Assessment of Apolipoprotein E Derived Peptides as Novel Antimicrobials for the Coating of Biomedical Devices

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Table of Contents

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List of Figures

List of Tables

List of Abbreviations

AMP	Antimicrobial peptide
ApoE	Apolipoprotein-E
BSA	Bovine serum albumin
CAMP	Cationic antimicrobial peptide
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CFU	Colony forming unit
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
IEF	Iso-electic focusing
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MHA	Muller-Hinton Agar
MHB	Muller-Hinton Broth
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PHMB	Polyhexamethylene biguanide
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate buffer
TEMED	Tetramethylethylenediamine
w/v	Weight by volume

THE UNIVERSITY OF MANCHESTER

ABSTRACT OF THESIS submitted by Sarah Forbes for the Degree of Doctor of Philosophy and entitled Assessment of Apolipoprotein E Derived Peptides as Novel Antimicrobials for the Coating of Biomedical Devices. October 2012

The microbial contamination of biomedical devices is a leading cause of hospital-acquired infection. A number of strategies aimed at developing device coatings that are refractory to microbial adhesion, colonisation and biofilm formation have been developed, but the problem remains. The incorporation of biocides into biomedical device surface coatings has shown promising results in preventing the establishment of infection. Current controversy over the possibility that extensive use of biocides could potentially lead to antimicrobial resistance has fuelled the search for new actives with good antimicrobial activity and low cytotoxicity, that maintain marked efficacy after prolonged use. This doctoral thesis aims to evaluate the antimicrobial potential of a novel peptide based on human apolipoprotein E receptor binding region (apoEdpL-W). The spectrum of antimicrobial activity and anti-biofilm efficacy of apoEdpL-W was compared to that of common biocides polyhexamethylene biguanide, triclosan, cetrimide and chlorhexidine. The potential to induce bacterial insusceptibility towards these agents after long-term sub-lethal level exposure was assessed. Initial examination against 18 test microorganisms, commonly associated with device infection, showed that apoEdpL-W displayed broad-range antimicrobial and anti-biofilm efficacy. ApoEdpL-W also maintained marked antibacterial activity after incorporation onto various biomaterial polymers, often used in device surface coatings. Alterations in bacterial susceptibility after prolonged exposure to apoEdpL-W, as well as to the other biocides, were often temporary and partially reverted once the bacteria had been grown in the absence of the antimicrobial agent. The adaption of *Staphylococcus aureus* to the presence of triclosan resulted in the formation of small colony variants (SVCs) with reduced triclosan susceptibility. Analysis of the physiological characteristics of the triclosan induced SCVs revealed the loss of virulence determinants and potentially reduced pathogenic capability, when compared to the parent strain. The biocompatibility index values of the test actives were determined by the parallel assessment of their antibacterial activity and *in vitro* cytotoxicity. ApoEdpL-W showed good antibacterial efficacy whilst remaining relatively less toxic to mammalian cells than triclosan or chlorhexidine. We studied the interactions of the test antimicrobials with a preformed phospholipid bilayer using the quartz crystal microbalance device and dual polarisation interferometry, to better understand potential mode of action. Analysis revealed that ApoEdpL-W and PHMB induced the highest level of bilayer disruption, of all the antimicrobials tested. These data suggest that apoEdpL-W demonstrates antibacterial activity; biocompatibility and long-term efficacy on a level that compares favourably to that of currently used biocides. The peptide demonstrates good antimicrobial efficacy when incorporated into a range of biomaterial polymers and shows the potential to be developed as an effective coating for the reduction of device associated infections.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Research Contributions

First author papers
Latimer, J., S. Forbes and A.J McBain (2012). Attenuated Virulence and Biofilm Formation in *Staphylococcus aureus* following Sublethal Exposure to Triclosan. *Antimicrobial Agents and Chemotherapy.* 56:3092-3100.

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Chapter 1

General Introduction

1.1 Antimicrobials

Antimicrobial agents have been used for centuries to control microbial growth in healthcare, domiciliary and industrial environments. Ever since Girolamo Fracastoro's concept of 'the seeds of disease' in 1543 and Antonie Philips van Leeuwenhoek's description of 'animalcules' during the 17th century, there has been an awareness of contagion. By the time Louis Pasteur and Robert Koch proved the presence of microorganisms, over 300 years later, antimicrobial chemotherapy was already becoming established. Historically, compounds with naturally occurring antimicrobial activity, such as honey or vinegar were used in attempt to reduce the incidence of infection, for instance in the treatment of wounds (Cooper, 1999). Today, some of the most important antimicrobials, such as penicillin are still derived from naturally occurring compounds. The use of synthetic agents, however, has increased due to their breadth of antimicrobial activity. Biocides, which are active against a range of bacteria, viruses, fungi and protozoa, have shown significant application during the 20th century for the purpose of disinfection, sterilisation and preservation (Russell, 2008).

With an on-going battle between man and microorganism there is a constant demand to produce new antimicrobials. An increase in antibiotic resistance and the problem of infections caused by antibiotic resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Davis*, et al.*, 2004) and vancomycin-resistant enterococci (Linden, 2002), has further spurred the search for novel antimicrobial strategies. Over the past 20 years, there has been over 100 new antibacterial agents developed, however, these largely fall into pre-existing categories and whilst proving promising, it has been criticised that they are a short term solution (Meyer &

Cookson, 2010). To stay ahead of the evolutionary arms race between man and microbe there is a need to produce antimicrobial agents that exploit novel antibacterial targets or that are less prone to the development of microbial resistance over time.

1.2 Biocides

The term biocide refers to a chemical antimicrobial agent with a broad-spectrum of activity and generally low cellular target specify. Biocides have been used extensively as antiseptics (agents that kill or inhibit microbial growth whilst causing limited toxicity to human tissue), disinfectants (agents that kill microorganisms, usually on hard surfaces) and preservants (agents that prevent microbial growth, often in pharmaceuticals and cosmetics) (McDonnell, 1999). Whilst biocides have traditionally been used in cleaning products, antimicrobial soaps, hand washes and toothpastes, more recently their incorporation into the coatings of copolymer materials used in medical devices, such as wound dressings, catheters and prosthetic joints, has shown promise in preventing the establishment of infection (Gaonkar*, et al.*, 2003, Silver, 2006, Kugel, 2009). When considering the potential application of a biocide, it is important to consider both its antimicrobial activity and its cytotoxicity.

1.3 Mechanism of Action of Biocides

Biocides tend to have a broader-spectrum of activity than antibiotics, although this may not necessarily be due to the absence of a specific target site. The principal target site of many biocides is the bacterial cytoplasmic membrane, damage to other sites often resulting as a secondary event (Russell, 1996). Most biocides have a range

of microbial activity depending upon their concentration and may be deemed "bacteriostatic" whereby they inhibit microbial growth or "bactericidal" in which they kill the target organism. In the past 100 years, we have seen the development of cationic biocides such as quaternary ammonium compounds (QACs) and biguanides, as well as aldehydes and phenolics (Russell, 2008). The activity of a biocide depends on a multitude of factors both inherent to the biocide or the targeted microorganism. The next section will discuss the currently proposed modes of action of the biocides used throughout this study.

1.3.1 Polyhexamethylene biguanide

Polyhexamethylene biguanide (PHMB) is a broad-spectrum cationic biocide with both bacteriostatic and bactericidal capabilities (Rose & Swain, 1956). The synthesis of Polyhexamethylene biguanide (PHMB) was first described by Rose and Swain in 1956 (Rose & Swain, 1956), subsequently it has been used in various consumer applications, largely as an industrial biocide used to disinfect water-cooling towers (Vantocil IB^{TM}), as a swimming pool sanitizer (Baquacil^{TM}) and as the active ingredient in a range of hard surface cleansers (Hansmann, 2004). It has recently been incorporated into coatings for wound dressings and used as an antimicrobial agent in contact lens solutions, due to its ability to exhibit long lasting antimicrobial activity without the development of local or systemic intolerance (Hansmann, 2004).

Structurally PHMB is a synthetic polymeric biguanide containing alternating hexamethylene and biguanide residues. PHMB possesses amine, guanidine and cyanoguadine end-groups, due to the basicity of the biguanide groups PHMB has an overall positive charge at physiological pH. This charge results in a rapid attraction

of PHMB to the negatively charged sugar residues on the bacterial cell surface or the phosphate groups of the phospholipid bilayer, helping PHMB bypass the outer-defensive layers of the bacterial cell (Broxton*, et al.*, 1984).

The initial interaction of PHMB with the bacterial cell has been followed using micro-electrophoresis (Gilbert*, et al.*, 1990), showing that the negative surface charge of the bacterial cell is rapidly neutralised when sufficient PHMB is added. Electron micrographs have demonstrated that bacteriostatic concentrations of PHMB cause changes in the outer membrane integrity of Gram-negative cells (Ikeda, 1984). During sub-lethal PHMB exposure, there appears to be a displacement of divalent cations such as Ca^{2+} from the cell surface, because the PHMB competes for the negative sites on the underlying peptidoglycan. This leads to membrane destabilisation and the breakdown of the outer-membrane leaving the cell susceptible to further attack by PHMB on the inner cytoplasmic membrane (CM) (Gilbert*, et al.*, 1990). Since the hexamethylene groups are inflexible, it has been suggested that the biocide does not interdigitate into the hydrophobic core of the membrane, like other cationic biocides such as QACs (Gilbert, 2005). Rather, adjacent acidic phospholipids within the membrane are bridged by the PHMB molecule, altering the membrane integrity and potentially impairing neighbouring protein function, such as ATPases, whilst producing a 'fluid mosaic' of phospholipid domains (Broxton*, et al.*, 1984). The bacterial cells response to PHMB may manifest as a loss in biosynthetic, catabolic and transport capabilities (Gilbert, 2005). Exposure of the CM to increasing levels of PHMB may lead to a progressive leakage of Ca^{+} and potentially plasmolysis of the cell. Purportedly, bacteria treated with bacteriostatic levels of

PHMB may be able to recover from this loss and reform the membrane, despite temporary impaired function of membrane bound proteins (Broxton*, et al.*, 1984).

At bactericidal levels, PHMB has been shown to cause leakage of intracellular components, due to the lack of membrane integrity. Once the cell has leaked over 15% of its nucleotides it becomes irreversibly damaged and undergoes cytoplasmic precipitation, as the nucleic acids within the cytoplasm interact with the PHMB causing the cytoplasm to appear to coagulate when observed using electron microscopy (Gilbert, 2005). After exposure to high levels of PHMB, the membrane breaks up into destabilised zones, which quickly aggregate into a favourable hexagonal arrangement, causing total loss of membrane function and potential cell death (Broxton*, et al.*, 1984).

1.3.2 Chlorhexidine

Chlorhexidine is a cationic biocide with activity against a range of both Gram-positive and Gram-negative organisms (Hansmann, 2004). Chlorhexidine is used in a wide variety of topical pharmaceutical applications, including as a pre-operative skin antiseptic (Hibitane ^{TM}), as a surface wound treatment and for use on burns patients (O'Malley, 2008). It is also an effective anti-plaque agent with the ability to bind strongly to the tooth surface inhibiting pellicle formation and is therefore used as an active ingredient in a range of dental formulations, principally mouthwashes (Charles*, et al.*, 2004).

Structurally, chlorhexidine is a bisbiguanide containing two cationic groups separated by a hydrophobic hexamethylene group. This contrast with PHMB, a

polymeric biguanide, which contains multiple cationic groups separated along a hexamethylene chain backbone (Gilbert, 2005). Chlorhexidine is a also membrane active compound, displaying both bacteriostatic and bactericidal effects at different concentrations (Hennessey, 1973).

With respect to mode of action, the cationic chlorhexidine molecule rapidly absorbs to the anionic bacterial cell surface causing a change in membrane integrity and allowing the biocide access to the inner membrane. Chlorhexidine purportedly bridges adjacent phospholipid head groups, destabilising the membrane and increasing fluidity (Kuyyakanond, 1992). This increase in cell membrane permeability may lead to the leakage of cell contents and cause the displacement of potassium ions and protons from the membrane, which impacts the osmoregulatory and metabolic capacity of the cell, impairs respiration and solute transport leading to cessation in cell growth (Hugo & Hugo, 2011). At higher bactericidal concentrations, chlorhexidine is believed to form phosphate complexes causing cytoplasmic precipitation and cell death (Kuyyakanond, 1992). Both chlorhexidine and PHMB have been proposed to exhibit self-promoted uptake onto the bacterial cell, during which the antimicrobials destabilise the divalent cations assocated with the cell envelope, causing a re-organisation of lipopolysaccharides (LPS) further facilitating cell entry (Wilkinson, 1987).

1.3.3 Triclosan

Triclosan (2,4,4'-trichloro-2'hydroxydipheneylether) is a chlorinated antibacterial and antifungal compound containing both ester and phenol functional groups. Since it was introduced in 1968 triclosan has been used as an adjunct to many oral and

skincare products, such as soaps, toothpastes, deodorants and shaving creams (Bhargava & Leonard, 1996). It is especially effective against Gram-positive microorganisms, which may be associated with skin infections, such as staphylococci. Consequently, it has been implemented as an active agent in hand washes that are frequently used in healthcare settings to prevent the transmission of MRSA (Zafar*, et al.*, 1995).

It was initially believed that the primary mode of action of triclosan was due to non-specific interaction with the bacterial cytoplasmic membrane. However, studies have since shown that at bacteriostatic concentrations, triclosan functions by inhibiting fatty acid synthesis via binding to the enoyl-acyl carrier protein reductase enzyme (ENR) encoded by the *fabI* gene (Heath & Rock, 1995). This binding results in the formation of a stable tertiary complex, which is unable to participate in fatty acid synthesis and therefore bacterial growth is inhibited (Heath & Rock, 1995, Heath, 1999). At bactericidal levels, triclosan is thought to act through multiple non-specific mechanisms, including damage to the cytoplasmic membrane, leading to the leakage of cytoplasmic components (Villalaín, 2001), loss of protons (Phan, 2006) and the uncoupling of oxidative phosphorylation from respiration (Levy*, et al.*, 1999). This leads to cessation of growth and eventual cell death.

1.3.4 Cetrimide

Cetrimide (alkyltrimethylammonium bromide) is a quaternary ammonium compound effective against a wide range of bacteria and fungi (Gilbert, 1985). It is used frequently as a topical antiseptic, is the active agent in the antimicrobial skin cream

Savlon™ and is often used in hair conditioning products due to its surfactant properties (Gilbert, 1985).

QACs are a diverse group of antimicrobial compounds that share the presence of a central positively charged nitrogen atom, covalently bonded to four carbons. This central quaternary ammonium group, in part, helps determine the physicochemical properties of the biocide (Daoud*, et al.*, 1983). Cetrimide is comprised of a mixture of *n*-alkyltrimethyl ammonium bromides where the *n*-alkyl group is a hydrophobic region between 8 and 18 carbons long. This polar head group, along with the non-polar tail, allows QACs to behave in a detergent-like manner.

The mode of action of cetrimide is thought to be targeted towards the bacterial cytoplasmic membrane, causing generalised leakage of cytoplasmic components (Guerin Mechin, 2000). At low QAC concentrations, it has been suggested that the positively charged quaternary nitrogen ion binds to the bacterial cell surface anionic regions, such as the acidic phospholipid head groups, within the cytoplasmic membrane. The hydrophobic tail can interdigitate into the hydrophobic core of the membrane, causing an increase in membrane core hydrophobicity and promoting the rearrangement of the membrane phospholipids into a hexagonal conformation (Daoud*, et al.*, 1983). The membrane is believed to loose its fluidity and transitions into a liquid crystalline state, impairing osmoregulatory and metabolic functions (Guerin Mechin, 2000). Similarly to the biguanides, the surface binding of QACs to the bacterial cell is thought to cause the displacement of protons and potassium ions, which damages the cells' respiratory and biosynthetic capabilities (Gilbert, 1985). At higher concentrations, QACs are thought to rapidly kill the bacterial cell by

solubilising the membrane, causing cell lysis. The length of the *n*-alkyl chain correlates to the overall hydrophobicity of the compound and may influence the activity of the biocide. A chain length of 12-14 has been shown to maximise activity against Gram-positive organisms and yeasts, whereas 14-16 has been shown to be more effective against Gram-negative organisms (Gilbert, 1985). When the n-alkyl chain length is above 10, it is thought that the level of attraction between the hydrophobic chains exceeds the level of electrostatic repulsion from the nitrogen head groups, this leads to the formation of dimers (Daoud*, et al.*, 1983). Dimers interact more readily with the cytoplasmic membrane than their monomeric counterparts, resulting in a stronger antimicrobial efficacy (Daoud*, et al.*, 1983, Gilbert, 1985).

1.4 Biocide Resistance

The extensive usage of biocides in recent years is partially accredited to an increase in customer awareness of microbial contamination and of the importance of community hygiene in preventing the transmission of infectious microorganisms. However, an increase in the use of antimicrobial agents and an escalation in products containing low concentrations of biocides, such as medical device coatings, have led to concern over the potential selection of bacterial populations with decreased susceptibilities towards common antimicrobials. It has been suggested that the exposure of bacteria to sub-lethal concentrations of biocides might lead to the clonal expansion of intrinsically less susceptible populations, as well as inducing genotypic changes in individual bacteria that will further decrease their biocide susceptibility (Braoudaki & Hilton, 2004). This decrease may develop to the primary antimicrobial agent, to which the bacteria are exposed and potentially also to third party agents

(other biocides or antibiotics), a phenomenon referred to as cross-resistance (Guerin Mechin, 2000, Russell, 2000).

Biocides, in general, are less target-specific than antibiotics, and whilst antibiotics may have a singular pharmacological target, biocides tend to act concurrently on multiple sites in a concentration dependent manner and often target the cell membrane (McDonnell, 1999). There has been some concern regarding the development of cross-resistance between biocides and other antimicrobials, due to the presence of shared target sites, or due to induced changes in the bacterial cells physiology making the bacteria less susceptible. Several *in vitro* studies have previously demonstrated a link between the decrease in biocide and antibiotic susceptibilities (Guerin Mechin, 2000, Denyer & Maillard, 2002, Joynson, 2002, Braoudaki & Hilton, 2004, Karatzas, 2007), however, similar studies have indicated that this is not a universal attribute (McBain, 2004). Importantly, it has been suggested that although there may be a theoretical relationship between biocide exposure and the development of cross-resistance, there is currently little evidence that this is the case outside of the laboratory. However, if biocides are thought to potentially play even a minor role in the development of resistance, then this is an area that requires further investigation.

Many factors can contribute towards a reduction in activity of an antimicrobial, such as the use of inappropriate biocide concentrations, the presence of organic materials able to inactivate the agent, as well as reduced exposure times and changes in pH. These can all result in a fall in antimicrobial efficacy, potentially leading to treatment

failure (Russell, 2008). Therefore, the indiscriminate use of biocides, particularly in a healthcare setting, needs to be carefully monitored.

1.5 Microbial Physiology and Biocide Action

There are multiple mechanisms that determine the susceptibility of a particular bacterium to a biocide (Maillard, 2002). The interaction of a biocide with the bacterial cell occurs through a series of events including (i) diffusion onto the cell surface, (ii) interaction with the cell surface components, (iii) transversing the cell wall, (iv) interacting with and transversing the cytoplasmic membrane and (v) diffusion into/reaction with the cytosol (Gilbert, 2001). The term intrinsic insusceptibility, when referring to an antimicrobial, refers to a natural determined property of the bacterium and is often due to variations in the cellular structure and permeability of the bacterial cell to the active agent. This may be due to defensive outer cell layers (Bloomfield & Arthur, 1994), the presence of efflux pumps (Ziha Zarifi, 1999), the absence of a target site or an inability of the active agent to accumulate at the necessary targets at sufficient concentrations (Zhu*, et al,* 2010). The following section will discuss the different components of the bacterial cells' physiology that may influence its antimicrobial susceptibility.

1.5.1 The bacterial cell envelope

The outer layers of the bacterial cell represent a permeability barrier, limiting the rate of entry of the biocide. The cell envelope forms the initial point of bacterial contact and in order to be effective, the biocide must accumulate outside the bacterial envelope and then bypass this outer layer to make contact with the cell membrane.

This initial interaction is believed to be largely influenced by the charge, hydrophobicity and amphiphilicity of the antimicrobial compound (Nikaido, 1976, Denyer & Maillard, 2002). Antimicrobial uptake may occur via a porin-mediated hydrophilic pathway (Frenzel, 2011), by the diffusion of the molecule across the bacterial outer membrane (hydrophobic) (Nikaido, 1976) or by self-promoted uptake (Gilbert, 1990). In general, Gram-negative spores show lower biocide susceptibility than Gram-positive (Russell, 1996), although within each group there is a wide range in susceptibilities. This relative insusceptibility is due to the fact that Gram-negative bacteria contain an outer membrane that Gram-positive do not, which is important in helping to prevent biocide penetration of the cell (Gilbert, 1990). Bacterial spores and mycobacteria also have a low intrinsic susceptibility to biocides due to their impermeable outer layers (Martin, 1969, Selvaraju, 2008).

