18 hour cultures of either S.aureus LAC or MW2 in the presence of varying concentrations of exudate extracted from intact blisters of burn patients.](image)

As can be seen above (Figure VI-1), the fluorescence response of vesicles decreases with increasing concentrations of supernatant. Further experiments demonstrated that the
fluorescence of the dye 5(6)-carboxyfluorescein was not affected by the inclusion of serum. As the experiment was performed using supernatant rather than actively growing bacteria the result demonstrated an interaction between exudate and the PSMs. A search of recent literature uncovered a paper which demonstrated that the PSMs are sequestered by lipoproteins found in blood and serum [7]. This was confirmed through exposure of DPPC53 vesicles to a range of synthetic toxin concentrations in the presence of high-density lipoprotein (HDL), (Figure VI-2).

![Graph A](image1)

![Graph B](image2)

**Figure VI-2** The response of DPPC53 vesicles to *S.aureus* PSMs in the presence of high density lipoprotein. Vesicles were exposed to a concentration range of either A) PSM α3 or B) δ haemolysin in the presence of 10µg ml⁻¹ high density lipoprotein purchased from Sigma Aldrich.
Whilst the difference between HDL and non HDL treated samples clarified the role of this serum protein in reduction of sensing efficacy, it was also noted that vesicle sensitivity was further decreased when exposed to supernatant from cells grown in the presence of serum. This suggested another interaction this time between the bacterial cells and a serum component that might effect PSM/haemolysin production. As no difference was seen between growth rates of cultures exposed to serum, it was hypothesised that some component was affecting production of the PSMs at a transcriptional level. As such the effect of serum on AGR expression was investigated using \textit{S.aureus} LAC with and associated GFP reporter element (Figure VI-3).

![Figure VI-3 Growth and AGR activity of \textit{S.aureus} USA300 LAC in the presence of varying concentrations of human serum. A) Growth of \textit{S.aureus} in the presence of Serum concentrations ranging from 0% to 10%. B) GFP production as a marker of AGR activity in \textit{S.aureus} in the presence of Serum concentrations ranging from 0% to 10%.

This experiment suggested that AGR expression was affected even by very low levels of serum. Further investigation into the literature suggested the ApoB1 a protein component of some HDLs has the capacity to sequester the auto-inducing peptide component of the staphylococcal AGR quorum sensing system [8].
With the understanding that human serum or exudate is likely to interact with toxins responsible for vesicle lysis it becomes important to determine to what extent this suppression will affect the sensor system. Moving forward in the understanding of this sensor system will then require determination of several factors. Primarily the level of expression of individual toxins in a wound environment would be very useful in tuning the system. Secondly an understanding of how individual toxins are affected by lipoprotein sequestration may help in designing a vesicle that shows higher susceptibility to those toxins with lower sequestration rates. Finally tuning of the vesicles to increase susceptibility but maintain stability may negate much of the sequestration effect. Once these factors are known, a proper assessment of the system and how applicable it would be in a clinical setting can be made.

(c) Modification of the sensor system for the detection of alternate toxins.
As well as narrowing the vesicles systems sensitivity to a few key toxins, changes in the vesicle bilayer also show the potential to broaden specificity. As such a system could be produced which is more sensitive to bacterial infection through sensitivity to multiple toxins. Alternatively vesicles with enzyme sensitivity as opposed to amphiphile sensitivity could be produced in order to create a slow release sensor which could act either in tandem or as an alternate to an amphiphile based sensor.

Preliminary work in developing such a system was attempted through the addition of sphingomyelin to the vesicle systems bilayer. Sphingomyelin is known to play an important role in the binding of Staphylococcal β haemolysin to the membrane of host cells [9]. The response of vesicles containing no sphingomyelin and 10% sphingomyelin were compared when challenged with a β haemolysin knockout strain of S.aureus (Figure VI-4).
A comparison of the lytic activity of *S.aureus* supernatant with and without β haemolysin on DPPC vesicles containing sphingomyelin. A) the response of DPPC53 vesicles to *S.aureus* 83254 and its isogenic mutant 83254 Δhlb. B) shows the response of DPPC43% vesicles containing 10% sphingomyelin extracted from pig neurones to the same two *S.aureus* strains.

As can be seen a slight decrease in lytic activity is seen from the β haemolysin mutant when challenging sphingomyelin-containing vesicles. This indicative of β haemolysin conference of a lytic advantage to *S.aureus* when sphingomyelin is included into the vesicle bilayer. In order to confirm this hypothesis such sphingomyelin containing vesicles would need to be tested against Δhld and Δαβpsm *S.aureus* mutants. Regardless however the result of this experiment highlights the possibility of generating vesicles which are sensitive to multiple toxins. As such a further cord of experimentation to be followed would be the modification of vesicle bilayers in line with what is known of the requirements of other *S.aureus* toxins.
Section 6.03 In Conclusion

A sensor system based on the interaction of amphiphilic bacterial products with a vesicle bilayer has been developed for the detection of toxin producing bacteria. This system shows promise in that amongst organisms tested interacting compounds are produced across a broad swath of clinical isolates. Additionally in the case of both organisms investigated in-depth the amphiphiles involved in bilayer interaction are produced as a result of a quorum sensing mechanism. As such the sensor would have the capacity to distinguish between organisms in an infective Vs a non-infective state. Such differentiation would be advantageous in regards to a clinician’s decision as to whether to provide antibiotic treatment for a patient. A particularly prescient decision in the case of patient who could be susceptible to the development of multi-drug resistant infections.

While development of surface bound antimicrobial using the zinc Schiff base was not successful as a whole, a degree of effectiveness can be drawn from the sum of its parts. It has been established that the Schiff base architecture shows promise as a moiety for surface attachment. In future however assessment of the surface bound compound must be assessed as an antimicrobial in the first instance to ensure that developed antimicrobials show the highest degree of efficacy.

In the case of both the sensor and the antimicrobial surface concept there is still much work to be done and much work that can be done. This is especially true in expanding the investigation to the more clinical aspects of the study and the practical potential of such systems.

Section 6.04 References