1.5.2 Gram-positive bacteria

The cell wall of Gram-positive bacteria is largely composed of a 20-80 nm layer of peptidoglycan, covalently linked to linear anionic polymers known as teichoic acids, which give the cell its net negative charge (Figure 1.1). Lipoteichoic acids, consisting of a glycerolphosphate teichoic acid chain covalently linked to a glycolipid, are located on the outer surface of the cytoplasmic membrane and associate with a number of functionally important proteins, which mediate interactions between the cell and its surrounding environment (Kristian, 2005). High molecular weight substances (30,000 to 57,000 Da) can readily transverse the Gram-positive cell wall (Lambert, 2002), for example, biocides such as alcohols QACs and bisbiguanides are small enough to easily penetrate the Gram-positive cells' network

of peptidoglycan and associated polymers, making the bacteria relatively susceptible to these compounds (Scherrer, 1971). Peptidoglycan helps confer strength and rigidity to the bacterial cell. It is comprised of long glycan strands made up of repeating units of two amino acid sugars N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), which are linked by β-1,4 glycosidic bonds via the action of transglycosylase enzymes. Peptides, which extend at right angles from the glycan chains, are linked by transpeptidases forming cross bridges which connect the strands producing a mesh like network, which helps prevents cell lysis. Growth and nutrient limitation may also impact the physiological state of the bacterial cell, and thus its antimicrobial susceptibility, by influencing the level of peptidoglycan cross-linking and the resulting cell wall thickness, potentially making it more difficult for antimicrobial agents to reach the inner cell membrane target site (Brown & Williams, 1985, Gilbert, 1990). Additionally, some Gram-positive organisms are able to produce extracellular polysaccharides that can form an external capsule that may help protect the cell from external stresses, such as antimicrobial exposure (Russell, 1988).

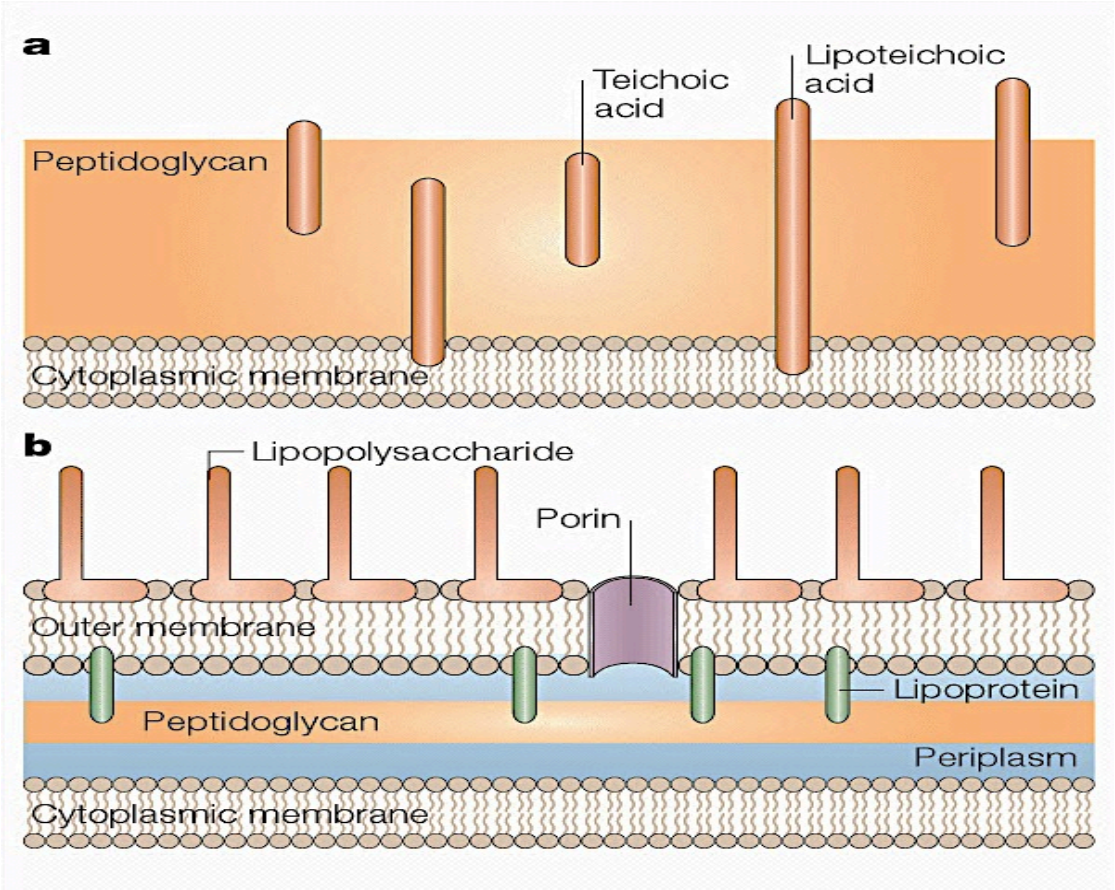

Figure 1.1. Diagrammatic example of the a) Gram-positive and b) Gram-negative bacterial cell envelope. The Gram-positive cell wall consists of a thick layer of peptidoglycan (PG) outside the cytoplasmic membrane, in which teichoic acids are embedded. The Gram-negative cell wall has a protective outer membrane composed of a phospholipid bilayer, encasing globular proteins such as porins that allow the diffusion of small hydrophilic molecules across the membrane. Below this layer is the cell wall consisting of a thin PG layer that helps maintain the cells integrity. The cytoplasmic membrane (CM), composed of phospholipids and embedded proteins, controls the molecules that enter and leave the cytoplasm. Divalent cations such as calcium and magnesium found within the cell envelope partially neutralise the negative charge on the cell surface due to the peptidoglycan layer and play an important role in the maintenance of membrane stability (Cabeen & Jacobs-Wagner, 2005).

1.5.3 Gram-negative bacteria

The outer layer of Gram-negative bacteria, consists of a layer of peptidoglycan (1-7 nm) and both a cytoplasmic and outer membrane enclosing a periplasmic space (Figure 1.1), which can act as a barrier decreasing the rate of antimicrobial diffusion into the cell (Nikaido, 1985). Inside the cell, the peptidoglycan layer is firmly linked to the outer membrane by the covalent attachment of glycan strand side chains to

murein lipoprotein in the outer membrane and this helps maintain the cell structure (Hammond, 1984). The outer envelope contains phospholipids, lipopolysaccharide (LPS) and embedded proteins, such as porins, that all influence bacterial antimicrobial susceptibility (Nikaido, 1985). *Pseudomonas aeruginosa (P. aeruginosa)* treated with QACs has previously showed alterations in outer membrane fatty acid composition, which has been linked to its decreased susceptibility towards cationic biocides (Guerin Mechin, 2000). Studies into mutants of *Escherichia coli* (*E. coli*) defective in outer membrane proteins and LPS have shown reduced biocide susceptibilities when compared to their wild-type counterparts, which may indicate that it is a collaboration of various cell surface components, not just the outer membrane, that determines antimicrobial susceptibility (Nikaido, 1985).

1.5.4 Spore-forming bacteria

Certain Gram-positive bacteria, such as those from the bacillus genus, have life cycles that allow them to exist in two forms' metabolically vegetative cells and dormant spores (Bloomfield & Arthur, 1994). In these bacteria, biocide susceptibility depends upon the stage of their life cycle that they are within. During the spore cortex and coat development stages, bacteria show a decreasing biocide susceptibility, compared to the vegetative cell state (Shaker, 1986). Gram-positive bacteria such as Bacillus and Clostridium are known to produce spores, which are resistant to many chemical agents (Leggett, *et al*., 2012). This is largely due to low biocide penetration of the thick protective spore coat and their metabolic inactivity. Some biocides such as QACs and chlorhexidine lack sporicidal effect but can be aided by an increase in temperature, which helps the biocide to penetrate the defensive spore coat and reach its target site (Shaker, 1986).

1.5.5 Lipopolysaccharide

Lipopolysaccharides (LPS) (Figure 1.2) are a principal component of the Gram-negative cell wall and are, in part, responsible for cell impermeability to antimicrobial agents (Hancock, 2002). LPS is a large molecule endotoxin, consisting of polysaccharide and lipid covalently linked chains. It has three regions (i) Lipid A- a hydrophobic phosphorylated glucosamine disaccharide unit, deletion of which has previously shown to increase bacterial susceptibility to hydrophobic antibiotics (Vaara, 1992); (ii) the central core polysaccharide and (iii) the outer O-polysaccharide chain made up of repeating oligosaccharide units that are used in the serotyping of a species (Nikaido, 1985).

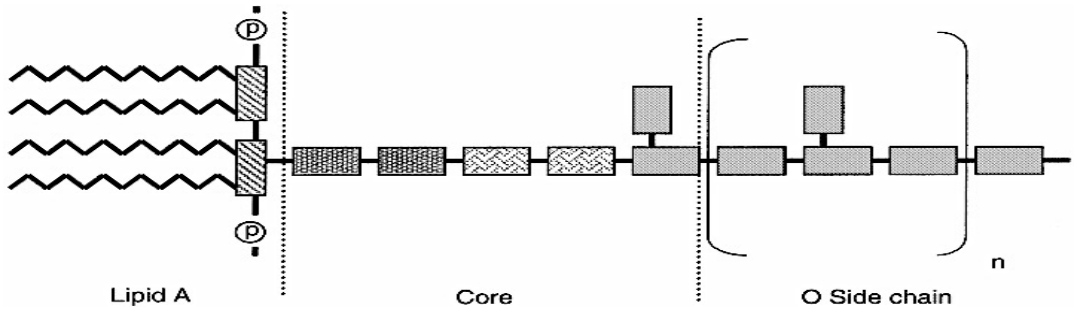

Figure 1.2. Structural composition of lipopolysaccharide showing the lipid A, central core polysacchairde and the o-polysaccharide chain (Denyer & Maillard, 2002).

LPS strands are cross-linked and held in place due to the attracting forces from divalent cations, giving the outer membrane structural integrity and decreasing permeability to biocides (Nikaido, 1985). LPS has previously been linked to a decrease in biocide susceptibility in bacteria, for example, some members of the *Proteus* genus are believed to have a less acidic form of LPS that reduces binding affinity and confers a lower susceptibility to cationic biocides, such as chlorhexidine

and some quaternary ammonium compounds (Martin, 1969). Alterations in LPS composition in *P. aeruginosa* and *Burkholderia cepacia (B. cepacia)* have been associated with insusceptibility towards certain cationic biocides, this is believed to be due to an alteration in LPS charge, resulting in an inability of the cationic molecule to efficiently bind to the LPS and associate with the bacterial cell surface (Cox & Wilkinson, 1991).

1.5.6 Porins

Proteins found in the bacterial cell outer membrane, such as porins, form an entry point for hydrophilic molecules into the cell. Porins can contribute towards the intrinsic susceptibility of bacteria by influencing the rate of cell influx and efflux. Porins are generally classified into two groups (i) non-specific porins, which allow the general diffusion of molecules and (ii) specific porins that only allow entry to certain compounds, that cannot enter the cell via diffusion (Frenzel, 2011). Previous studies have shown *P. aeruginosa* to be an extremely antimicrobial insusceptible Gram-negative microorganism (Winder, 2000). It is considered, in *P. aeruginosa*, that high levels of Mg^{2+} within the cell envelope promote strong bonds between lipopolysaccharide molecules, that decrease porin size and decrease the diffusion of biocides into the cell, lowering its biocide susceptibility (Nikaido, 1985).

1.5.7 Efflux pumps

Efflux pumps are widely distributed in bacteria and play a major role in both intrinsic and acquired bacterial resistance mechanisms towards antibacterial agents (Poole, 2004). It is thought that efflux systems were originally evolved for the removal of

endogenous metabolites from the cell in co-transport with harmful compounds. Their ability to be induced by a range of substances and to remove a wide variety of structurally unrelated molecules suggests that they may have evolved to protect cells from natural occurring environmental toxicants (Marquez, 2005). Substances that have previously been shown to induce the expression of efflux pumps include antibiotics, QACs, pine oil and salicylate (Moken, 1997). Induced expression of efflux pumps, due to exposure to an antimicrobial agent, can result in a decrease in susceptibility towards the antimicrobial to which it is exposed and also potentially to other drugs and biocides via the expression of a broad substrate range drug efflux mechanisms (Chuanchuen*, et al.*, 2001).

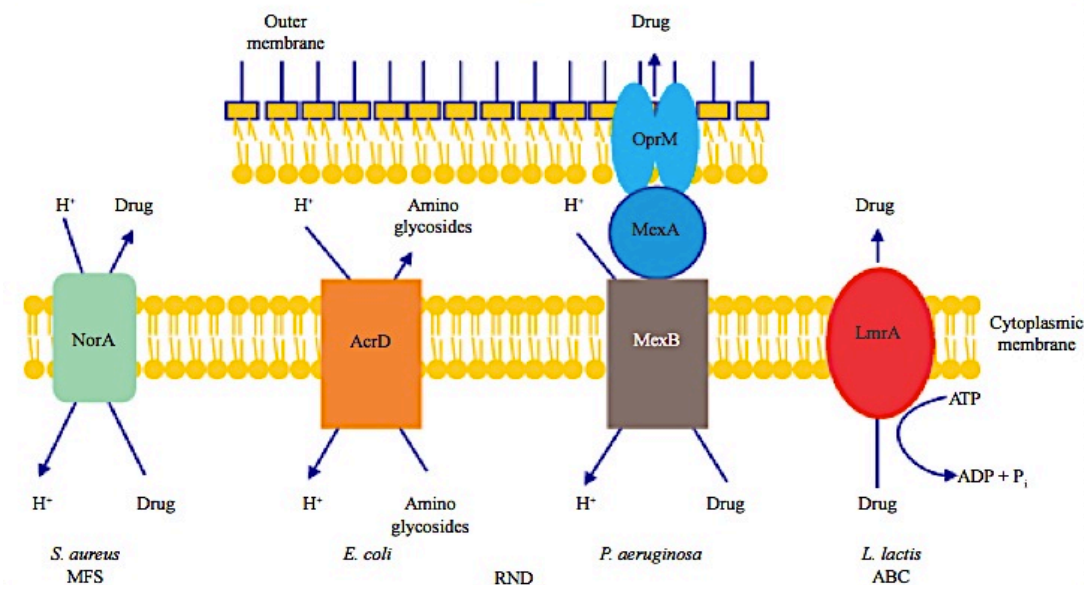

Figure 1.3. An illustration showing key efflux systems in *Pseudomonas aeruginosa, Staphylococcus aureus* and *Lactobacillus lactis.* Shown are NorA part of the major facilitator superfamily, AcrD and MexAB-OprM part of the resistance nodulation-division and LmrA part of the ATP-binding cassette family (Schweizer, 2003).

The periplasm is a region found within the inner and outer membrane in Gram-negative bacteria that consists largely of protein and polysaccharides. Its main roles

include maintaining cell shape, osmoregulation, transport of substances involved in nutrition and the detoxification of harmful compounds (Beveridge, 1999). The periplasm plays a key role in the efflux of agents that may be toxic to the bacterial cell. Five identified groups of bacterial efflux systems have currently been described; these are (i) the resistance nodulation-division family; (ii) the small multidrug resistance family; (iii) the major facilitator superfamily; (iv) the ATP-binding cassette family and (v) the multidrug and toxic compound extrusion family. These have been reviewed in detail previously (Schweizer, 2003, Poole, 2004, Marquez, 2005). Figure 1.3 shows some of the families of efflux systems found in Gram-positive and Gram-negative organisms. All of these systems either use the proton motive force or utilise ATP to allow active efflux. Antimicrobial resistance due to mutations in efflux systems may be due to one of two mechanisms, either (i) expression of the efflux system is increased or (ii) the efflux protein contains an amino acid substitution that increases the pumps ability to export (Schweizer, 2003). An increase in the rate of efflux results in a lowered concentration of antimicrobial within the cell cytoplasm, allowing the bacteria to survive for a greater length of time or in the presence of a higher antimicrobial concentration. The increased expression can be triggered by mutations in regulatory genes or induced by various stresses such as exposure to antibiotics and other antimicrobial agents. For example, point mutations in *mexR,* the regulator gene in the *mexA-mexB-oprM* operon in *P. aeruginosa* have been linked to an over-expression of the efflux system, rendering the bacteria resistant to many antibiotics (Ziha Zarifi, 1999). Since many efflux pumps have such a broad range of expression inducers, key metabolites may be pumped out of the cell along with the harmful substance. Ideally, expression of an efflux system would be induced only when needed, therefore if an antimicrobial acts

as an inducer and a substrate then it should be quickly removed and have minimal impact on the cell. If a biocide is a pump substrate but not an inducer then exposure of bacteria to the biocide may select for mutants that constitutively express an appropriate efflux system, whilst a selective pressure is maintained (Gilbert, 2003), resulting in a bacterial population with reduced antimicrobial susceptibility.

1.6 Inductive Change in Bacterial Susceptibility

Resistance may develop due to an induced phenotypic change in an organism, reflecting the conditions to which they have been exposed. These changes may include expression of efflux pumps in the cell membrane (Maseda, 2009), the synthesis of protective enzymes (Thomson & Smith Moland, 2000), or chromosomal changes in the form of mutations in the genes encoding or regulating the antimicrobial target site (Liu*, et al.*, 2009). Acquired resistance in bacteria may also arise by the acquisition of genetic material such as plasmids or transposons (Alton, 1979). Bacteria may acquire biocidal resistance due to genetic changes in the cell that usually develop in response to persistent sub-lethal exposure to a chemical agent. Evidence of such decreases in susceptibility have been provided by the development, for example, of highly QAC-insusceptible *Serratia marcescens (S. marcescens)* (Prince, 1978) and chlorhexidine-insusceptible *E. coli*, *P. aeruginosa* and *S. marcescens* (Fitzgerald*, et al.*, 1992, Braoudaki & Hilton, 2004). This form of inductive change may, however, be unstable and bacteria may revert to their more susceptible forms once grown in a biocide-free medium (Fitzgerald*, et al.*, 1992).

1.6.1 Target modification

Chromosomal mutations in antibiotic target sites can lead to the development of antibiotic resistance in bacteria (Thomson & Smith Moland, 2000). This is largely due to their pharmacological specificity, meaning any mutation altering the primary target site is likely to lead to a resistant clone, which when exposed to the antibiotic would be selected for at the expense of its more susceptible congeners. It is, however, possible that the mutation will result in a cost to the fitness of the microorganism and therefore may have a detrimental impact on its survival (Rozen*, et al.*, 2007). Since biocides, in general, tend to act upon multiple bacterial target sites, it is not often that a mutation in a single target will induce resistance. In comparison, it has been reported that exposure of *E. coli* to sub-lethal concentrations of triclosan over an extended period has lead to the production of mutants in the *fabI* gene, encoding the triclosan target enzyme, which display an enhanced resistance towards the biocide (McMurry, 1998). Similar reports have been documented for triclosan insusceptible *S. aureus (*Jang, *et al.,* 2008)*,* suggesting that the relative specificity of triclosans' target site, increases the likelihood of developing an insusceptible microorganism, when compared to biocides with a broader mechanism of action.

1.6.2 Changes in outer membrane composition

Changes in outer membrane permeability have been related to acquired resistance in many Gram-negative bacteria (McDonnell, 1999). Mechanisms responsible include changes in outer membrane structure (Tattawasart, 2000), outer membrane protein composition (Winder, 2000) and outer membrane fatty acid composition (Guerin Mechin, 2000). Such changes in the outer membrane have been linked to a decrease

in bacterial susceptibility to certain biocides, in particular QACs (Guerin Mechin, 2000). For instance, studies by Winder and colleagues (Winder, 2000) showed the serial passage of *P. aeruginosa* in the presence of increasing concentrations of the biocide chloromethylisothiazolone (CMI) resulted in the loss in expression of outer membrane protein T-OMP, resulting in a decrease in biocide susceptibility (Winder, 2000). How it achieves this change in susceptibility remains unclear, since serial passage of the newly developed resistant strain in the absence of CMI resulted in the re-appearance of T-OMP, even though the MIC values remained elevated.

1.6.3 Horizontal gene transfer

Mobile genetic elements such as plasmids and transposons allow the global transfer and dissemination of genes that confer antimicrobial resistance (Gillespie, 1986, Paulsen, 1993). Integrons containing multiple antibiotic resistance cassettes have been recovered from clinical bacterial isolates, consequently gene transfer between bacteria has greatly contributed towards the problem of multidrug-resistance (MDR) (Paulsen, 1993). In terms of plasmid-mediated resistance, genetic elements are able to fuse to form large multi-resistance plasmids, that enable bacteria that carry the plasmid to persist, for instance, in clinical environments where selection pressure from antibiotics can be considerable (Gillespie, 1986). As well as encoding a reduced susceptibility to antibiotics, there is also the potential for the plasmid-mediated transfer of biocidal insusceptibility. In this respect, QAC resistance genes have been discovered in a variety of plasmids and as conserved elements within integrons in Gram-positive and Gram-negative bacteria (Gillespie, 1986, Paulsen, 1993, Kupferwasser, 1999). Changes in biocide susceptibility due to plasmid transfer have been reported, which included the acquisition of the genes encoding efflux pumps,

for example, plasmid-mediated silver efflux has been described for *Salmonella* (Gupta, 1999). Plasmid-carrying methicillin resistant *S. aureus* have shown low susceptibility to a range of biocides including chlorhexidine, cetrimide and triclosan (Al-Masaudi*, et al.*, 1991). Although the transfer of biocide susceptibility traits via plasmid acquisition has been suggested, so far it has been unable to provide resistance to in use biocide concentrations.

1.7 Biocide Toxicity

Many commonly used biocides are relatively inexpensive to synthesise, however, their toxicity profile makes them most suited for use as disinfectants or biocidal surface coatings, as they often display low level toxicity towards human cells. In addition, biocides have reportedly had adverse effects on the environment, particularly contaminating aquatic settings (Singer*, et al.*, 2002).

1.7.1 Triclosan

Triclosan is an antimicrobial used in health-care products and household items including plastics, textiles and food packaging materials. It may enter the human body frequently through an oral route due to the use of triclosan containing mouthwashes and toothpastes. Triclosan has been identified in the human milk of some European populations and in human urine in the USA (Calafat*, et al.*, Allmyr*, et al.*, 2006). It is considered that triclosan may have a cytotoxic mechanism of action linked to its ability to solubilise lipids within the plasma membrane, leading to membrane disruption and cell death (Bhargava & Leonard, 1996). Studies have previously demonstrated the cytotoxicity of triclosan against various mammalian cell

lines, although results showed that it was not cytotoxic at concentrations approved by FDA for use in customer care products (Udoji*, et al.*, 2010)**.** It has been considered that exposure to higher levels of triclosan may occur through the release of the biocide from triclosan impregnated food packaging (Chung, 2003). This was demonstrated by showing the release of triclosan from an impregnated polymer using 1.2 to 10 % aqueous ethanol, which simulates the conditions food packaging would be exposed to if containing acidic or aqueous foods and in 65 % heptane, which simulates the presence of fatty foods. Triclosan has shown cytotoxicity towards human gingival cells *in vitro* at concentrations of 0.1 mM, as determined by the leakage of lactic acid dehydrogenase from the cell (Zuckerbraun, 1998). Furthermore, a study by Liu (Liu, 2002) demonstrated that triclosan was a slow binding inhibitor of human type 1 fatty acid synthase, inhibiting the growth of MCF-7 and SKBr-3 human breast cancer cells in culture. Additionally, triclosan is reported to have immunosuppressive activity by inhibiting the lytic function of natural killer cells (Udoji*, et al.*, 2010).

1.7.2 Chlorhexidine

Several studies have reported that chlorhexidine may be toxic against a range of eukaryotic cells, with cytotoxicity mechanisms being related to the biocides interaction with the cell surface. The inhibition of human erythrocyte membrane bound Na^+ K^+ ATPases after exposure to chlorhexidine has been reported (Helgeland, 1971). An increase in cell permeability to Ca^{2+} and leakage of the lactate dehydrogenase enzyme from human gingival cells, post chlorhexidine exposure, has also been noted by Babich, suggesting a mechanism for eukaryotic membrane disruption (Babich, 1995). Cell proliferation is also believed to be negatively

impacted by chlorhexidine, with 100% cytotoxicity demonstrated for human fibroblast cells undergoing exposure to chlorhexidine for 24 h (Lucarotti, 1990). Furthermore, *in vitro* studies indicate that chlorhexidine can inhibit mitochondrial activity and cell proliferation (Hidalgo & Dominguez, 2001), as well as protein synthesis (Faria, 2009).

1.7.3 Polyhexamethylene biguanide

PHMB is an antimicrobial often used in contact lens solutions or incorporated into wound dressings. It has been shown to display a high tolerance in cell and tissue cultures (Müller & Kramer, 2008). PHMB has a weaker attraction to the neutral phospholipids found in mammalian cell membranes compared to bacterial ones. This difference in selectivity allows PHMB to exhibit low cytotoxicity, whilst maintaining marked antibacterial activity (Broxton*, et al.*, 1984). However, studies by Rosenthal and Juven showed PHMB to be toxic towards a mammalian cell line (V79), at doses similar to those required for antibacterial activity suggesting a potential harmful effect on eukaryotic cells (Rosenthal *et al*, 1982).

1.7.4 Cetrimide

Cetrimide is often used as a topical antiseptic and in ophthalmic preparations. High QAC concentrations have previously been suggested to lead to cell necrosis and ocular surface damage after long-term eye drop usage (Debbasch*, et al.*, 1999). Additionally, there have been reports of cetrimide application causing skin necrosis in neonates (Gathwala, 2006) and inducing chemical peritonitis, due its disruptive effects on the cell membrane (Gilchrist, 1979).

1.8 Cationic Antimicrobial Peptides

Cationic antimicrobial peptides (CAMPs) have proven effective against various bacteria, viruses and parasites. A wide variety of organisms produce CAMPs as part of an innate defence mechanism, including plants, mammals, amphibians, birds and fish (Martin *et al*., 1995). Expression of CAMPs can be induced by infectious stimuli, for example, the presence of bacterial molecules such as LPS, it is therefore unsurprising that many have strong antimicrobial properties (Cunliffe & Mahida, 2004). Extensive research into the function of CAMPs during the past decade has revealed a number of abilities, including that to confer protection against pathogens (Park, 1998) and limit sepsis (Wildman & Henzler, 2003). Host antimicrobial peptides are often cationic in nature with diverse sequences and structures. They are able to interact with and insert into lipid membrane bi-layers, an association which is enhanced by their conformational flexibility and secondary structure (Sitaram & Nagaraj, 1999). Cationic antimicrobial peptides are generally categorized into four classes based on their structure i.e., α-helical, β-sheet, loop or extended structures, however, there are peptides that do not fit into these categories (Powers, 2003). For many CAMPs the secondary structure is only observed when the peptide interacts with its target membrane (Hsu, 2005). Many naturally produced peptides can be altered by the substitution of amino acids to change their structure and optimise their level of activity (Kelly, 2007). CAMPs can also be developed from synthetic sequences often comprising of cationic residues (Arg, Lys or His) or host anti-infective proteins may be used as a template to design small peptides (Kelly, 2007). Overall, antimicrobial peptides provide a valuable reservoir that could be exploited for use in various anti-infective strategies.

1.9 Antibacterial Mode of Action of Cationic Peptides

Studies into the structure activity relationships of CAMPs show that there are many possible peptides with antibacterial and antiviral activities. In terms of antiviral activity, peptides have previously been shown to inhibit the cell-to-cell spread of certain viruses by blocking cell entry via interactions with specific mammalian cellular receptors, via viral glycoproteins or through other associations with the viral envelope or host cell membrane (Andersen*, et al.*, 2003, Sinha, 2003, Andersen*, et al.*, 2004). It was initially believed that CAMPs only acted on bacteria via permeabilisation of the bacterial cell membrane, however, multiple target sites are now considered (Brogden, 2005). Regardless, the initial stage involves the attraction of the peptide towards the target cell. This is facilitated by the opposing charges between the peptide and the negative components of the outer envelope such as phosphate groups within the LPS of Gram-negative organisms or lipoteichoic acids on the surface of Gram-positives (Hancock, 2002). There are several models that predict how peptides may interact with the cytoplasmic membrane, such as the barrel-stave, carpet, detergent, toroidal pore and aggregate models (Brogden, 2005). Each providing a different intermediate route which leads to one of three events (i) the formation of a transient channel (ii) translocation across the membrane or (iii) micellarisation/dissolution of the cytoplasmic membrane (Matsuzaki, 1998). Studies have shown that certain AMPs do not cause membrane permeabilisation at all but instead translocate into cells and target intracellular processes (Powers, 2003). Figure 1.4 gives an overview of the currently discussed models for peptide-mediated killing of bacterial cells, either via membrane permeabilising or non-membrane permeabilising mechanisms.

1.9.1 Membrane-permeabilising peptides

There are various models that may explain how peptides can insert themselves into the bacterial cytoplasmic membrane, resulting in an increase in membrane permeability, often due to the formation of pores (Figure 1.4 A-D). In all models, the cationic peptide initially interacts with the negatively charged head groups of the lipid bilayer and orientates parallel to the surface of the membrane. In the aggregate model, the peptides re-orientate and span the membrane as an aggregate, with micelle like complexes of peptides and lipids (Figure 1.4A). It is though that the peptides are able to translocate across the bilayer as the aggregates collapse and the membrane undergoes negative curvature strain (Matsuzaki, 1999). The 'toroidal pore' model states that the peptide inserts into the bilayer at a perpendicular angle forming a pore (Figure 1.4B), during which the hydrophilic regions of the peptide interact with the phospholipid head groups and the hydrophobic regions with the lipid core. The lipid membrane is thought to curve inwards around the peptide containing toroidal hole, so that the bilayer forms the lining of the pore. In the 'barrel stave' model the peptides insert themselves into the membrane, at an orientation that is perpendicular to the plane of the bilayer, forming a barrel shaped cluster of staves, with the hydrophilic peptide regions facing the lumen and hydrophobic regions interacting with the lipid bilayer (Figure 1.4C) (Ehrenstein & Lecar, 1977). The 'carpet model' proposes that the peptides aggregate in a parallel orientation to the bilayer, forming a carpet like structure on the membrane surface (Figure 1.4D) (Pouny, 1992). Once a threshold concentration of peptide is reached they act in a detergent like manner and cause the formation of micelles, decreasing membrane stability and resulting in possible holes forming in the membrane. Many CAMPs are capable of behaving in this manner when at high enough concentrations due to their

amphipathic nature. It is important to acknowledge, when considering the varying modes of action of these CAMPs, that these proposed models may only have validity under certain conditions *in vivo*. Additionally, examination of a wide range of peptides with different structures and sizes has shown that they all leave very different signatures of membrane insertion, suggesting that exact mode of action may be unique to the individual peptide (Hancock, 2002).

1.9.2 Non-membrane permeabilising peptides

Initially all antibacterial peptides will interact with the bacterial cytoplasmic membrane, however, it has been shown that some of the peptides do not cause membrane permeabilisation at minimal effective concentrations but instead act upon intracellular processes (Figure 1.4E- I). These peptides translocate across the membrane and accumulate intracellularly, where they target essential intracellular processes leading to cell death. Processes targeted include nucleic acid, protein and cell wall synthesis and inhibition of enzymic activity (Steinberg, 1997, Scott, 2002, Brogden, 2005). It is thought that during infection many antimicrobial peptides may use more than one of the mechanisms discussed. A multi-target model has been proposed which hypothesises that after initial membrane attachment and cell entry, the CAMPs bind to anionic molecules within the cytoplasm, such as nucleic acids, and interfere in the processes which these molecules are themselves involved in (Powers, 2003). The complexity of the mechanisms of action of CAMPs makes it difficult to select cationic peptide resistant mutants, as has been shown by *in vitro* studies (Tew, 2002).

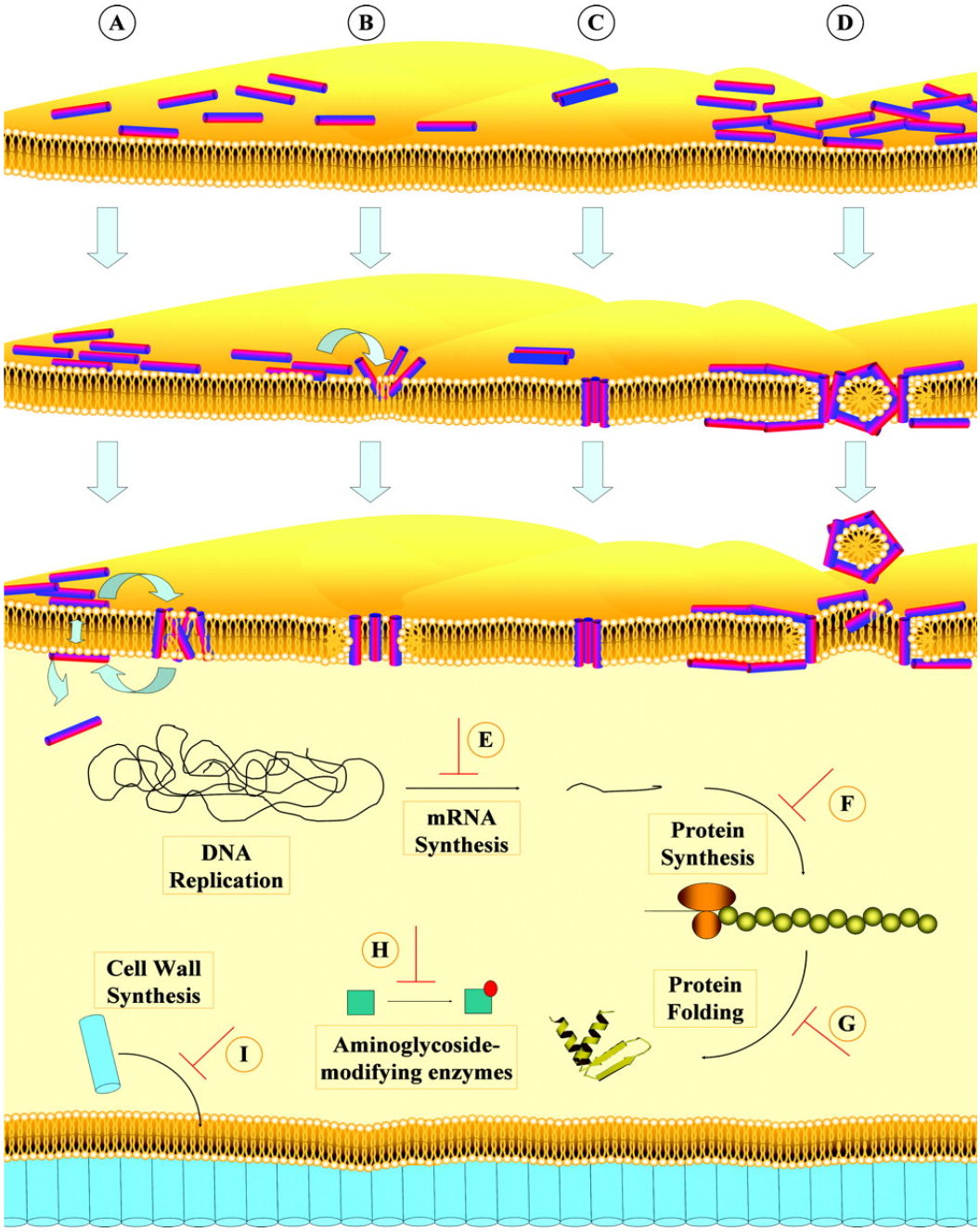

Figure 1.4. Mechanisms of action of antibacterial peptides on the bacterial cell membrane. The cell membrane is represented in yellow with the peptides shown as cylinders with pink hydrophilic regions and purple hydrophobic regions. A-D shows the mechanisms of action for membrane permeabilising peptides. A) Represents the A) 'Aggregate' model, B) 'toroidal pore' mechanism, C) 'barrel-stave' model and D) 'carpet' model. E-I shows the potential targets of non-membrane permeabilising peptides (Jenssen, 2006).

1.10 Bacterial Susceptibility to Cationic Antimicrobial Peptides

Bacteria undergo repeated exposure to CAMPs either from host cells during infection or from competing microorganisms, therefore, it is unsurprising that they have developed various strategies to avoid CAMP mediated killing. Research is providing increasing numbers of examples of CAMP resistant bacteria including microorganisms found on the skin, such as staphylococci, intestinal bacteria such as salmonellae and oral bacteria such as streptococci, all of which have been exposed to high CAMP concentrations (Peschel, 1999, Nizet, 2006). Extensive research examining different bacterial species has provided several possible mechanisms that allow bacteria to achieve such resistance, which are discussed in the following section.

1.10.1 Composition of bacterial outer-layers

The specificity of antimicrobial peptides for bacterial cells over mammalian ones is due to the strong attraction between cationic peptides and the electronegative bacterial envelope. CAMPs are thought to interact with the cytoplasmic membrane and disrupt the lipids, altering the membrane structure and potentially gaining entry to the interior of the bacterial cell (Hancock, 2002). Therefore, the composition of the membrane, as the initial site of contact for the peptide, will greatly influence the peptides overall effectiveness. For example, the presence of cholesterol in the bacterial membrane is thought to decrease the activity of certain CAMPs due to it stabilizing the lipids within the membrane, or by directly interfering with the peptide itself (Matsuzaki, 1999).

1.10.2 Bacterial cell surface electrostatic charge

Many bacteria are able to alter their cell surface composition, modulating the negative charge responsible for the attraction of the CAMP to the bacterial cell envelope, thus decreasing the chance of the CAMP binding and achieving membrane penetration. In Gram-positive microorganisms, the incorporation of positively charged D-alanine esters into the polyionic phosphate groups, found within teichoic acid, has been demonstrated to partially neutralise the polymers and limit the CAMPs interaction with the cell wall (Gunn, 2000). This has been shown to decreases the susceptibility of *S. aureus* to a range of CAMPs, including human defensins (Peschel, 1999). This ability to alter the bacterial cell surface charge has also been shown in further studies involving bacterial species such as *Streptococcus pyogenes* and *Listeria monocytogenes* (Kristian, 2005). In *Salmonella enterica* the outer-membranes charge may be regulated by the PhoPQ regulon and this can affect the sensitivity of the bacteria towards CAMPs, via modulation of the PmrA regulon. The PmrA regulon controls a set of genes that mediate the presence of positively charged compounds ethanolamine and 4-aminoarabinose on the bacterial outer membrane, reducing the net negative charge and thus the attraction of the peptide to the bacterial cell (Gunn, 2000).

1.10.3 Proteolytic cleavage of CAMPS

Peptidases and proteases, which are produced by bacteria, have been shown to be able to cleave CAMPs rendering them inactive. For example, *P. aeruginosa* and *Enterococcus faecalis (E. faecalis)* are all able to produce proteases capable of cleaving liner peptides (Schmidtchen, 2002). In addition, *S. aureus* has been shown

to synthesise serine proteases and the metaloprotease autolysin V8, which are able to cleave the antimicrobial peptide LL-37 rendering it inactive (Sieprawska-Lupa*, et al.*, 2004).

1.10.4 CAMP exporters

Energy dependent exporters in the bacterial cell membrane, referred to as multiple drug resistance exporters (MDRs), may have broad substrate specificity and have been shown to protect the cell from small amphipathic drugs (Van Veen, 1997). As CAMPs are also often small and amphipathic in nature, some of the known MDRs are able to conifer a resistance towards them also. *Neisseria gonorrhoea* contains an efflux pump MtrCDE MDR, which has been shown to contribute towards resistance to a β-sheet porcine CAMP protegrin 1 and to LL-37 a human α-helical peptide (Shafer, 1998).

1.11 Therapeutic Potential of Antimicrobial Peptides

With the increasing problem of antibiotic resistance in bacteria, antimicrobial peptides have provided a new approach at treating microbial infection. Antimicrobial peptides offer certain advantages over currently used antimicrobials, they provide a naturally occurring alternative to help combat infection, have a high level of antimicrobial activity and often display low cytotoxicity (Kelly, 2007). The likelihood of the development of CAMP resistance seems low compared to that of other, more site-specific agents, such as antibiotics and certain biocides. This is due to the fact that their mode of action exploits fundamental features of the bacterial cell as well as usually acting on more than one target site. This hypothesis is supported

by several *in vitro* studies that show despite serial passage in sub-inhibitory concentrations of antimicrobial peptides, resistant bacterial strains did not develop (Steinberg, 1997, Ge, 1999, Tew, 2002). Current knowledge regarding peptide structure and function relationships gives the potential to synthetically develop antimicrobial peptides with a broad-spectrum of activity against bacteria, viruses and fungi, allowing a range of organisms to be targeted in a single treatment. With the development of new high throughput screening techniques there is the potential to examine a high quantity of peptides, for their respective antimicrobial activities, in a short period of time, making this approach economically advantageous (Hilpert, 2009).

Despite these promising attributes there are still considerable challenges in the clinical application of CAMPs. Their modes of action seem to depend strongly upon the experimental conditions to which they are exposed. Any proteolytic degradation *in vivo* would negatively impact pharmokinetics and there have been doubts concerning the peptides ability to reach high bactericidal activity under the pH and salt conditions present in serum, thus potentially making them difficult to use in systemic applications (Sitaram & Nagaraj, 1999, Hancock, 2002). Additionally, the cost of manufacturing peptides is exceptionally high and strategies to lower production costs, for example, by using bacteria, fungi, plant or animal expression systems, have not proven any feasible results to date (Hancock & Sahl, 2006).

Recent work has indicated that CAMPs can act as potent modulators of innate immunity, providing an alternative route to combat infection. The innate immune system is often triggered through activation of host pattern recognition receptors via

bacterial signature molecules, such as LPS, which can lead to sepsis due to over-stimulation. Certain CAMPs have been shown to supress toll-like receptor (TLR) signalling responses and the subsequent production of proinflammatory cytokines, such as TNFα, reducing septic shock in animal models (Bowdish*, et al.*, 2005). Additionally, human LL-37, a small naturally occurring AMP, has been shown to protect from infection in animal models without having any direct antibacterial activity (Bowdish*, et al.*, 2005), this is thought to be due to a selective upregulation of innate immunity, whilst specifically inhibiting proinflammatory cytokine responses. The use of AMPs as anti-infective agents that act through manipulation of the innate immune system has additional value as it limits the chance of the development of bacterial resistance. This is because the peptides act via immune modulation, rather than having a direct impact on the microorganisms themselves.

1.12 The ApoE Antimicrobial Peptide

Human apolipoproteins are amphipathic detergent-like molecules usually studied in relation to lipid metabolism, as they are known to bind lipids forming lipoproteins that help transfer dietary fats through the blood stream (Mahley, 1988). They have previously been linked to cardiovascular disorders and as a contributor to Alzheimer's disease but recent work now indicates a possible link to infection (Itzhaki, 1998, Burgos*, et al.*, 2005, Itzhaki & Wozniak, 2006). Apolipoproteins fall into six classes A-J, within which are several subclasses. Human apolipoprotein E (apoE) is a soluble protein with a role in lipid transport in plasma and CNS. Apolipoprotein E has 3 common structural isoforms, the *APOE-ε4* allele (which codes for the apoE- *ε4* isoform) has been associated with the greatest risk of developing Alzheimer's disease (Dobson*, et al.*, 2003, Burgos*, et al.*, 2005). The

APOE-ε4 allele additionally protects from liver damage in those infected with hepatitis C (Wozniak, 2002).

The molecular mechanisms of how the *APOE* allele influences infection are largely unknown but it is likely that it involves a direct interference of apoE with the infection process. This may include the modulation of inflammation during infection (Lynch, 2003), an influence in tissue repair post infection (Itzhaki, 1998), by impacting virus transport in the blood (Burgos*, et al.*, 2005) or an antimicrobial mode of action similar to human AMPs, such as defensins, lysozyme and lactoferrin (Andersen*, et al.*, 2003). In terms of viral infection, direct interactions between viruses and lipoproteins have been previously reported (Mehdi, 1994). Viruses may enter cells directly attached to lipoproteins, an interaction possibly mediated through apolipoproteins (Mehdi, 1994). Both viruses and lipoproteins initially bind to the extracellular matrix e.g. heparin sulphate proteoglycans (HSPGs) and then often to various low-density lipoprotein cell receptors (LDLRs) before entering cells (Bates*, et al.*, 1993). ApoE also uses these HSPGs and LDLR receptors during cell entry. It is therefore considered that apoE may be able to compete for these attachment sites with the virus particles, competitively inhibiting viral cell entry (Itzhaki*, et al.*, 1997, Itzhaki, 2004).

There has been strong evidence to support the theory that apolipoprotein E acts as an immunomodulatory agent. Studies have demonstrated that both apoE containing lipoproteins and synthetic apoE peptides prevent the proliferation of cultured lymphocytes by inhibiting DNA synthesis and also by reducing the turnover of phospholipids in T cells (Hui*, et al.*, 1980, Dyer*, et al.*, 1991). *In vivo* studies have

shown that apoE knockout mice have abnormally low antigen-specific delayed type hypersensitivity responses, after immunization with tetanus toxoid, compared to the wild-type mice (Laskowitz*, et al.*, 2000). Additionally, injection of LPS into the brains of apoE deficient mice stimulated a lower production of inflammatory cytokines, such as TNFα, than the control population, suggesting that apoE may be able to bind LPS limiting the inflammatory response (Laurat*, et al.*, 2001, Davenport & Tipping, 2003). ApoE deficient mice were also shown to have an increased susceptibility to bacterial challenge with *Listeria monocytogenes* (Roselaar & Daugherty, 1998, De*, et al.*, 2000) and *Klebsiella pneumoniae* infection (de Bont*, et al.*, 1999).

1.12.1 ApoE binding region anti-infective activity

The receptor-binding region of apoE has previously been shown to have direct anti-infective activity (Dobson*, et al.*, 2006). It is located between residues 130 and 150 close to the two sites that determine the isoform of the protein and influence its structure. The sequence between residues 142-147 gives rise to the heparin-binding domain, which mediates the attachment of apoE to cellular HSPGs and the LDLR receptor family (Dobson*, et al.*, 2006). A stabilized, octadecamer, tandem repeat peptide of apoE141-149 referred to as apoEdp has been used to investigate the biological activity and anti-infective activity of the apoE binding region in more detail (Kelly, 2007). This short tandem repeat peptide was found to have antiviral activity against HSV1 and HIV, by blocking viral attachment *in vitro* – possibly by binding the cellular HSPG sites – and potentially by exerting a virucidal action on the herpes virus. Additionally, apoEdp displayed high antibacterial activity against *S. aureus* and *P. aeruginosa*, which may be due to its cationic nature (Dobson*, et al.*,

2006). Studies by Kelly into the structure/activity relationships of other apoEdp derived AMPs revealed antimicrobial activity was abolished by the replacement of cationic residues (Kelly, 2007). The substitution of some or all of the Leu residues with the aromatic Trp residue increased potency for the majority of species, however, unsubstituted apoEdp showed the highest activity against *P. aeruginosa*, whereas Phe substitutions increased activity against *S. aureus*. In general, higher activity was observed with peptides that contained bulkier residues separating the cationic amino acids (Kelly, 2007). Potential mode of action of these cationic peptides against bacterial cells could be to disrupt the negative charge of the cell surface promoting attachment, as is often seen with small cationic α-helical peptides. Following attachment, the previously discussed peptide modes of action could take effect. Both the 'carpet model' and the 'aggregate pore mechanism' appearing possible in the case of the apoEdp peptide, (Figure 1.4) whereas the 'barrel stave' mechanism is unlikely as it is linked to peptides above 22 amino acids in length, which apoEdp AMPs are not (Sato & Sato, 2006). As apoEdp has broad anti-infective properties and shows very low haemolytic activity it is possible that it could be modified to obtain a safe and effective AMP against human pathogens (Kelly, 2007).

1.12.2 ApoE antimicrobial peptide toxicity towards mammalian cells

Many CAMPS show selectivity towards microbial membranes in comparison to the host organism (Sitaram & Nagaraj, 1999). This selectivity is believed to be due the spatial arrangement of the hydrophobic and hydrophilic components of the CAMP that are able to distinguish between the negatively charged bacterial membrane and the more neutral and cholesterol rich mammalian cell membranes. Whilst there have been reports of cytotoxicity in CAMPs (Lichtenstein, Maher & McClean, 2006),

peptide derivatives of the apoE have demonstrated antibacterial activity whilst showing low-haemolytic activity towards mammalian cells (Kelly, 2007, Kelly*, et al.*, 2010).

1.13 Biofilms

The term biofilm is defined as a matrix-enclosed bacterial population adherent to each other and/or surfaces or interfaces. This definition includes microbial aggregates and floccules and also adherent populations within the spaces of porous media (Costerton*, et al.*, 1995). In natural environments, the majority of bacteria can be found in a close association with a surface. They form complex multispecies communities with intricate architecture and distinct chemical properties. A single biofilm consists of a large number of tower and mushroom shaped micro colonies (Figure 1.5), encased in a hydrated matrix (Sauer, 2002). This matrix of exopolymeric substances (EPS) is a key component in biofilm formation and consists largely of cells, water and secreted macromolecules from the resident microorganisms (Gilbert*, et al.*, 2002). The matrix forms a site of enzymic and regulatory activities that allow the structure and integrity of the biofilm to be greatly influenced by the surrounding macro-environment, for example, the fluid dynamics of an area (Allison, 2003). In essence, a biofilm can be seen as an organised multi cellular ecosystem. It is thought that exposure to antimicrobials may alter the biofilms community structure either by clonal expansion of innately insusceptible bacteria or a through an induced adaption of the original population (Gilbert*, et al.*, 1997).

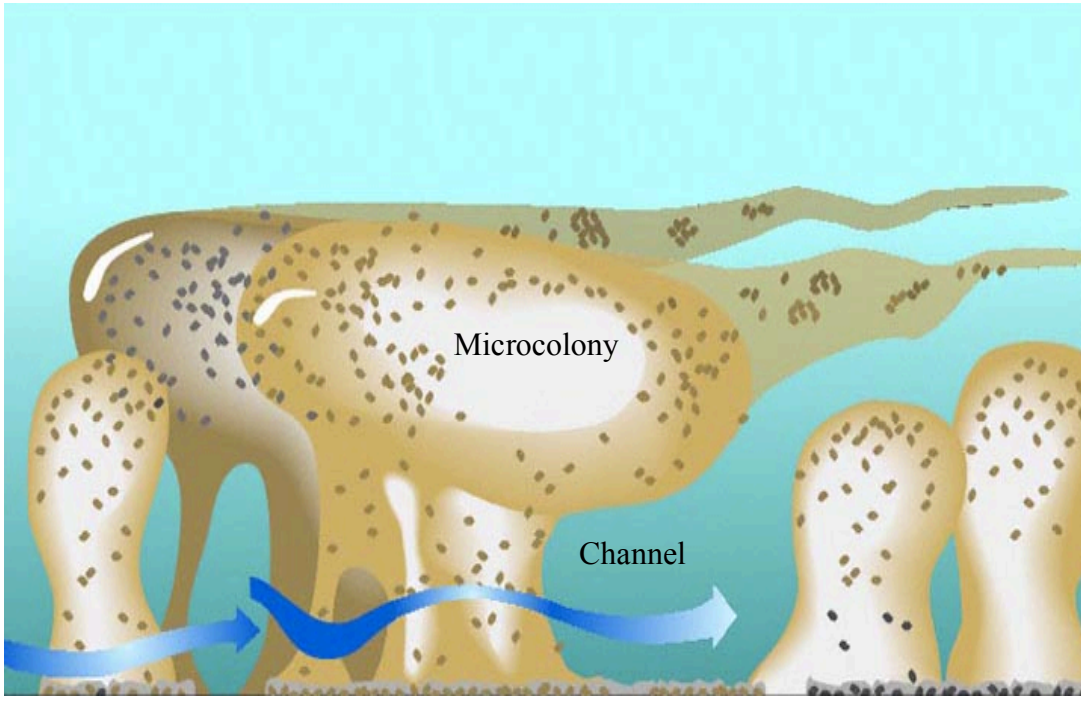

Figure 1.5. A diagrammatic representation of the structure of a biofilm. Mushroom shaped stacks (colonies) contained in an EPS matrix form an anionic polymer diffusion barrier. Water channels shape the structure as well as providing a route for nutrient distribution and waste product removal (Dirckx, 1997).

1.14 Biofilm Formation

The formation of a biofilm can be viewed as a step-by-step process, as shown in Figure 1.6. The first event is the formation of a conditioning film and the attraction and reversible attachment of planktonic bacteria to a surface via weak Van der Waals forces. In a flowing system, organisms tend to be drawn within a close proximity of a surface due to fluid dynamics (Towler, 2006). In comparison, in a non-flowing system, this surface attraction is determined by the condition of the adhering surface. This initial conditioning film provides new sites for bacterial attachment such as mucin and fibronectin (Gilbert*, et al.*, 1997). The second stage of development involves the anchorage of the initial planktonic colonisers to the surface via cell adhesion molecules such as pili. The conversion from planktonic cell to attached one involves a great deal of gene regulation and consequent production of proteins that

play a role in the motility and chemotaxis of the cell (Costerton*, et al.*, 1995). The third stage of biofilm development is microcolony formation, in which the early colonisers facilitate the adhesion of the subsequent cells due to an increased diversity in adhesion sites and the production of an extracellular matrix commences. EPS comprises of a mesh of polysaccharide fibres that increase the biofilms mechanical stability and anchors it to a surface via the occurrence of non-covalent interactions, either directly between EPS chains or via multivalent cation bridges (Dirckx, 1997). Once the bacteria become irreversibly attached to a surface, if the environment is nutritionally sufficient, the biofilm begins to mature. Coaggregation interactions are thought to contribute to the biofilms development. During coaggregation bacterial single cells recognize and adhere to genetically distinct cells, either already within the biofilm or by the prior coaggregation of secondary colonizers followed by the adhesion of this coaggregate to the developing biofilm. This process is known as co-adhesion and allows the newly co-adhered cells to become part of the mature biofilm community (Rickard, 2003). As the biofilm enters the fourth stage of maturation, stack shaped colonies, as shown in Figure 1.5, start to develop due to the cell-to-cell communication and organisation between the bacteria (Rice, 2005). This signalling between the cells is referred to as quorum sensing and has a key role in the biofilms development (Rice, 2005, Kong, 2006). Quorum sensing instigates the alteration of bacterial gene expression in response to cell density. Bacteria that undergo quorum sensing secrete signalling compounds, such as N-Acyl homoserine lactones (AHL) in Gram-negative organisms, oligopeptides in Gram-positive ones or Auto-inducer 2 (Ai2) that may be used by both Gram-types. These molecules can be detected by certain receptors found on the bacterial cell surface, which allows the bacteria to

measure the cell density of the biofilm and may induce density dependent changes the expression of genes (Greenberg, 2003).

Over time the biofilm may mature producing nutrient, pH and gaseous gradients throughout. This will provide varying areas with different selective pressures and influence the interactions with the neighbouring cells. The final climax communities are very resistant to further colonisation (Vroom*, et al.*, 1999). Once nutrient availability becomes depleted, small clusters of cells detach from the stacks and move to find a new nutrient rich environment, thereby allowing the movement of the cells to begin a new biofilm cycle (Figure 1.6) (Costerton*, et al.*, 1995).

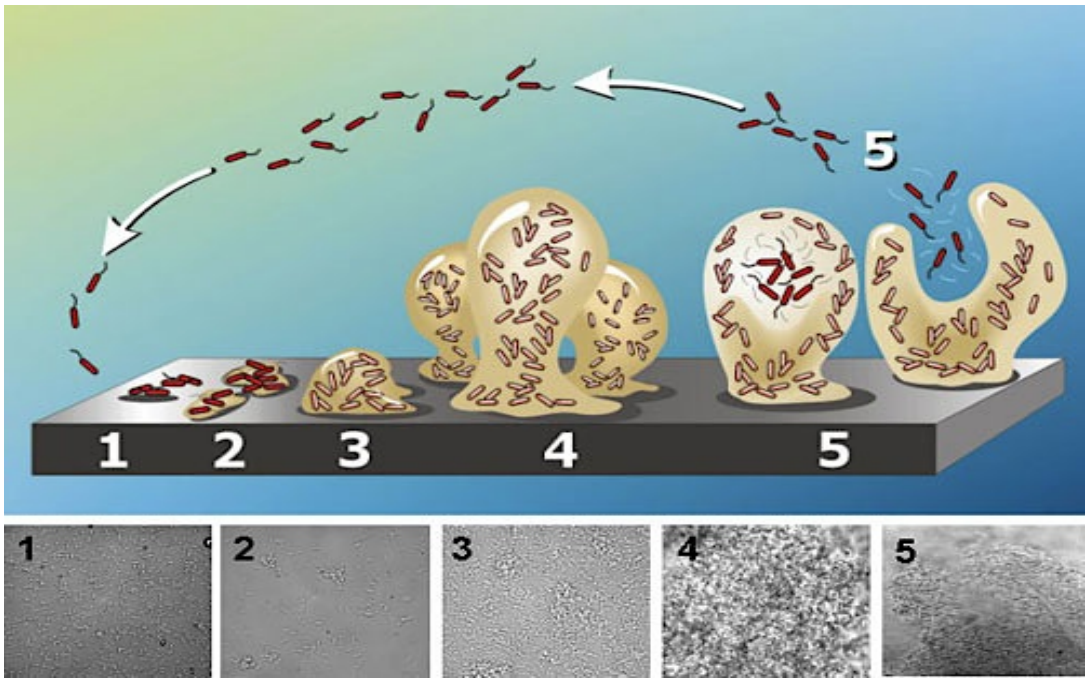

Figure 1.6. The development cycle of biofilm formation in *Pseudomonas aeruginosa*. A diagrammatic representation of the individual stages that occur from the transition of a planktonic bacterial cell to an adhered biofilm. The image represents the initial attachment of the bacterial cell to a surface (1), leading to the gradual formation of permanently attached microcolonies (2). These develop and mature into larger more stable stack like colonies due to quorum sensing between the cells (3-5) and lead to the release of cells from the biofilm, once nutrient levels deplete, and the final stage of detachment occurs (5) (Sauer, 2002).

1.15 Biofilm Insusceptibility to Antimicrobials

There are numerous advantages for bacteria to exist in a biofilm. These advantages include an increased resistance to sheer forces, accelerated rate of gene transfer and significantly an increased resistance to antimicrobials (Zhu*, et al.*, 2010, Gilbert, 2002). Bacteria within a biofilm may show up to 100 times more resistance to antimicrobial treatments, when compared to their planktonic counterparts (Cochran*, et al.*, 2000, Gilbert, 2001). Biofilm resistance, in general, will tend to develop as the biofilm matures. Cell co-aggregation and adhesion to a surface will alter the bacterium's initial susceptibility (Gilbert, 2001), subsequent EPS production and the thickening of the biofilm results in further substantial reductions in antimicrobial susceptibility (Gilbert, 2002). Research has identified several innate factors that may alter the biofilms susceptibility towards antimicrobial agents, which have been reviewed in detail (Mah & O'Toole, 2001, Donlan & Costerton, 2002, Patel, 2005). These factors are summarised in the following section.

1.15.1 Aggregation of bacteria and initial surface attachment

Once bacterial cells begin to cluster, the cells in the centre of the cluster become shielded from external stresses, such as exposure to antimicrobials, and therefore the antimicrobial is seen to be less effective (Gilbert & Gilbert, 2002). Once cells attach to a surface there may be a minor change in antimicrobial susceptibility, due to a change in gene regulation, including the up regulation of operons associated with the biofilm phenotype that may confer resistance, for example, EPS production (Whiteley, 2001). These induced factors may work in a synergistic fashion with innate factors to enhance bacterial survival in the face of an antimicrobial stress.

1.15.2 Exopolymeric matrix production

The biofilm matrix may act as a permeability barrier limiting the antimicrobials diffusion towards its target site (Donlan & Costerton, 2002). This thick layer of EPS, DNA and protein may limit biocide diffusion and protect bacteria embedded in the deeper layers of the biofilm (Gilbert*, et al.*, 2002). The alginate EPS produced by *P. aeruginosa* has been studied in depth due to its ability to trap antimicrobials, an ability which is thought to be due to its strong anionic nature. *P. aeruginosa* biofilms are extremely recalcitrant to many antimicrobial therapies, especially towards cationic biocides (Bagge*, et al.*, 2004), as the cationic molecules are attracted towards the negatively charged matrix where they become trapped and cannot interact with the bacterial cell. In addition, EPS may chemically interact with an antimicrobial rendering it inactive and allowing the biofilm to survive any antimicrobial chemotherapy (Mah & O'Toole, 2001).

1.15.3 Slow growth and stress within the biofilm

Bacteria located deep inside the biofilm experience a lack of nutrients and oxygen, as well as a build up of waste products such as carbon dioxide. They therefore show a decrease in their metabolic activity and often display a cessation of growth (Donlan & Costerton, 2002, Dunne, 2002). Since many antimicrobials target actively growing cells, bacteria in the deeper layers of the biofilm may be less susceptible to antimicrobial action, due to their relative metabolic inertness. For example, Gilbert and colleagues examined the impact of active growth on the antimicrobial susceptibility of planktonic and biofilm cultures of *E. coli*, *P. aeruginosa* and *S. epidermidis* and observed that susceptibility towards tobramycin or ciprofloxacin increased with an increasing growth rate (Gilbert*, et al.*, 1997). The slow growth and

altered metabolism of the biofilm has lead researchers to hypothesise that the cells are in fact in a constant stationary phase state (Anderl*, et al.*, 2003). Studies into biofilm formation in *K. pneumoniae* revealed increased catalase expression, not only in stationary phase cells in planktonic culture, but also in biofilms when compared to exponentially growing counterparts (Anderl*, et al.*, 2003). Catalase is able to break down hydrogen peroxide, thereby decreasing the oxidative stress that the biofilm is exposed to, thus it appears a stress response may become activated within bacterial biofilms that could confer a protective impact to the bacteria imbedded within it (Gilbert*, et al.*, 1997).

1.15.4 Presence of persisters

Within a biofilm lies a small population of bacterial cells that may differentiate into a resistant spore-like state, these are referred to as persisters. These mutant persisters are believed to be an additional innate mechanism that contributes towards antimicrobial resistance in biofilms (Lewis, 2005). This differentiated state is often believed to be due to phenotypic variation and not a stable genetic change (Keren, 2004). Persisters typically display a slow rate of growth thereby making them less susceptible to antimicrobials, as discussed previously. However, studies have revealed persisters can survive treatments with bactericidal levels of antimicrobials that are able to kill non-growing microorganisms (Kaldalu, 2004), indicating that decreased growth rate alone is not able to account for this increased resistance. It has been hypothesised that persister cells are able to achieve this stasis by shutting down certain antibiotic targets, via the actions of persister/MDT proteins, resulting in a dormant, antimicrobial tolerant cell (Lewis, 2005)

1.15.5 Efflux within the biofilm

Antibiotic treatment of a biofilm is thought to induce the expression of various bacterial virulence factors. Efflux pumps play a role in antibiotic resistance within planktonic bacteria and are also thought to influence bacterial survival within a biofilm (Patel, 2005). Analysis of the change in gene expression during biofilm formation, in the presence of a particular antimicrobial treatment, helps establish which induced mechanisms play a role in the biofilms survival. For example, *P. aeruginosa* contains three known multi-drug efflux pumps, the study of which has suggested the importance of one of these pumps in the biofilms resistance towards ofloxacin. By using strains of *P. aeruginosa* lacking or overexpressing the MexAB-OprM pump it was demonstrated that, in the presence of the antibiotic, the biofilms lacking the pump were far more susceptible than those over expressing it (Ziha Zarifi, 1999).

1.16 Biofilm Formation on Indwelling Medical Devices and Preventative Strategies

Biofilms display low antimicrobial susceptibility and are rarely resolved by immune defence mechanisms. The formation of biofilms on implanted medical devices such as catheters, artificial joints as well as wound dressings and gauzes can lead to persistent bacterial infections that are difficult to treat (Costerton*, et al.*, 1999). Complications such as the development of a systemic infection or the need for device removal are common. Artificial implant contamination can arise via direct inoculation of bacteria to the device surface or from the blood. Organisms commonly found on the patients skin such as *S. aureus* and *S. epidermidis* can easily establish a foothold once within the host, due to a lack of competition (Hume*, et al.*, 2004,

Vuong*, et al.*, 2004). Other bacteria commonly associated with device related infections are summarised in Table 1, adapted from (Donlan, 2001) .

Table 1. Microorganisms commonly associated with biomedical device infections.

Biomedical device	Organisms
Urinary catheter	*Escherichia coli, Klebsiella pneumoniae, Staphylococcus epidermidis and Enterococcus faecalis*
Central venous catheter	*Coagulase-negative Staphylococci, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae and Pseudomonas aeruginosa.*
Artificial hip prosthesis	*Coagulase-negative Staphylococci, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa.*
Intrauterine device	*Staphylococcus epidermidis, Corynebacterium species, Staphylococcus aureus, Micrococcus species and Enterococcus species.*
Prosthetic heart valve	*Coagulase negative Staphylococci, Staphylococcus aureus and Enterococcus species*

Current strategies to limit the bacterial contamination of biomedical device surfaces involve the incorporation of various agents, which either prevent physical adherence of the microorganism (passive inhibition) or contain a compound capable of killing the bacteria through direct contact (active inhibition) (Monteiro*, et al.*, 2009). The substratum of the device greatly influences the rate and extent of microbial adherence, with rougher more hydrophobic surfaces tending to show a higher level

of biofilm development (Fletcher & Loeb, 1979). The fluid dynamics surrounding the device, for instance within the urinary tract or blood stream, and the formation of a conditioning film comprised largely of host proteinaceous material, further controls attachment and biofilm establishment (Fletcher & Loeb, 1979).

Polymers that have previously shown to reduce short-term bacterial attachment include polyethylene glycol (PEG) (Harbers, 2007) and certain peptide mimic polymers (Statz*, et al.*, 2008). Surface coatings containing components capable of killing bacteria may involve a covalently attached antimicrobial agent or one that is leachable into the surrounding environment. Previous examples of active coatings include cationic polymers such as chitosan (Fu, 2005), quaternary ammonium compounds (Murata*, et al.*, 2007) and triclosan (Edmiston*, et al.*, 2006). A worry with the use of biocidal coatings is their level of toxicity towards the mammalian cells, to which they are exposed, and the potential for the development of cross-resistance to third party agents such as other biocides and antibiotics. Another approach widely considered in the prevention of device assocated infections is the use of antibiotic coatings, such as penicillin (Aumsuwan*, et al.*, 2007) and vancomycin (Jose, 2005) but this approach is under scrutiny due to the possibility of the selection of antibiotic resistant organisms (Boucher*, et al.*, 2010). A newer approach is to use antimicrobial peptides, which offer a wide range of activity against microorganisms whilst functioning with some selectivity for bacteria over mammalian cells (Matsuzaki*, et al.*, 1995). Peptide mimics of host AMPs have been artificially synthesised with high activity, high stability and low cytotoxicity (Statz*, et al.*, 2008). They show considerable chemical diversity, biocompatibility and

resistance to protease degradation making them promising device coatings for the inhibition of microbial device contamination within the host (Kelly*, et al.*, 2010).

1.17 Aims

This project compares the antiseptic potential of a novel 18 amino acid long, tryptophan rich, cationic apoE derived peptide (apoEdpL-W) to that of commonly used biocides PHMB, chlorhexidine, triclosan and cetrimide. This evaluation will include detailed analysis of (i) the broad-range antimicrobial activity against microorganisms frequently associated with device-related infections, (ii) the anti-biofilm potency of the test compounds, (iii) the effects that long-term antimicrobial exposure has on bacterial susceptibility and physiology (iv) the antimicrobials biocompatibility with a mammalian cell line and (v) the association of the test agents with different biomaterial polymers and their resulting antimicrobial activity.

Chapter 2

General Experimental Methods

2.1 Bacterial Strains

Pseudomonas aeruginosa ATCC 9027*, Staphylococcus aureus* ATCC 6538 and *Serratia marcescens* ATCC 13880 were obtained from Oxoid (Basingstoke, UK). *Burkholderia cepacia* ATCC BAA-245*, Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 13883 were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Micrococcus luteus* MRBG 9.25*, Staphylococcus caprae* MRBG 9.3, *Staphylococcus capitis* MRBG 9.34*, Staphylococcus lugdunensis* MRBG *9.36, Staphylococcus warneri* MRBG 9.27*, Staphylococcus epidermidis* MRBG 9.33 and *Staphylococcus haemolyticus* MRBG 9.35 were obtained from the axillae of three healthy male volunteers, ranging from 25 to 30 years old. *Bacillus cereus* MRBG 4.21, *Stenotrophomonas maltophilia* MRBG 4.17 and *Chryseobacterium indologenes* MRBG 4.29 were isolated from a domestic kitchen drain biofilm (McBain, 2003). *Enterococcus faecalis* and *Corynebacterium xerosis* were both wild-type strains isolated from a chronic wound. Species were identified using 16s rRNA sequencing prior to use.

2.2 Chemicals and Bacterial Growth Media

Triclosan, cetrimide and chlorhexidine were obtained from Sigma-Aldrich (Poole, UK). Vantocil^{TM} a 20 % aqueous solution of PHMB was obtained from Arch Chemicals Inc. (Manchester, UK). Peptides were purchased from Alta Bioscience (Birmingham, UK) having been synthesized using 9-fluorenylmethyl carbamate chemistry and purified by high performance liquid chromatography. Bacteriological media was purchased from Oxoid (Basingstoke, UK). All other chemical reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.3 Bacterial Culture Growth and Cryopreservation

Bacterial growth medium was sterilised at 121˚C, 15 psi for 15 min using a Prestige Medical bench top autoclave (Prestige Medical, Sutton Coldfield, UK). Bacteria were cultured onto Muller-Hinton Agar (Oxoid, UK) and incubated aerobically at 37˚C for 18 h unless stated otherwise. Bacterial planktonic cultures were grown in Muller-Hinton Broth (Oxoid, UK), incubated at 37˚C and 100 rpm for 18 h unless otherwise stated. Bacterial cryogenic stocks were produced and archived at -80˚C.

2.4 Minimum Inhibitory Concentrations (MICs)

Single colonies of test bacteria from 18-24 h old agar plates were inoculated into 10 ml of sterile Muller-Hinton Broth and incubated for 18-24 h at 37˚C and 100 rpm until an approximate OD_{600} of 0.8 was reached. Cultures were diluted to 1:100 into sterile media to use as an inoculum for biocide susceptibility tests. All biocide stock solutions were prepared at 1.16 mg ml^{-1}. Stock solutions of chlorhexidine and PHMB were prepared in sterile distilled water. Triclosan and cetrimide stock solutions were prepared in 25% ethanol and 25% methanol respectively. ApoEdp and apoEdpL-W peptide stock solutions were prepared using 5% DMSO. All solutions were filter sterilised using 0.2 µm porosity nitrocellulose filters (Millipore, Watford, UK) and stored at –80˚C until use. To determine minimum inhibitory concentrations (MICs) of antimicrobials, diluted overnight culture (200µl) was delivered to each test well of a 96-well microtiter plate (Becton Dickinson, Oxford, UK). Doubling dilutions of each antimicrobial were prepared at five times test concentration, in sterile water, then diluted 1:5 into fresh medium in the test wells of the plate, to give a final volume of 250 µl. The plates were incubated for 24 h at 37˚C and 100 rpm. MICs were determined as the lowest concentration for which bacterial growth did not occur.

Growth was assessed according to turbidity (496 nm) in comparison to an uninoculated well (negative control) detected using a microtiter plate reader (Anthos HTII; Anthos-Labtec Instruments. Salzburg. Austria). Controls for 25 % ethanol, 25 % methanol and 5% DMSO were included. Each assessment was carried out in three technical replicates (in the same plate) and two biological replicates.

2.5 Minimum Bactericidal Concentrations (MBC)

To determine MBCs, 10 µl aliquots were taken from each well of the previously performed MIC plate and spot plated onto a Muller-Hinton Agar plate in triplicate. Plates were incubated overnight at 37˚C. MBCs were determined as the lowest concentration of biocide at which no growth occurred after 4 d of incubation. Neutralisation of the biocide using Den/Engley universal neutralising broth, prior to spot plating, provided no significant change in MBC as determined by previous validation studies and was therefore deemed unnecessary.

2.6 Determination of Minimum Biofilm Eradication Concentration (MBEC)

Single species biofilms were grown on the pegs of a Calgary Biofilm Device (CBD) (Ceri, 1999). The CBD is a two-part reaction vessel; the base of the vessel consists of a standard 96-well microtiter plate that underlies a pegged lid. The lid has 96 pegs, each of which protrudes into the centre of a single underlying well. To produce inoculant for the biofilm susceptibility testing, single colonies of test bacteria from 18-24 h old culture plates were inoculated into 10 ml of sterile Muller-Hinton Broth and incubated at 37˚C in a shaking aerobic incubator (100 rpm) for 18 h. Cultures were diluted to 1 McFarland Standard, then further diluted 1:30 using fresh growth

medium. 200 µl of bacterial inoculum was added to each well of the CBD base, plates were then incubated at 37˚C and 30 rpm for 48 h to allow biofilm formation on the pegs. After 48 h the lid was removed and the pegs were rinsed twice in 250 µl of sterile PBS, contained within the wells of a sterile 96-well wash plate. Doubling dilutions for biocide stocks to be tested were prepared at five times test concentration and were then diluted 1:5 into sterile Muller-Hinton Broth in the test wells of a 96-well 'antimicrobial challenge plate' (250 µl). Biofilms were exposed to antimicrobials by transferring the CBD lid to the 'challenge plate' and incubating for 24 h at 37˚C and 100 rpm. After incubation the lid was removed and each peg was rinsed three times in 300 µl of sterile PBS, before being transferred to a 96-well 'regrowth plate', containing 300 µl of sterile Muller-Hinton Broth. Once the lid was transferred, the regrowth plates were sonicated on high (approximately 30kHz) for 15 min and incubated for 24 h at 37˚C and 100 rpm. Minimum biofilm eradication concentrations (MBECs) were determined as the lowest concentration for which bacterial growth did not occur after 18 h of incubation. Growth was viewed as turbidity in comparison to an uninoculated well (negative control). Each MBEC was carried out with three technical replicates (in the same plate) and two biological replicates.

2.7 Bacterial Training to Sub-Lethal Level of Antimicrobial Exposure

Reproducible antimicrobial concentration gradients were created on Muller-Hinton Agar plates using a spiral plater (Whitley automated spiral plater, Don Whitley Scientific, Shipley, UK). The plater dispenses liquid in an Archimedes spiral onto the surface of a rotating agar plate creating a 100-fold concentration gradient decreasing from the centre of the plate towards the edge. Petri dishes (10 cm diameter) were

filled with 27.5 ± 1.0 ml of Muller-Hinton Agar to produce a mean depth of 3.5 mm, before being left for 24 h at room temperature to dry, prior to use. Initial MIC concentration antimicrobial stock solutions (50 µl) were deposited on the agar surface using the spiral plater. Plates were dried for 1 h at room temperature prior to radial deposition of bacterial pure cultures. Each plate was divided into 9 segments, each segment containing a different test microorganism. Plates were then inverted and incubated for 3 d at 37˚C in a static aerobic incubator. After incubation, growth observed at the highest biocide concentration was aseptically removed using a sterile loop and streaked onto a fresh plate, containing the same antimicrobial concentration gradient. If growth was observed across the whole antimicrobial gradient, a loopfull of bacteria was transferred to a new plate containing a twice as high stock solution concentration. This process was repeated until 10 passages onto a new plate had occurred. Bacteria were then passaged a further 10 times in the absence of any antimicrobial (X10). Bacteria at P0, P10 and X10 were archived as cryogenic stock at -80˚C for subsequent MIC and MBC testing, against the antimicrobial it had undergone prolonged exposure to.

2.8 Crystal Violet Biofilm Assay

Overnight cultures of test bacteria were grown to an approximate OD_{600} of 0.8 and diluted 1:100 into sterile Muller-Hinton Broth to provide a bacterial inoculum for the assay, following which 150 µl of inoculum was delivered to each test well of a 96-well microtiter plate. Plates were incubated for 48 h at 37˚C and 20 rpm to promote biofilm growth. After incubation wells were washed twice with 200 µl of sterile PBS. After washing, 200 µl of 0.5% crystal violet solution was added to the test wells and the plates were incubated for 30 min at room temperature. Subsequently, wells were

washed twice with 250 µl of phosphate buffered saline (PBS) and left to dry at room temperature for 1 h. Attached crystal violet was solubilised within the well by adding 250 µl of ethanol and plates were agitated at room temperature at 20 rpm for 1 h. After solubilisation, biofilm growth was viewed as change in OD_{600} relative to a sterile negative control.

2.9 Determination of Planktonic Growth Rate

Overnight suspensions of bacteria were diluted 1:100 in 200 ml of Muller-Hinton Broth in a sterile conical flask and incubated at 37˚C with shaking at 100 rpm. 1 ml samples were removed at regular intervals and diluted as appropriate. Cultures were transferred into 1 ml semi-micro cuvettes and optical density was measured at 600 nm using a Helios spectrophotometer (Pye Unicam Ltd, Cambridge, UK). Sterile controls were included. Growth rate was plotted as OD_{600} over time (h).

2.10 Proteomic Analysis of Bacteria via Two-Dimensional Electrophoresis

2.10.1 Protein Extraction

To extract protein from strains of interest, 10 ml bacterial cultures were grown in Muller-Hinton Broth at 37˚C and 100 rpm for 18 h. Cultures were then diluted 1:100 into 200 ml of sterile Muller-Hinton Broth in a conical flask and incubated at 37˚C and 100 rpm until approximate mid-log phase of growth was reached (OD_{600} of 0.4). The culture was spun at 12,000 x g using a Denley DL136 bench centrifuge (DJB Labcare LTD, Buckinghamshire, UK) to pellet the bacterial cells. The cells were washed in three times in 3 ml of ice cold PBS before being resuspended in 1 ml of PBS. Resuspended cells were disrupted via sonication using the MSE Ultransonic

disintegrator Soniprep 150 (Fisher Scientific Ltd, Loughborough, UK) at an amplitude of 10 microns in 6 x 30 second bursts, followed b y 30 seconds of cooling on ice at each interval. After sonication, protein was precipitated by mixing a 1:1:8 solution of cell lysate with trichloroacetic acid and ice-cold acetone. The protein solution was left to precipitate at -20˚C for 1 h. Protein was pelleted by centrifugation at 16,000 x g using a Sigma 1-14 microcentrifuge (Poole, UK) before being washed in three times in 1 ml of ice-cold acetone. The protein pellet was dissolved in 2 ml of rehydration buffer (9M urea, 2% CHAPS, 1% DTT, 2% Carrier ampholytes, 0.5% protease inhibitor, 0.001% bromophenol blue) via incubating for 1 h at room temperature and vortexing for 30 seconds every 15 min. Soluble protein concentration was quantified using the Bradford assay (Sigma, Poole, UK). In brief, 20 µl of rehydrated protein solution was added to 1 ml of Bradford's reagent and was incubated for 4 min at room temperature before OD_{595} was measured. Protein concentration was determined using a standard curve, produced using bovine serum albumin standards of known concentrations.

2.10.2 Protein loading and isoelectric focusing

Protein concentration was adjusted using rehydration buffer to the required level. Between 250 µg and 500 µg of protein per 200 µl total volume of buffer was loaded into an 11cm ReadyStrip^{TM} immobilised protein gradient (IPG) strip (Bio-Rad, Hertfordshire, UK). Each sample of rehydrated protein solution was pipetted along an individual lane in the PROTEAN IEF Focusing Tray, avoiding bubbles (Bio-Rad, Hertfordshire, UK). A single IPG strip was laid gel side down into the tray to allow contact with the rehydrated protein solution. Strips were then covered in 2 ml of sterile mineral oil and rehydrated under active conditions overnight using a

PROTEAN IEF cell (Bio-Rad, Hertfordshire, UK). After strip rehydration, paper wicks dampened with 10 µl of deionised water were placed underneath the strips to cover the electrodes. IPG strips were covered with 2 ml of sterile mineral oil before isoelectric focusing was conducted as follows; Conditioning step S1, 250 V for 15 min; Voltage ramping S2, voltage increases linearly until 8000 V is reached; finally a 500 V is maintained until the run is stopped. In the first dimension of electrophoresis, the proteins were separated along an IPG strip depending upon their isoelectric points.

2.10.3 Equilibration and second dimension electrophoresis

After the first dimension of focusing, IPG strips were removed from the PROTEAN IEF Focusing Tray and placed on dampened filter paper for 1 min to remove any excess oil from the gel side of the strip. At this point strips could be wrapped in cling film and stored at -80˚C until required for use. To equilibrate, strips were placed gel side up in a disposable plastic tray and 5 ml of equilibration buffer 1 (6M urea, 2 % SDS, 50 mM Tris-HCL pH 8.8, 2 % glycerol and 1 % DTT) was added, immersing the strip. Trays were agitated at 20 rpm at room temperature for 20 min before buffer was decanted and the process was repeated with 5 ml of equilibration buffer 2 (6M urea, 2 % SDS, 50 mM Tris-HCL pH 8.8, 2 % glycerol and 2.5 % iodoacetamide). In the second dimension run, proteins were separated by size, via SDS-denaturing electrophoresis. Polyacrylamide casting gels (34 ml distilled water, 25 ml 1.5 M Tris-HCL pH 8.8, 0.5 ml of 20 % SDS and 40 ml of 30 % bis-acrylamide) were polymerised by the addition of 0.5 ml of 10 % Ammonium persulphate and 100 µl of TEMED. The gel was constructed as follows; two glass plates separated by a 1 mm thick plastic spacer were assembled in a Bio-Rad gel caster (Bio-Rad, Hertfordshire,

UK), approximately 50 ml of gel solution was poured between the plates immediately after addition of the APS/TEMED and the gels were left at room temperature for 1 h to polymerise. After polymerisation, approximately 15 ml of stacking gel solution (34 ml distilled water, 6.25 ml of 1 M Tris-HCL, 0.25 ml of 20 % SDS, 8.5 ml of 30 % bis-acrylamide, 0.25 ml APS and 50 µl TEMED) was poured above the set casting gel. A few drops of isopropanol were added to the top of the gel to obtain a bubble free surface layer. IPG strips were loaded above the stacking gel with the gel side of the strip contacting the larger glass plate. 10 µl of Bio-Rad broad range protein marker was added to a paper wick, which was inserted to the top left corner of the stacking gel to act as a molecular weight ladder. Approximately 3 ml of overlay agarose was poured over the IPG strip and wick to obtain a smooth top layer. Gels were run at 20 V for 1-2 h until the bromophenol blue dye front could be seen to have loaded onto the gel. Gels were then run at 55 V for 15-18 h until the dye front had migrated approximately 80 % through the length of the gel.

2.10.4 Gel staining

Gels were fixed for 8 h in fixing solution (500 ml ethanol, 400 ml water and 100 ml acetic acid) at room temperature. Fixing solution was decanted and replaced by 500 ml of coomassie blue stain (0.8g coomassie blue R350, 400 ml of 40 % methanol and 400 ml 20 % acetic acid) and left for 18 h at room temperature and 20 rpm. After staining, the coomassie blue stain was decanted and gels were destained using a destain solution (500 ml methanol, 400 ml water and 100 ml acetic acid), which was continuously replaced until the gel appeared clear and the blue spots could easily be visualised. Gel spots of interest were excised and proteins were identified using

tandem mass spectrometry, performed at The Biomolecular Analysis Facility within The University of Manchester. Gels were stored in a storage solution (25 ml acetic acid and 500 ml water) for up to 1 month.

2.11 16S rRNA Sequencing

Bacterial species were subjected to 16s rRNA sequencing to confirm identification. Single bacterial colonies were aseptically transferred to a microcentrifuge tube containing 100 µl of nanopure water, vortexed for 30 seconds and boiled at 100˚C for 15 min to lyse cells. Microcentrifuge tubes were centrifuged at 16,000 x g using a bench top centrifuge for 10 min to remove cellular debris. Supernatant was retained as DNA template for the polymerase chain reaction (PCR). PCR was performed using the primers 8FLP (5'-GAG TTT GAT CCT GGS TCA G-3') and 806R (5'-GGA CTA CCA GGG TAT CTA AT-3') at 5 µM per reaction {Rickard, *et al*., 2003}. PCR mixture consisted of 5 µl of forward and reverse primers, 25 µl of Red Taq Ready Mix ™, 5 µl of template DNA and 16 µl of PCR water. PCR was conducted using a Perkin Elmer Thermal Cycler model 480 and run for 35 thermal cycles: 94˚C (1 min), 53˚C (1 min) and 72˚C (1min). A 15 min elongation step was included in the final cycle. PCR products were purified using a QIAquick PCR purification kit (Qiagen, West Sussex, UK) according to manufacturers instructions and the resulting DNA yield was quantified using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). A reaction mixture containing 4 pM forward or reverse primer and 40-50 ng of DNA in 10 µl total volume was used for DNA sequencing. DNA sequencing was performed using the Applied Biosystems 3730 DNA Analyse system at the DNA Sequencing Facility within The University of Manchester.

2.12 Agarose Gel Electrophoresis

PCR products were visualised using agarose gel electrophoresis. In brief, 0.8 % agarose gels were prepared by dissolving 0.4 g of agarose powder in 50 ml of Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (50 x stock: 40 mM Tris base, 20 mM glacial acetic acid and 1mM EDTA, pH 8.0). The gel solution was microwaved on high for 3 min to ensure the solute had dissolved, before being allowed to cool for 10 min prior to the addition of 5 µl of GelRed ^{TM} (Biotium, Middlesex, UK), to allow visualisation of nucleic acids under ultra violet (UV) illumination (312 nm). Molten gels were poured into a Bio-Rad electrophoresis tank cast (Bio-Rad, Hempstead, UK) and allowed set for 1 h at room temperature before use. PCR products were supplemented with loading buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 10 % glycerol and 0.01 % (w/v) bromophenol blue) and 5 µl samples were loaded into a single lane within the gel, before being run for 1 h at 70 V. Molecular weight markers Hyperladder I or IV ^{TM} (Bioline Reagents Ltd, London, UK) were used to determine band size. Gels were visualised under UV illumination using a T2201 transilluminator (Sigma, Poole, UK).

2.13 Microscopy

2.13.1 Transmission electron microscopy (TEM)

Cultures (50 ml) were grown to an OD_{600} of 0.3 and bacterial cells were pelleted via centrifugation at 16,000 x g for 10 min. Cells were resuspended in 1 ml of 0.25 % gluteraldehyde at 4˚C, further fixed in 2 % osmium tetroxide, passed through an ethanol dehydration series and sectioned (80 nm). TEM was conducted using a FEI Polara 300kV FEG transmission electron microscope (FEI, Oregon, USA) at The University of Manchester imaging suite.

2.13.2 Confocal scanning laser microscopy

Bacteria were stained using the BacLight™ LIVE/DEAD staining kit (Invitrogen Ltd, Paisley, UK), before conducting fluorescent microscopy. BacLight™ uses green fluorescent SYTO9 stain and red fluorescent propidium iodide stain to differentiate between live and dead organisms respectively. Confocal scanning laser microscopy was used to conduct 3-dimensional micrograph analysis of fluorescently dyed samples. All confocal imaging was performed using the Nikon C1 Upright system (Nikon, Amsterdam, Netherlands) with 20x; 60x or 100x plan apochromatic objectives. Confocal settings were as follows; pinhole 30 μm, scan speed 400 Hz, unidirectional format 1024 x 1024. Images for FITC, DAPI and Texas red were excited by 488 nm, 405 nm and 543 nm laser lines respectively. The optical sectioning of samples allowed the acquisition of images at different focal planes. The confocal software was used to determine the optimal number of Z sections per sample. Only the maximum intensity projections of these 3D stacks are shown in the results. 3D images were reconstructed from the resulting Z-stack file using Imaris software (Bitplane AG, Zurich, Switzerland).

2.13.3 Widefield microscopy

2-dimensional fluorescent imaging was performed using the Zeiss Axioscope system (Carl Zeiss Ltd, Cambridge, UK) with a 10x, 20x or 100x objective. Samples were treated with appropriate fluorescent markers and visualized at the correct wavelength. For the BacLight™ LIVE/DEAD system bacterial cells stained with SYTO 9 or propidium iodide fluoresced green (viable) or red (dead) at 480 and 490 nm respectively. Images were captured using a digital microscope eyepiece and

exported as JPEGs for analysis with ImageJ software (National Institute of Health, Maryland, USA).

Chapter 3

The Effect of Long-Term Antimicrobial Exposure on Bacterial Susceptibility *in vitro*

Abstract

In the current chapter, the effect of repeated exposure of a test panel of bacteria (12 genera, 18 species) to the apoEdpL-W peptide, on bacterial susceptibility, was compared to the biocides cetrimide, chlorhexidine, polyhexamethylene biguanide and triclosan. A previously validated gradient plating technique was used to sub-lethally expose bacteria to the antimicrobials over ten passages. Susceptibilities (MICs and MBCs) were determined before and after antimicrobial exposure. Exposed bacteria were subsequently passaged ten times in the absence of antimicrobial, to determine the stability of the susceptibility changes. Minimum biofilm eradication concentrations (MBECs) were determined using the Calgary Biofilm Device. Biofilm-forming ability, before and after antimicrobial exposure, was determined using a crystal violet biofilm assay. Following exposure, susceptibility decreases (MIC or MBC) of over 2-fold occurred for apoEdpL-W (8/18 strains), cetrimide (7/18 strains), chlorhexidine (10/18 strains), polyhexamethylene biguanide, (9/18 strains) and triclosan (12/18 strains). Following growth in the absence of any antimicrobial, susceptibilities partially reverted to pre-exposure values for the majority of strains, however, certain organisms maintained reduced susceptibility even after the selective pressure was removed. Bacteria that demonstrated a 4-fold or greater alteration in MIC or MBC were examined for changes in biofilm forming ability. Significant alterations in biofilm forming capability occurred for ApoEdpL-W (2/18 strains), chlorhexidine (3/18 strains) and triclosan (3/18 strains). Exposure to polyhexamethylene biguanide or cetrimide did not result in any significant change in biofilm formation.

3.1 Introduction

Cationic antimicrobial agents have been used for over a century to control microbial growth (Gilbert, 2005). The mode of action of cationic biocides, such as quaternary ammonium compound cetrimide and biguanides which include chlorhexidine and polyhexamethylene biguanide (PHMB), relies largely on interaction with the bacterial cytoplasmic membrane, leading to membrane disruption and the leakage of cytoplasmic components (Broxton*, et al.*, 1984). Alternatively, biocides may have specific intracellular targets, such as enoyl-acyl carrier protein reductase FabI, a specific target for triclosan (McMurry, 1998, Gilbert, 2005). Whilst biocidal compounds have previously been used to achieve antisepsis in the clinical environment (Gilbert, 2005), they have also been used as hygienic adjuncts in soaps (Johnson, 2002), hand washes, and as hard surface disinfectants (Tanner, 1989). More recently they have been incorporated into medical device coatings (Gaonkar*, et al.*, 2003) and dressings (Silver, 2006), with the intention of inhibiting bacterial colonization and resulting biofilm formation (Mah & O'Toole, 2001, Campanac*, et al.*, 2002, Donlan & Costerton, 2002, Bjarnsholt, 2008).

Concerns have been raised that the extensive use of biocides may potentially select for bacterial lineages with antimicrobial resistance (Ledder*, et al.*, 2006). This may occur through the selection of target site adapted mutants (McMurry, 1998), non-susceptible lineages (Chuanchuen*, et al.*, 2003) or through induced phenotypic adaption (Gandhi, 1993). Despite the demonstrable benefits of some applications of biocides (Gaonkar*, et al.*, 2003, Zabramski*, et al.*, 2003), there are concerns about the potential generation of cross-resistance between biocides and antibiotics, due to the expression of broad-range efflux systems or mutations in shared pharmacological

targets (Moken, 1997, Poole, 2001). Additionally, possible issues with biocidal toxicity to human cells (Hidalgo & Dominguez, 2001) (Babich, 1995) has lead to the search for alternative antimicrobial actives. Whilst laboratory studies have demonstrated links between the exposure of pure cultures of bacteria to sub-lethal concentrations of various biocides and changes in antimicrobial susceptibility (Joynson, 2002, Karatzas, 2007), there is currently little evidence that this is the case in clinical practice (Gilbert, 2003, Rupp, 2004).

Cationic antimicrobial peptides (CAMPs), originating as components of the innate immune system, are attractive therapeutic antimicrobials and have a proven ability to prevent and eliminate infection (Zasloff, 2002). CAMPs display long-term stability, low toxicity and are proposed to present a low risk of the development of bacterial insusceptibility (Steinberg, 1997, Tew, 2002), which makes them candidate molecules for use in the production of antimicrobial device coatings (Statz*, et al.*, 2008).

Human apolipoprotein E (apoE) has been largely researched for its influence on the outcome of viral infection. Further studies by Kelly (Kelly, 2007) into the structure/activity relationships of apoE derived antimicrobial peptides, revealed antibacterial activity against *S. aureus* and *P. aeruginosa,* which is believed to be due to their cationic nature, although their exact mode of action currently remains unclear.

There are claims in the literature that antimicrobial peptides represent promising new agents for therapy, prophylaxis and are less prone to selecting for reduced bacterial susceptibility than more site specific agents (Tew, 2002). Few studies have

systematically examined the effects of repeated exposure of bacteria to antimicrobial peptides, in comparison to commercially used biocides. This chapter therefore aims to compare the ability of a tryptophan-substituted derivative of the apoEdp peptide (apoEdpL-W) in the generation of bacterial insusceptibility, to that of commonly used biocides cetrimide (CET), chlorhexidine (CHX), polyhexamethylene biguanide (PHMB) and triclosan (TCS), against a test panel of bacteria that are commonly associated with medical-device infection. Additionally, the influence of antimicrobial exposure on bacterial biofilm viability, as well as any consequence that long-term exposure may have on biofilm forming ability, will be evaluated.

3.2 Materials and Methods

3.2.1 Bacterial strains

Pseudomonas aeruginosa ATCC 9027*, Staphylococcus aureus* ATCC 6538 and *Serratia marcescens* ATCC 13880 were obtained from Oxoid (Basingstoke, UK). *Burkholderia cepacia* ATCC BAA-245*, Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 13883 were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Micrococcus luteus* MRBG 9.25*, Staphylococcus caprae* MRBG 9.3, *Staphylococcus capitis* MRBG 9.34*, Staphylococcus lugdunensis* MRBG *9.36, Staphylococcus warneri* MRBG 9.27*, Staphylococcus epidermidis* MRBG 9.33 and *Staphylococcus haemolyticus* MRBG 9.35 were obtained from the axillae of three healthy male volunteers, ranging from 25 to 30 years old. *Bacillus cereus* MRBG 4.21, *Stenotrophomonas maltophilia* MRBG 4.17 and *Chryseobacterium indologenes* MRBG 4.29 were isolated from a domestic kitchen drain biofilm (McBain, 2003). *Enterococcus faecalis* and *Corynebacterium xerosis* were both wild-type strains

isolated from a chronic wound. Species were identified using 16s rRNA sequencing prior to use.

3.2.2 Chemical reagents and bacterial growth media

Triclosan, cetrimide and chlorhexidine were obtained from Sigma-Aldrich (Dorset, UK). Vantocil a 20 % aqueous solution of PHMB was obtained from Arch Chemicals Inc. (Manchester, UK). Peptides were purchased from Alta Bioscience (West Midlands, UK) having been synthesized using 9-fluorenylmethyl carbamate chemistry and purified by high performance liquid chromatography. Bacteriological media was purchased from Oxoid (Basingstoke, UK). All other chemical reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Bacterial growth media was sterilised at 121°C and 15 psi for 15 min prior to use. All bacteria were cultured onto Muller-Hinton Agar (Oxoid, UK) and incubated aerobically at 37˚C for 18 h unless stated otherwise.

3.2.3 Determination of bacterial minimum inhibitory concentrations (MICs)

Overnight bacterial cultures were grown to an approximate OD_{600} of 0.8 and diluted 1:100 into sterile media to use as an inoculum for biocide susceptibility tests. All biocide stock solutions were prepared at 1.16 mg ml^{-1}, filter sterilized and stored at –80˚C until use. To determine minimum inhibitory concentrations (MICs) of antimicrobials, diluted overnight culture (200µl) was delivered to each test well of a 96-well microtiter plate (Becton Dickinson, Oxford, UK). Doubling dilutions of each antimicrobial were prepared at five times the highest test concentration, in sterile

water, then diluted 1:5 into fresh media in the test wells of the plate, to give a final volume of 250 µl. The plates were incubated for 24 h at 37 ˚C and 100 rpm. MICs were determined as the lowest concentration for which bacterial growth did not occur. Growth was viewed as turbidity (496 nm) in comparison to an uninoculated well (negative control) and was detected using a microtiter plate reader (Anthos HTII; Anthos-Labtec Instruments. Salzburg. Austria). Each MIC was carried out in three technical replicated and two biological ones.

3.2.4 Determination of bacterial minimum bactericidal concentrations (MBC)

To determine MBCs, 10 µl aliquots were taken from each well of the previously performed MIC plate and spot plated onto Muller-Hinton Agar plates in triplicate. Plates were incubated overnight at 37˚C. MBCs were determined as the lowest concentration of biocide at which no growth occurred after 4 d of incubation.

3.2.5 Determination of minimum biofilm eradication concentration (MBEC)

Single species biofilms were grown on the pegs of the Calgary Biofilm Device (CBD) (Ceri, 1999). To produce inoculum for the biofilm susceptibility testing overnight bacterial cultures were grown to an approximate OD_{600} of 0.8 diluted to 1 McFarland Standard, then further diluted 1:30 using fresh growth medium. 200 µl of bacterial inoculum was added to each well of the CBD base, plates were then incubated at 37˚C and 30 rpm for 48 h to allow biofilm formation on the pegs. After 48 h the lid was removed and the pegs were rinsed twice in 250 µl of sterile PBS, contained within the wells of a sterile 96-well wash plate. Doubling dilutions for biocide stocks to be tested were prepared at five times the highest test concentration

and diluted 1:5 into sterile Muller-Hinton Broth in the wells of a 96-well antimicrobial "challenge plate" (250 µl). Biofilms were exposed to the challenge plate and incubated for 24 h at 37˚C and 100 rpm. After incubation the lid was removed and each peg was rinsed three times in 300 µl of sterile PBS, before being transferred to a 96-well "recovery plate", containing 300 µl of sterile Muller-Hinton Broth. Recovery plates were solicited on high for 15 min and incubated for 24 h at 37˚C and 100 rpm. MBECs were determined as the lowest concentration for which bacterial growth did not occur after 18 h of incubation. Growth was viewed as turbidity in comparison to an uninoculated well (negative control). Each MBEC was carried out in three technical replicates and two biological ones.

3.2.6 Crystal biofilm violet assay

Overnight cultures of test bacteria were diluted 1:100 into sterile Muller-Hinton Broth to use as a bacterial inoculum for the assay. 150 µl of inoculum was delivered to each test well of a 96-well microtiter plate. Plates were incubated for 48 h at 37˚C and 20 rpm to promote biofilm growth. After incubation wells were washed twice with 200 µl of sterile PBS. After washing, 200 µl of 0.5% crystal violet solution was added to the test wells and plates were incubated for 30 min at room temperature. Wells were washed twice with 250 µl of PBS and left to dry at room temperature for 1 h. Attached crystal violet was solubilised within the well by adding 250 µl of 70 % ethanol and plates were agitated at room temperature at 20 rpm for 1 h. After solubilisation, biofilm growth was viewed as change in OD_{600} relative to a sterile negative control.

3.2.7 Bacterial training to sub-lethal level of antimicrobial exposure

Reproducible 100-fold antimicrobial concentration gradients were created on Muller-Hinton Agar plates using a Whitley automated spiral plater (Don Whitley Scientific, Shipley, UK). Initial MIC antimicrobial stock solutions (50 µl) were deposited on the agar surface using the spiral plater. Plates were dried for 1 h at room temperature prior to radial deposition of bacterial pure cultures. Plates were incubated for 4 d at 37˚C in a static aerobic incubator. After incubation, growth observed at the highest biocide concentration was aseptically removed using a sterile loop and streaked onto a fresh plate, containing the same antimicrobial concentration gradient. This process was repeated until 10 passages onto a new plate had occurred. Bacteria were then passaged a further 10 times in the absence of any antimicrobial (X10). Bacteria at P0, P10 and X10 were archived for subsequent MIC and MBC testing.

3.3 Results and Discussion

3.3.1 Evaluation of susceptibility changes in bacteria

The mean, median and range MIC and MBC values before and after prolonged exposure to antimicrobials are shown in Table 3.1. The hierarchy of bactericidal activity (average MBC) pre-exposure was CHX> PHMB> apoEdpL-W> TCS> CET. ApoEdpL-W, CET and TCS showed a similar range in MICs and MBCs, whereas those for PHMB and CHX were narrower. After 10 passages in the presence of an antimicrobial, the average fold changes in MICs were apoEdpL-W (3.8), CET (3.2), CHX (3), PHMB (2.7) and TCS (32.2). The average fold changes in MBCs were apoEdpL-W (5.5), CET (2.1), CHX (4.6), PHMB (2.7) and TCS (9.7) (Table 3.1). This changed the hierarchy of bactericidal activity (MBC), post antimicrobial

exposure, to PHMB> CHX> apoEdpL-W> CET> TCS. From P10 to X10, when passaged in the absence of any antimicrobial, insusceptibilities partially reverted to unexposed values in the majority of microorganisms. When comparing P0 to X10 the average fold changes in MIC were as follows; apoEdpL-W (1.6), CET (3.1), CHX (2.6), PHMB (1.3) and TCS (3.5). The average fold changes in MBC were apoEdpL-W (3.4), CET (1.6), CHX (1.0), PHMB (1.2) and TCS (3.8) (Table 1). This resulted in a final hierarchy of activity (MBC) of PHMB> CHX> apoEdpL-W> TCS> CET, for the recovered microorganisms.

Table 3.1. Mean, maximum, minimum and median values for minimum inhibitory and minimum bactericidal concentrations before and after sub-lethal level exposure to antimicrobials, for all test bacteria.

	MIC ($\mu g\ ml^{-1}$)					MBC ($\mu g\ ml^{-1}$)				
	P0	P10	X10	Fold P0-P10	Fold P10-X10	P0	P10	X10	Fold P0-P10	Fold P10-X10
ApoEdpL-W										
Mean	24.1	43.6	24.7	3.8	1.6	46.4	77.5	55.2	5.5	3.4
Max	232	464	232	21.4	4.0	464	464	464	31.8	13.8
Min	0.9	3.6	0.9	0.9	0.2	1.8	7.3	7.3	0.3	0.5
Med	7.3	21.8	10.9	2.0	1.0	14.5	29	29	2.0	1.0
CET										
Mean	29.4	41	26.2	3.2	3.1	70.9	129.0	74.5	2.1	1.6
Max	232	232	232	18.3	18.3	464	464	464	8.1	8.1
Min	0.4	1.8	1.8	0.5	0.7	0.9	7.3	7.3	0.3	0.5
Med	9.7	10.9	10.9	1.5	1.2	29.0	26.6	29	1.1	1.0
CHX										
Mean	7.2	16.5	13.3	3.0	2.6	20.2	67.3	48.3	4.6	1.0
Max	29	116	58	9.6	13.8	58	232	116	28.4	2.0
Min	0.9	3.0	1.8	0.1	0.4	1.7	7.3	7.3	0.5	0.5
Med	4.2	7.3	7.3	1.4	1.1	15.4	38.7	29	2.2	1.0
PHMB										
Mean	13.6	17.6	11.6	2.7	1.3	34.1	54.1	32.6	2.7	1.2
Max	58	58	58	8.1	5.4	116	232	116	8.9	4.1
Min	0.9	3.6	1.8	0.5	0.5	1.8	7.3	7.3	0.3	0.5
Med	3.6	7.3	7.3	1.9	1.0	29.0	48.3	29	2.0	1.0
TCS										
Mean	33	58.0	33.5	32.2	3.4	58.8	134.9	60.1	9.7	3.8
Max	232	232	232	145	16.1	464	464	464	58	29.0
Min	0.2	3.6	0.4	0.5	0.5	0.5	7.3	3.6	0.2	0.5
Med	5.3	29	6.1	12.0	1.0	19.4	58.0	14.5	2.7	1.0

3.3.2 Effects of apoEdpL-W exposure on bacterial susceptibility

Before bacterial exposure to apoEdpL-W, MICs ranged from 0.9 μg ml^{-1} to 232 μg ml^{-1} with an average value of 24.1 μg ml^{-1}. Average MBC was 46.4 μg ml^{-1}, ranging from 1.8 μg ml^{-1} to 464 μg ml^{-1} (Table 3.1). After long-term apoEdpL-W exposure, *C. indologenes, E. faecalis, P. aeruginosa, K. pneumoniae S. caprae, S. epidermidis* and *S. haemolyticus* all exhibited over a 2-fold increase in MIC and MBC, giving a raised average P10 MIC of 43.6 μg ml^{-1} (3.6 μg ml^{-1} to 464 μg ml^{-1}) and MBC of 77.5 μg ml^{-1} (7.3 μg ml^{-1} to 464 μg ml^{-1}) (Table 3.2). Frequently, MIC and MBC decreased in value once the bacteria were passaged in the absence of the peptide, reducing average X10 MIC to 24.7μg ml^{-1} (0.9 μg ml^{-1} to 232 μg ml^{-1}) and MBC to 55.2 μg ml^{-1} (7.3 μg ml^{-1} to 464 μg ml^{-1}) (Table 3.2).

Table 3.2. MICs and MBCs of bacteria before and after treatment with apoEdpL-W.

Species	MIC (μg/ml^{-1})			MBC (μg/ml^{-1})		
	Before treatment	P10	X10	Before treatment	P10	X10
Bacillus cereus	14.5	29	29	58	58	58
Burkholderia cepacia	29	29	29	58	58	58
Chryseobacterium indologenes	1.4 (0.4)	**14.5**	**3.63**	3 (1)	**14.5**	**14.5**
Corynebacterium xerosis	14.5	29	14.5	29	24.2 (8)	29
Enterococcus faecalis	7.3	**29**	**29**	7.3	**232**	**58**
Escherichia coli	58	29	29	58	96.7 (34)	29
Klebsiella pneumoniae	7.3	**29**	7.3	7.3	**29**	12.1
Micrococcus luteus	7.3	12.1 (4)	7.3	14.5	29	29
Pseudomonas aeruginosa	14.5	**48.3 (17)**	14.5	58	**193.3 (67)**	58
Serratia marcescens	232	464	232	464	464	464
Staphylococcus aureus	7.3	3.6	7.3	14.5	29	14.5
Staphylococcus capitis	13.3 (3)	24.2 (8)	14.5	29	9.8 (4)	29
Staphylococcus caprae	0.9	**19.3 (8)**	**3.6**	3.6	**29**	7.3
Staphylococcus epidermidis	0.9	**7.3**	**3.6**	4.2 (2)	**58**	**58**
Staphylococcus haemolyticus	1.3 (1)	**3.6**	**3.6**	3.6	**29**	**38.7**
Staphylococcus lugdunensis	7.3	3.6	1.2	7.3	7.3	7.3
Staphylococcus warneri	1.8	3.6	0.9	1.8	**19.3 (8)**	**14.5**
Stenotrophomonas maltophilia	14.5	7.3	14.5	14.5	14.5	14.5

[a] Data shows the means of duplicate experiments repeated in triplicate. When data varied between replicates standard deviations are given in the parenthesis. Bold font indicates over a two fold change in values (P0-P10) and (P0-X10).

Induced bacterial defence mechanisms to CAMPS have been previously documented, which may provide insight into deceases in apoEdpL-W susceptibility observed in this study. These include alterations of the cell surface electrostatic charge, efflux mechanisms (Padilla*, et al.*, 2010) and the secretion of CAMP degrading proteases (Guina, 2000), or binding molecules. It has been shown that CAMP exposure in certain Gram-negative bacteria may induce protein, phospholipid and LPS modifications due to activation of the PhoP/PhoQ regulon (Guo*, et al.*, 1998), potentially decreasing the attracting force between the positively charged peptide and the negative bacterial cell wall (Gunn, 2000) (Kristian, 2005). In *K. pneumoniae,* a bacterial capsular polysaccharide (CPS) is thought to mediate CAMP resistance. *K. pneumoniae* has been shown to increase CPS transcription when grown in the presence of polymyxin B and lactoferrin, causing a fall in susceptibility (Campos*, et al.*, 2004). *K. pneumoniae* was one of the few organisms that showed a widespread decrease in susceptibility to all the antimicrobials tested in this study, it is therefore plausible that upregulation of capsule synthesis in *K. pneumoniae* may confer a broad-range defence mechanism, when experiencing an antimicrobial stress.

Of the 8 test organisms that exhibited a decrease in susceptibility to apoEdpL-W, four were staphylococci. It has previously been documented that staphylococci produce extracellular proteases that play a role in their pathogenesis (V8 proteases). It is thought that certain cationic peptides may be substrates for such proteases and therefore when expressed confer CAMP resistance to the bacteria (Sieprawska-Lupa*, et al.*, 2004). Expressions of efflux systems have also been associated with CAMP resistance in staphylococci, such as the *qacA* mediated efflux system in *S. aureus*. When *qacA* is expressed, it is thought to contribute towards thrombocidin

insusceptibility, as well as insusceptibility towards other cationic antimicrobials including cetrimide and chlorhexidine (Paulsen, 1996, Kupferwasser, 1999). These mechanisms help alleviate peptide-mediated toxicity and could have a role in the development of apoEdpL-W insusceptibility seen in this investigation.

3.3.3 Effects of cetrimide exposure on bacterial susceptibility

Initial MICs for cetrimide ranged from 0.4 μg ml^{-1} to 232 μg ml^{-1} with an average of 29.4 μg ml^{-1}. MBCs ranged from 1.8 μg ml^{-1} to 464 μg ml^{-1} with an average of 70.9 μg ml^{-1} (Table 3.1). Post exposure MIC (P10) was on average 41 μg ml^{-1} (1.8 μg ml^{-1} to 232 μg ml^{-1}) and MBC was 129 μg ml^{-1} (7.3 μg $ml^{-1}$to 464 μg ml^{-1}) (Table 3.1). After exposure to cetrimide, *E. coli, K. pneumoniae, S. epidermidis, S. haemolyticus* and *S. lugdunensis* all displayed over a 2-fold increase in MIC (Table 3.3), out of which only *E. coli, K. pneumoniae* and *S. epidermidis* reverted when passaged in the absence of the biocide. In terms of MBC, *B. cereus, E. coli, S. marcescens* and *S. epidermidis* all showed over a 2-fold increase in MBC from P0 to P10 (Table 3.3). All MBCs reverted in the absence of cetrimide with the exception of *S. epidermidis* (Table 3.2). This gave a final average MIC (X10) value of 26.2 μg ml^{-1} (1.8 μg ml^{-1} to 232 μg ml^{-1}) and MBC of 74.5 μg ml^{-1} (7.3 μg ml^{-1} to 464 μg ml^{-1}) for the microorganisms, after recovery in a cetrimide free medium.

Table 3.3. MICs and MBCs of bacteria before and after treatment with cetrimide

Test bacterium	MIC (μg/ml^{-1})			MBC (μg/ml^{-1})		
	Before treatment	P10	X10	Before treatment	P10	X10
Bacillus cereus	7.3	14.5	7.3	14.5	**48.3 (8)**	29
Burkholderia cepacia	38.7 (17)	38.6 (8)	29	116	242	232
Chryseobacterium indologenes	12.1 (4)	14.5	14.5	29	29	29
Corynebacterium xerosis	3.6	3.6	3.6	14.5	9.7 (4)	14.5
Enterococcus faecalis	12.1 (4)	14.5	14.5	29	38.7 (16)	58
Escherichia coli	29.17 (8)	**116**	29	116	**464**	116
Klebsiella pneumoniae	29.3 (8)	**116**	29	29	58	58
Micrococcus luteus	14.5	7.3 (33)	14.5	58	19.3 (8)	29
Pseudomonas aeruginosa	232	232	232	464	464	464
Serratia marcescens	24.2 (8)	37.3 (14)	29	37.3 (14)	**116**	58
Staphylococcus aureus	4.8(2)	6.1	7.3	7.3	14.5	7.3
Staphylococcus capitis	3.6	7.3	7.3	14.5	7.3	7.3
Staphylococcus caprae	0.9	1.8	1.8	14.5	14.5	14.5
Staphylococcus epidermidis	1.8	**7.3**	1.8	3.6	**7.3**	**7.3**
Staphylococcus haemolyticus	0.4	**7.3**	**7.3**	14.5	14.5	14.5
Staphylococcus lugdunensis	0.4	**3.6**	**7.3**	29	14.5	29
Staphylococcus warneri	4.8 (2)	6.1 (2)	7.3	193.3	232	116
Stenotrophomonas maltophilia	19.3 (8)	29	29	58	24.2 (8)	58

[a] See footnote in table 3.2

Multidrug resistance efflux pumps have previously been shown to influence insusceptibility towards quaternary ammonium compounds in bacteria. This form of efflux mediated resistance has been reviewed in detail (Poole, 2004). Overexpression of the MFS efflux pump, NorA, in *S. aureus* has previously been linked to cetrimide insusceptibility. This may suggest a plausible explanation for the slight decrease in susceptibility of *S. aureus* and possibly other staphylococci, after cetrimide treatment, observed in this study (Kaatz & Seo, 1995).

3.3.4 Effects of chlorhexidine exposure on bacterial susceptibility

Before exposure to chlorhexidine, MIC averaged 7.2 μg ml^{-1} ranging from 0.9 μg ml^{-1} to 29 μg ml^{-1}, after exposure this average rose to 16.5 μg ml^{-1} (3 μg ml^{-1} to 116 μg ml^{-1}) (Table 3.1). Initial average MBC started at 20.2 μg ml^{-1} ranging from 1.7 μg ml^{-1} to 58 μg ml^{-1}. After exposure, average MBC increased to 67.3 μg ml^{-} (7.3 μg ml^{-1} to

232 μg ml^{-1}) (Table 3.1). *B. cepacia, E. faecalis, K. pneumoniae, S. marcescens, S. lugdunensis, S. warneri* and *S. maltophilia* demonstrated over a 2-fold increases in MIC when passaged in the presence of chlorhexidine, out of which only *B. cepacia, E. faecalis* and *S. warneri* reverted when passaged in a biocide free medium (Table 3.4). All of the above organisms showed a corresponding two-fold or above increase in MBC, with the exception of *E. faecalis* and *S. warneri* (Table 3.4). Additionally, *B. cereus*, *S. aureus* and *S. haemolyticus* also showed at least two-fold increase in MBC after chlorhexidine exposure. All changes in MBC remained elevated when passaged in the absence of chlorhexidine, apart from *S. aureus* and *S. haemolyticus* (Table 3.4). After regrowth in a chlorhexidine free environment, average MIC reverted to 13.3 μg ml^{-1} (1.8 μg ml^{-1} to 58 μg ml^{-1}) and MBC to 48.3 μg ml^{-1} (7.3 μg ml^{-1} to 116 μg ml^{-1}) (Table 3.1).

Table 3.4. MICs and MBCs of bacteria before and after treatment with chlorhexidine

Test bacterium	MIC (μg/ml^{-1})			MBC (μg/ml^{-1})		
	Before treatment	P10	X10	Before treatment	P10	X10
Bacillus cereus	14.5	14.5	14.5	29	**232**	**116**
Burkholderia cepacia	3.6	**29**	7.3	26.6 (6)	**232**	**116**
Chryseobacterium indologenes	7.3	7.3	7.3	7.3	14.5	7.3
Corynebacterium xerosis	3.3 (1)	3.6	3.6	21.8 (8)	14.5	14.5
Enterococcus faecalis	3.6	**24.2 (8)**	3.6	26.6 (6)	58	29
Escherichia coli	6.7 (1)	7.3	7.3	13.3 (3)	29	29
Klebsiella pneumoniae	2.1 (1)	**14.5**	**29**	16.3 (5)	**58**	**116**
Micrococcus luteus	3.6	3.6	3.6	14.5	7.3	14.5
Pseudomonas aeruginosa	7.3	14.5	7.3	14.5	29	14.5
Serratia marcescens	12.1 (4)	**116**	**58**	24.2 (7)	**232**	**116**
Staphylococcus aureus	8.5 (4)	3.6	3.6	13.3 (4)	**58**	29
Staphylococcus capitis	3.6	6 (2)	7.3	14.5	14.5	29
Staphylococcus caprae	3.6	3.6	7.3	29	29	29
Staphylococcus epidermidis	13.3 (3)	9.7 (4)	14.5	33.8 (12)	24.2(8)	29
Staphylococcus haemolyticus	1.4 (0.4)	3 (1)	1.8	4.2(1)	**14.5**	7.3
Staphylococcus lugdunensis	0.9	**3.6**	**4.8 (2)**	1.7 (0.3)	**48.3 (17)**	**58**
Staphylococcus warneri	29	**3.6**	29	58	58	58
Stenotrophomonas maltophilia	4.8 (2)	**29**	**29**	14.5	**58**	**58**

[a] See footnote in table 3.2

It has been previously suggested that the presence of a suitable efflux mechanism may confer chlorhexidine insusceptibility in bacteria. Fang documented the isolation of chlorhexidine insusceptible *K. pneumoniae,* which expressed a novel 903-nucleotide long locus with a sequence compatible with a cation efflux pump, designated *cepA* (Fang, 2002). *S. marcescens* grown in a chlorhexidine containing solution have shown alterations in outer membrane protein composition, which is believed to contribute towards its survival in the presence of the biocide (Gandhi, 1993). In addition, cloning of the SdeXY efflux pump from *S. marcescens* into *E. coli* resulted in an elevation in chlorhexidine MIC, when compared to the non SdeXY containing strain, further supporting the idea that the pump may have a role in the export of chlorhexidine from the bacterial cell (Chen*, et al.*, 2003). It is possible that the induction of such efflux mechanisms may contribute to the reductions in chlorhexidine susceptibility observed in our bacterial isolates.

3.3.5 Effects of PHMB exposure on bacterial susceptibility

Pre-exposure MIC values for PHMB were within the range 0.9 μg ml^{-1} to 58 μg ml^{-1} averaging at 13.6 μg ml^{-1}, whilst MBCs ranged from 1.8 μg ml^{-1} to 116 μg ml^{-1} with an average of 34.1 μg ml^{-1} (Table 3.1). Post exposure P10 average MIC was 17.6 μg ml^{-1} (3.6 μg ml^{-1} to 58 μg ml^{-1}) and MBC was 54.1 μg ml^{-1} (7.3 μg ml^{-1} to 232 μg ml^{-1}). Following exposure to PHMB, *C. indologenes, C. xerosis, E. faecalis, K. pneumoniae, M. luteus, S. capitis, S. epidermidis* and *S. haemolyticus* all showed over a 2-fold increases in MIC from P0 to P10, all of which reverted when passaged in PHMB free medium (Table 3.5). *C. indologenes, E. faecalis, K. pneumoniae, S. capitis* and *S. lugdunensis* showed at least a 2-fold increases in MBC, which also reverted in the absence of the biocide, with the exception of *C. indologenes* that

remained 4-fold higher than the initial value (Table 3.5). X10 MIC for PHMB averaged 11.6 μg ml^{-1} (1.8 μg ml^{-1} to 58 μg ml^{-1}) and MBC averaged 32.6 μg ml^{-1} (7.3 μg ml^{-1} to 116 μg ml^{-1}), after growth in the absence of the biocide.

Table 3.5. MICs and MBCs of bacteria before and after treatment with PHMB.

Test bacterium	MIC (μg/ml^{-1})			MBC (μg/ml^{-1})		
	Before treatment	P10	X10	Before treatment	P10	X10
Bacillus cereus	58	29	58	58	58	58
Burkholderia cepacia	58	58	29	116	58	58
Chryseobacterium indologenes	0.9	**3.6**	1.8	1.8	**14.5**	**7.3**
Corynebacterium xerosis	2.7 (1)	**7.3**	2.2 (0.4)	21.8 (8)	**7.3**	14.5
Enterococcus faecalis	1.8	**14.5**	9.7	7.3	**29**	7.3
Escherichia coli	13.3 (3)	24.2 (8)	7.3	26.6 (6)	58	14.5
Klebsiella pneumoniae	7.3	**29**	9.7 (4)	29	**96.7 (34)**	58
Micrococcus luteus	1.8	**7.3**	1.8	7.3	14.5	7.3
Pseudomonas aeruginosa	31.3 (6)	58	29	106.3 (27)	232	116
Serratia marcescens	38.7 (15)	29	29	38.7 (15)	29	29
Staphylococcus aureus	7.3	7.3	7.3	52 (11)	58	58
Staphylococcus capitis	1.1 (0.3)	**6 (2)**	1.8	7.3	**48.3 (17)**	7.3
Staphylococcus caprae	6.7 (2)	4.9 (2)	7.3	29	38.7 (17)	29
Staphylococcus epidermidis	3 (1)	**14.5**	3.6	26.6 (6)	38.7 (17)	29
Staphylococcus haemolyticus	1.8	**7.3**	1.8	29	58	29
Staphylococcus lugdunensis	3.6	7.3	1.8	5.4 (2)	**48.3 (17)**	7.3
Staphylococcus warneri	3.6	6 (2)	3.6	21.8 (8)	58	29
Stenotrophomonas maltophilia	3 (1)	3.6	3.6	29	29	29

[a] See footnote in table 3.2

Moore and colleagues examined the effect of sub-lethal PHMB exposure on a panel of bacteria, including *C. indologenes, S. lugdunensis* and *S. capitis,* however, they did not observe any decrease in susceptibility for these organisms (Moore *et al*, 2008). No other bacterial insusceptibility towards PHMB has been previously documented.

3.3.6 Effects of triclosan exposure on bacterial susceptibility

Initial MIC values for triclosan ranged from 0.2 μg ml^{-1} to 232 μg ml^{-1} with an average value of 33 μg ml^{-1}. MBC values ranged from 0.5 μg ml^{-1} to 464 μg ml^{-1} with an average of 58.8 μg ml^{-1} (Table 3.1). After exposure to triclosan, average MIC

increased to 58 μg ml^{-1} (3.6 μg ml^{-1} to 232 μg ml^{-1}) and MBC of 134.9 μg ml^{-1} (7.3 μg ml^{-1} to 464 μg ml^{-1}) (Table 3.1). *B. cereus, C. indologenes, C. xerosis, E. faecalis, E. coli, K. pneumoniae, S. aureus, S. epidermidis, S. haemolyticus, S. lugdunensis, S. warneri* and *S. maltophilia* all showed over a 2-fold increase in MIC after triclosan exposure, out of which *E. coli, K. pneumoniae*, and *S. aureus* remained elevated above initial values in the absence of the biocide (Table 3.6). After growth in a triclosan-free medium, average X10 MIC reverted to 33.5 μg ml^{-1} (0.4 μg ml^{-1} to 232 μg ml^{-1}) and MBC to 60.1 μg ml^{-1} (3.6 μg ml^{-1} to 464 μg ml^{-1}) (Table 3.1).

Table 3.6. MICs and MBCs of bacteria before and after treatment with triclosan.

Species	MIC (μg/ml^{-1})			MBC (μg/ml^{-1}		
	Before treatment	P10	X10	Before treatment	P10	X10
Bacillus cereus	7.3	**29**	7.3	58	116	58
Burkholderia cepacia	232	116	232	464	464	464
Chryseobacterium indologenes	0.9	**3.6**	0.9	3.6	7.3	3.6
Corynebacterium xerosis	7.3	**58**	7.3	7.3	**58**	7.3
Enterococcus faecalis	3.3 (1)	**58**	3.63	3.3 (1)	**96.7 (34)**	**14.5**
Escherichia coli	0.5	**29**	**4.82**	0.5	**29**	**14.5**
Klebsiella pneumoniae	0.9	**116**	**14.5**	29	**116**	14.5
Micrococcus luteus	7.3	12.1(4)	3.63	7.3	14.5	7.3
Pseudomonas aeruginosa	n/a	n/a	n/a	n/a	n/a	n/a
Serratia marcescens	232	116	232	232	464	232
Staphylococcus aureus	0.2	**29**	**2.4**	1.8	**58**	**12.1 (4)**
Staphylococcus capitis	24.2 (8)	29	14.5	29	**77.3 (33)**	29
Staphylococcus caprae	12.3 (4)	29	14.5	24.2 (8)	**58**	29
Staphylococcus epidermidis	13.3(3)	**38.7 (17)**	14.5	53.2 (12)	**116**	58
Staphylococcus haemolyticus	0.4	**29**	0.4	7.3	**58**	7.3
Staphylococcus lugdunensis	0.9	**29**	0.9	7.3	**58**	7.3
Staphylococcus warneri	0.9	**24.2 (8)**	0.9	14.5	**38.7 (17)**	14.5
Stenotrophomonas maltophilia	14.5	**232**	14.5	58	**463**	48.3

[a] See footnote in table 3.2

Bacterial insusceptibility towards triclosan has previously been observed which may, in part, help account for some of the changes in triclosan susceptibility seen in our bacterial isolates. Triclosan insusceptibility in *E. coli*, is believed to be due to the selection of bacteria with mutations in target enzyme FabI, or which are overexpression the multidrug efflux pump acrAB, or marA and SoxS, positive

regulators of acrAB (McMurry, 1998, McBain, 2004, Yu*, et al.*, 2010). Similarly, exposure of *S. maltophilia* to triclosan has been shown to select for an insusceptible population that overexpresses the SmeDEF multidrug efflux pump (Sanchez, 2005). Mutations in *fabI* in *S. aureus,* in response to triclosan, have also been reported (Jang*, et al.*, 2008). Jang and colleagues performed microarray analysis of *S. aureus* that has undergone prolonged exposure to triclosan, in which a down-regulation of ClpB chaperone-related genes were revealed. This is considered to play a role in its tolerance towards triclosan, however, the mechanism by which it does this remains unclear (Jang*, et al.*, 2008).

3.3.7 Anti-biofilm efficacy of test actives

The concentration of antimicrobial required to inhibit growth (MIC) was lower than that required to inactivate the bacteria (MBC). Concordantly, it required a higher concentration of antimicrobial to eradicate a bacterial biofilm (MBEC), than to inactivate a planktonic culture (MBC), demonstrating the relative recalcitrance of the bacterial biofilm towards antimicrobial chemotherapy (Table 3.7). Biofilm insusceptibility towards biocides and antimicrobial peptides has previously been documented (Nickel*, et al.*, 1985, Campanac*, et al.*, 2002, McBain*, et al.*, 2003, McBain*, et al.*, 2004), however, the anti-biofilm potency of the apoEdpL-W peptide has not been investigated prior to this study.

Table 3.7. Minimum biofilm eradication concentrations of test antimicrobials.

Species	MBEC (μg/ml^{-1})				
	A1	CHX	CET	PHMB	TCS
Bacillus cereus	1160	1160	145	1160	145 (20)
Burkholderia cepacia	580	290	242 (84)	580	580
Chryseobacterium indologenes	10.6 (4)	12.1 (5)	29	3 (1)	12.1 (4)
Corynebacterium xerosis	33.3 (7)	60.4 (19)	33.7 (7)	33.3 (7)	13.6 (5)
Enterococcus faecalis	145	36.3	18.2	145	54.4 (7)
Escherichia coli	580	145	36.3	132.9 (26)	193.3 (80)
Klebsiella pneumoniae	290	108.8 (40)	60.4 (21)	36.3	132.9 (75)
Micrococcus luteus	12.1 (5)	15.1 (5)	15.6	27.2 (10)	48.2 (20)
Pseudomonas aeruginosa	580	33.2 (7)	580	109 (40)	n/a
Serratia marcescens	1160	870 (318)	290	870 (318)	1160
Staphylococcus aureus	72.5	33.5 (7)	12.1 (5)	108.8 (40)	27.2 (10)
Staphylococcus capitis	72.5	13.6 (5)	18.1	36.3	27.2 (10)
Staphylococcus caprae	108.8 (40)	108.8 (40)	15.4 (6)	36.3	36.3
Staphylococcus epidermidis	145 (40)	36.3	9.1 (1)	108.8 (40)	54.4 (7)
Staphylococcus haemolyticus	108.8 (40)	6.8 (3)	14.5	108.8 (40)	24.2 (9)
Staphylococcus lugdunensis	15.1 (5)	15.1 (5)	14.5	7.6 (2)	9.1
Staphylococcus warneri	27.2 (10)	42.3 (15)	145	21.1 (7)	18.1
Stenotrophomonas maltophilia	241.7 (75)	218 (80)	145	241.7 (75)	580

[a] Data shows the means of duplicate experiments repeated in triplicate. When data varied between replicates standard deviations are given in the parenthesis.

The concentration of ApoEdpL-W required to eradicate a single species biofilm ranged from 10.6 μg ml^{-1} for *C. indologenes* to 1160 μg ml^{-1} for *B. cereus* and *S. marcescens* (Table 3.7). Some of the largest increases from MBC to MBEC occurred in staphylococci towards apoEdpL-W, which showed up to a 30-fold increase. Previous research has documented high CAMP tolerance in staphylococcal biofilms, due to the presence of polysaccharide intracellular adhesin (PIA), a major biofilm exopolysaccharide from *S. epidermidis* and *S. aureus* that has been found to protect against CAMPs from skin and neutrophil granules, such as human β-defensins and LL-37 (Vuong, 2004, Vuong*, et al.*, 2004). PIA is a positively charged exopolysaccharide and most likely repels the positively charged CAMP away from the bacterial cell, which may explain the low susceptibility of the staphylococcal biofilms to apoEdpL-W, observed in this study.

Previous work documenting the interactions of cationic agents with bacterial biofilms provides insight into their insusceptibility towards QACs and biguanides, such as those investigated in this work (Gilbert*, et al.*, 1997, Campanac*, et al.*, 2002). MBEC values for CET ranged from 9.1 μg ml^{-1} for *S. epidermidis* to 580 μg ml^{-1} for *P. aeruginosa* biofilms. *P. aeruginosa* biofilms are known to be relatively insusceptible towards QACs, believed to partly be due to the large amounts of mucoid glycocalyx production within the biofilm and alterations in bacterial cell envelope protein composition, during the transition from planktonic cell to biofilm state (Campanac*, et al.*, 2002). The largest change in value between MBC and MBEC, after exposure to CET, was observed in *B. cereus,* which was 10 fold. *B. cereus* biofilms have previously shown resilience towards treatment with QACs, as well as many other antimicrobials, which is believed to be due to the formation of biocide resistant spores, the production of which may be enhanced within a biofilm when the bacteria experience nutrient limitations, possibly inducing a stress response (Bloomfield & Arthur, 1994, Peng, 2002).

MBEC values for chlorhexidine ranged from 6.8 μg ml^{-1} for *S. haemolyticus to* 1160 μg ml^{-1} for *B. cereus* (Table 3.7). *S. marcescens, B. cepacia* and *S. lugdunensis* showed a large fold increase between MBC and MBEC. Interestingly, these were also the three organisms to show the largest decrease in susceptibility during the bacterial training procedure, possibly suggesting the presence of a broad defence system induced when the bacteria encounter chlorhexidine, both in planktonic and biofilm states.

The concentration of PHMB required to eradicate a single species biofilm ranged from 3 μg ml^{-1} for *C. indologenes* to 1160 μg ml^{-1} for *B. cereus*. *S. marcescens* biofilms showed widespread insusceptibility to all the antimicrobials tested in this study, however, the decrease in susceptibility of cells in their biofilm state compared to those in their planktonic shows the highest change for PHMB and chlorhexidine. Insusceptibility of *S. marcescens* biofilms to biguanides has been previously reported (Marrie, 1981) and has been attributed to a multitude of factors, including the possible expression of an appropriate efflux system.

The concentration of triclosan required to eradicate single species biofilms of the test bacteria ranged from 9.1 μg ml^{-1} for *S. lugdunensis* to 1160 μg ml^{-1} for *S. marcescens* (Table 3.7). *E. coli, E. faecalis* and *S. aureus* showed the highest fold increase from MBC to MBEC, after triclosan exposure. These three organisms also showed the largest decrease in triclosan susceptibility when undergoing the biocide "training procedure". This may indicate that the large increase between MBC and MBEC is not simply due to the physical shielding of the cells by formation of a biofilm but may be involve additional defence mechanisms induced by the presence of triclosan, which can occur in both planktonic and biofilm states. *P. aeruginosa* was shown to be insusceptible to triclosan even at the highest test concentration in both planktonic and biofilm states. *P. aeruginosa* is known to have intrinsic resistance towards triclosan in its planktonic state, due to expression of the MexAB-OprM efflux system, which may be maintained during biofilm formation (Chuanchuen, *et al.*, 2002).

3.4 The Impact of Antimicrobial Exposure on Biofilm Forming Ability of Selected Bacteria

The ability of test microorganisms, that had undergone a 4-fold or above change in MBC during the training procedure, to form biofilms before and after long-term antimicrobial exposure was assessed. Biofilm formation is often a key virulence determinant in bacteria, the clinical relevance of any development of antimicrobial insusceptibility must also take into account the virulence and fitness of the resulting insusceptible microorganism.

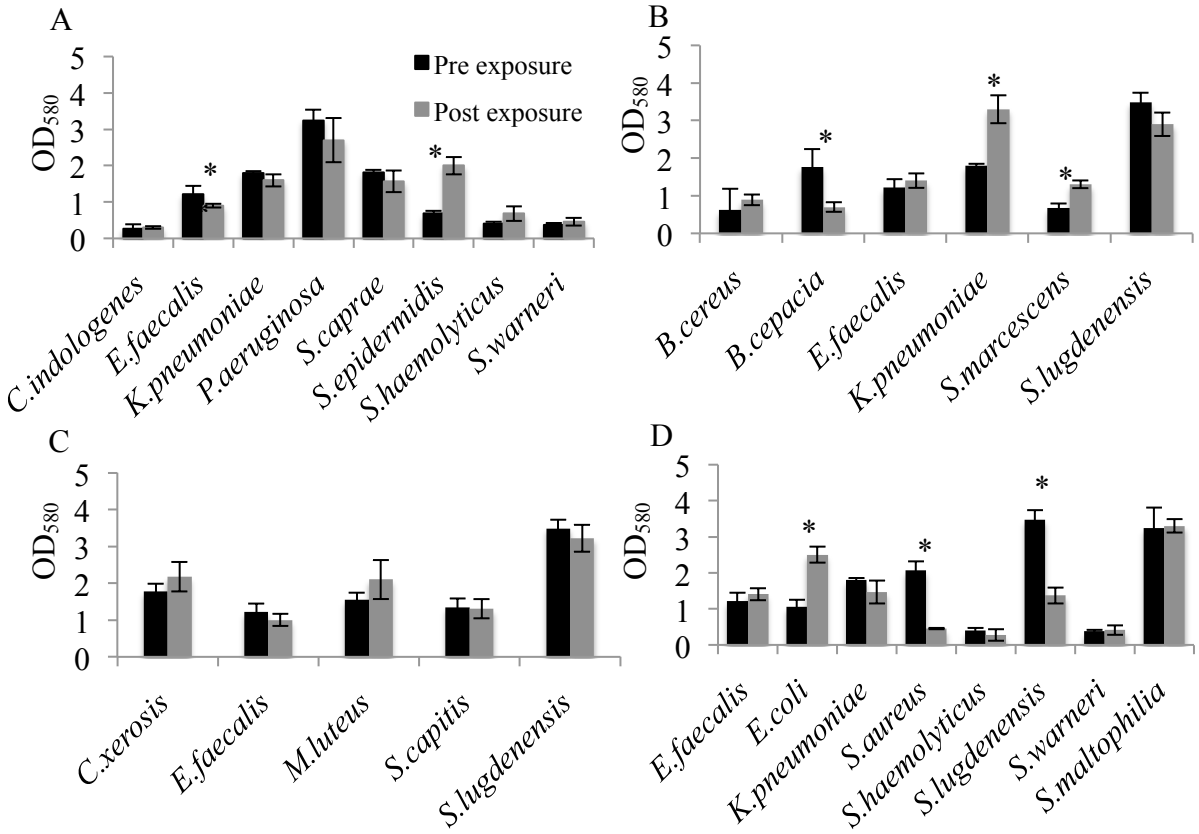

Figure 3.1. Crystal violet biofilm assay showing biofilm formation of bacteria before (black) and after (grey) long term exposure to A) ApoEdpL-W, B) chlorhexidine, C) PHMB and D) triclosan..*indicates a significant change in result P<0.001.

E. faecalis showed a significant decrease in its ability to form a biofilm after apoEdpL-W exposure, comparatively, *S. epidermidis* displayed an increase in

biofilm formation (Figure 3.1). Increased expression of PIA in *S. epidermidis* may account for the observed rise in biofilm production. Antimicrobial treatment of *S. epidermidis,* for instance with alcohol containing skin disinfectants, has previously resulted in an increase in biofilm formation, which is believed to be due to elevated PIA synthesis, to help protect the cell from antimicrobial attack (Vuong*, et al.*, 2004). A similar response in PIA production after exposure to apoEdpL-W, may help account for this observed increase in biofilm formation. Previous studies have shown a decrease in growth rate after bacteria have undergone exposure to an antimicrobial agent (Bayston*, et al.*, 2007, Latimer*, et al.*, 2012), this fall in growth rate may help account for the observed reductions in biofilm formation, seen in *E. faecalis*. This could be the consequence of a lower density of cells, within the slower growing culture, resulting in a decrease in the expression of cell density dependent genes, which are often involved in triggering biofilm formation (Kong, 2006).

S. marcescens exhibited increased biofilm forming ability after long-term chlorhexidine exposure, as did *K. pneumoniae,* which could indicate that any induced adaptions in the bacterial cells physiology, for example capsule formation in *K. pneumoniae* (Campos*, et al.*, 2004) or the expression of efflux mechanisms (Fang, 2002), could also play a role in the organisms ability to form biofilms (Marrie, 1981, Gandhi, 1993, Fang, 2002). In comparison, *B. cepacia* biofilms were reduced after chlorhexidine exposure, indicating that the changes in the bacterial cell that resulted in an increase in MIC and MBC may have had a detrimental impact on bacterial biofilm forming ability, potentially due to a reduction in fitness and growth rate (Rozen*, et al.*, 2007, Latimer*, et al.*, 2012).

E. coli showed a significant increase in biofilm forming ability after exposure to triclosan, whereas *S. aureus* and *S. lugdunensis* showed a decrease. A decrease in staphylococcal biofilm production has previously been attributed to alterations in PIA or Aap production, or due to changes in sarA, a regulatory gene which controls the expression of virulence determinants involved in biofilm development, such as DNase (Kiedrowski*, et al.*). We have previously reported reduced biofilm formation in *S. aureus* after repeated exposure to triclosan, which resulted in the formation of small colony variants with reduced growth rates, compared to that of the parent strain. This reduced growth alone did not, however, account for the decrease in biofilm formation observed in the triclosan exposed strains, suggesting the presence of further adaptions in the bacteria's' physiology that may play a role in the organisms ability to form a biofilm (Latimer*, et al.*, 2012).

Bacteria that underwent over a 4-fold decrease in susceptibility towards PHMB were also examined for alterations in biofilm production, however, no significant difference was seen between unexposed and exposed counterparts. No microorganism showed over a 4-fold change in susceptibility towards cetrimide.

3.5 Conclusion

In the current chapter, it was observed that repeated exposure of bacteria to antimicrobials may result in a decrease in susceptibility. Frequently, however, these changes were reversible, which may indicate predominantly phenotypic adaption rather than the selection of an intrinsically stable mutant. In cases where the change in susceptibility does not completely revert back to initial values in the absence of the antimicrobial, this may be indicative of the selection for a mutation that aids

survival of the bacteria, whilst in the presence of an antimicrobial. Out of all the antimicrobials tested triclosan induced the largest frequency and magnitude of changes in bacterial susceptibility, whereas PHMB showed the lowest. This may be due to the relatively site-specific mode of action of triclosan, when compared to the other test agents. The ApoEdpL-W peptide showed a similar average fold change in MIC to cetrimide and chlorhexidine across the test panel of bacteria, however fold change in MBC was slightly higher. MBCs towards triclosan and apoEpL-W did not completely revert when bacteria were passaged in the absence of biocide, this may indicate the selection of a stable mutant, potentially with an altered antimicrobial target site or that is expressing a mechanism of antimicrobial inactivation.

MBEC values were, in general, higher than those observed for MIC and MBC across the test panel of bacteria. Biofilm insusceptibility towards biocides may arise due to limited biocide penetration into the lower layers of the biofilm (Campanac*, et al.*, 2002), the protective effect of the biofilm extracellular polymeric substance (Neut, 2005), the presence of intrinsically insusceptible persisters (Lewis, 2005) or induced stress responses within the biofilm (Adnan*, et al.,* 2010). After exposure to antimicrobials several organisms demonstrated significant alterations in their ability to form biofilms. This may be due to the selection of mutants with alterations in factors directly involved in bacterial adhesion and biofilm maturation, or potentially due to the selection of mutants with altered fitness levels, leading to abnormal growth rates, which could effect the development of the biofilm (Latimer*, et al.*, 2012).

The data presented in this chapter, suggests that long-term bacterial exposure to antimicrobials may select for organisms with decreased susceptibilities, however, in adapting to an antimicrobial stress the resulting bacteria may potentially alter other physiological characteristics. A decrease in fitness in antimicrobial insusceptible bacterial mutants, when compared to wild-type strains, has been widely documented (Rozen*, et al.*, 2007). The acquisition of a mutation that renders the bacteria less susceptible to a particular antimicrobial may therefore also result in a fitness cost, this could present as a fall in growth rate and subsequent biofilm formation (Bayston*, et al.*, 2007). Since biofilm formation is often a key virulence determinant, this may indicate that the insusceptible organisms have an altered pathogenicity, when compared to the parent strains. Previous work has indicated changes in various virulence factors, in bacteria, after long-term antimicrobial exposure and these have resulted in significantly impaired bacterial pathogenesis (Latimer*, et al.*, 2012). It is possible that similar alterations may have occurred in many of the bacteria we have selected for in this study. Therefore, even though long-term biocide exposure may lead to the development of bacterial insusceptibility, there are a multitude of additional factors, which must be taken into account when considering how this adaption could impact bacterial virulence, thereby influencing the clinical relevance of these findings.

Chapter 4

Attenuated Virulence and Biofilm Formation in *Staphylococcus aureus* following Sub-Lethal Exposure to Triclosan

Abstract

Sub-effective exposure of *Staphylococcus aureus* to the biocide triclosan can reportedly induce a small colony variant (SCV) phenotype. SCVs are characterised by slow growth rates, reduced pigmentation and lowered antimicrobial susceptibility. Whilst they may exhibit enhanced intracellular survival, there are conflicting reports regarding their pathogenicity. The current study reports the characteristics of an SCV-like strain of *S. aureus*, created by repeated passage on sub-lethal triclosan concentrations. *S. aureus* (P0) was serially exposed ten times to concentration gradients of triclosan to generate strain P10. This strain was then further passaged ten times on triclosan-free medium (designated X10). The minimum inhibitory and bactericidal concentrations of triclosan for P0, P10 and X10 were determined and growth rates were measured in biofilm and planktonic culture. Haemolysin, DNase and coagulase activities were measured and virulence was determined using a *Galleria mellonella* pathogenicity model. Protein expression profiles were analysed before (P0) and after (P10) triclosan exposure via two-dimensional gel electrophoresis. Strain P10 exhibited decreased susceptibility towards triclosan and characteristics of an SCV phenotype, including reduced growth rate and the formation of pinpoint colonies. However, this strain also exhibited delayed coagulase production, impaired haemolysis, was defective in biofilm formation, DNase activity and displayed significantly attenuated virulence. Colony size, haemolysis, coagulase activity and virulence were only partially restored in strain X10, whereas planktonic growth rate was fully restored. However, X10 was at least as defective in biofilm formation and DNase production as P10. Proteomic analysis revealed an upregulation of triclosan target enzyme FabI, in the P10 strain, providing a possible explanation for the decrease in triclosan susceptibility. P10 was rapidly outcompeted by P0 when grown in competition, suggesting a lower competitive fitness in the

triclosan induced SCV than in the parent strain. These data suggest that although repeated exposure to triclosan may result in an SCV-like phenotype, this is not necessarily associated with increased virulence and adapted bacteria may exhibit other functional deficiencies

4.1 Introduction

Staphylococcus aureus is an important human pathogen that is responsible for a range of hospital and community-acquired infections (Davis*, et al.*, 2004). Successful treatment of such infections is complicated not only by the emergence of methicillin-resistant strains (MRSA) but also, it has been claimed, by the spontaneous generation of small-colony variants (SCVs) (von Eiff*, et al.*, 2000). Staphylococci are the organism most frequently associated with medical device infection and it has been claimed patients infected with staphylococci small colony variants often exhibit poor response to antimicrobial therapy (Acar, 1978, Proctor, 1995, von Eiff*, et al.*, 1997, Besier*, et al.*, 2007).

SCVs display a distinct phenotype, characterised by the formation of pinpoint colonies on agar, low growth rate and reduced pigmentation. Such characteristics may lead to their misidentification in clinical microbiology laboratories (Kipp*, et al.*, 2005), potentially complicating diagnosis (Proctor & Peters, 1998). SCVs in *S. aureus* are a slow growing sub-population that may be recovered from patients with persisting or relapsing infections (Proctor*, et al.*, 2006), particularly those undergoing treatment with aminoglycoside antibiotics (Schaaff*, et al.*, 2003), which may exhibit decreased susceptibility to antimicrobials. Over the past decade SCVs have been

associated with chronic osteomyelitis as well as skin and soft tissue infections (Table 4.1). Organisms previously reported to display the SCV phenotype, apart from *S. aureus*, include *S. epidermidis*, *S. capitis*, *S. lugdunensis*, *P. aeruginosa*, *B. cepacia* and *E. coli*.

Table 4.1. Clinical case studies in which *S. aureus* small colony variants have been isolated, adapted from (Von Eiff & von, 2006).

Type or site of infection	Study details
Sternoclavicular joint septic arthritis	First report SCV causing invasive disease in a child (Spearman, 1996).
Chronic osteomyelitis, septic arthritis	Persistent and relapsing infections over a period of several years (Proctor, 1998).
Osteomyelitis	SCVs recovered following gentamicin bead placement (von Eiff*, et al.*, 1997).
Osteomyelitis	Failed screw osteosynthesis of a proximal femur fracture in a patient with autosomal dominant osteoporosis type II (Rolauffs, 2002).
Deep hip abscess	Methicillin-resistant SCV isolated from a patient with AIDS with long-term trimethoprim/sulfamethoxazole prophylaxis (Seifert, 1999).

The SCV phenotype, in *S. aureus*, has been commonly attributed to defects in the electron transport chain, due to mutations in *hemB*, *ctaA* or *menD,* which inhibit haemin, haemin A and menadione biosynthesis, respectively (Bates*, et al.*, 2003). Defects in electron transport lead to a reduction in ATP production, which is required for cell wall synthesis, thus bacterial growth rate decreases, potentially leading to a

smaller colony size (Kriegeskorte*, et al.*, 2011). It has been shown that the SCV phenotype in such mutants, can be reversed via supplementation with menadione or haemin, as is typical for auxotrophic defects (Balwit*, et al.*, 1994). Furthermore, the menadione auxotrophic phenotype can be reversed via supplementation with O-succinylbenzoate but not with isochorismate, suggesting that the defect in the menadione biosynthetic pathway lies between these two compounds (Balwit*, et al.*, 1994).

Some SCVs result from thymidine auxotrophy, due to mutations in genes encoding thymidine synthesis or transport, leading to impaired DNA synthesis (Kahl*, et al.*, 2003). Thymidine-dependent SCVs may emerge after long-term treatment with trimethoprim sulphamethoxazole (SXT), a cystic fibrosis therapy which inhibits dihydropteroate synthetase, an enzyme involved in folic acid thus thymidine production. Such SCVs can survive on exogenous thymine, for instance in the airway secretions of CF patients, which are rich in necrotic cells (Kahl*, et al.*, 1998). Thymidine is also required for menadione biosynthesis, suggesting that menadione auxotrophy may be a common factor in SCV formation, in menadione and thymidine mutants. However, SCVs isolated from infections can result from mechanisms other than menadione and thymidine auxotrophy (von Eiff*, et al.*, 2000), suggesting diversity in SCV mechanisms that have not been systematically investigated and that SCVs which emerge from various sources are not necessarily functionally equivalent.

Reduced susceptibility to antibiotics in some SCVs is likely to be multifactorial, depending on whether the particular SCV is attributable to aminoglycoside-selected auxotrophy or was generated by other means. Since electron transport chain defects

result in reduced electrochemical gradients, this may impair the uptake of aminoglycosides and other cationic molecules (Chuard*, et al.*, 1997). Additionally, lowered growth rate in SCVs could reduce antimicrobial susceptibility by non-specific mechanisms associated with reduced expression of pharmacological targets. Alternatively, enhanced survival within the host cells could shield the bacteria from inhibitory concentrations of antibiotics and biocides (Vesga*, et al.*, 1996). The clinical significance of these attributes, however, depends on whether SCVs can revert to full virulence following cessation of treatment, whether the expression of SCV phenotypes influences the ability to form recalcitrant biofilms on surfaces and importantly the relative fitness of the SCV.

The generation of the SCV phenotype in *S. aureus* has been induced *in vitro* by exposure to triclosan (Bayston*, et al.*, 2007), a trichlorinated diphenyl ether biocide. Effective concentrations of triclosan are highly bactericidal towards susceptible species where the molecule causes membrane damage through direct interaction and/or by perturbing lipid biosynthesis (Escalada*, et al.*, 2005). Triclosan is used as an antibacterial in a variety of consumer and clinical applications (Braid & Wale, 2002), including triclosan wash solutions, which have been used to reduce carriage of methicillin-resistant *S. aureus* (MRSA) in hospitals (Kampf & Kramer, 2004, Rashid*, et al.*, 2006, Al-Mukhtar*, et al.*, 2009). The enoyl-acyl carrier protein reductase FabI has been identified as a primary pharmacological target for triclosan (McMurry, 1998, Levy*, et al.*, 1999). Inhibition of FabI renders the cell unable to synthesise fatty acids and growth is therefore inhibited and depending on concentration and time, bacteria can be permanently inactivated (Heath, 1999). It has been suggested that the use of triclosan and other antiseptics should be limited to

applications where a clear health benefit can be demonstrated, due the possibility of reduced bacterial susceptibility to the molecule and potentially also to chemically-unrelated compounds (Braoudaki & Hilton, 2004).

Reduced susceptibility to triclosan is reported in a number of *in vitro* studies but not, to date, in the environment (Levy, 2001, Meyer & Cookson, 2010). In one study, SCVs generated by the *in vitro* exposure of bacteria to sub-lethal concentrations of triclosan-impregnated in silicone disks exhibited reduced susceptibility to the molecule and altered colony morphology. Since triclosan-exposed *S. aureus* has the potential to display an SCV-like phenotype and some clinical SCV isolates appear to be able to cause disease (von Eiff*, et al.*, 2000), it has been inferred that triclosan use might generate SCVs, which could potentially be of clinical significance (Berends*, et al.,* 2010). However, these *in vitro* studies do not necessarily reflect the clinical impact of triclosan-selected SCVs (Berends*, et al.,* 2010). For example, in a previous report, SCVs generated using triclosan-impregnated disks demonstrated slow growth rate, reduced coagulase and DNase activity and decreased haemolysis (Bayston*, et al.*, 2007), which is suggestive of decreased virulence.

There is a clear need to determine the ability of triclosan-generated SCVs to initiate infection (von Eiff*, et al.*, 2000). To our knowledge, there are currently no published reports regarding the impact of triclosan exposure on the virulence of resulting SCV-like strains. Additionally, whilst antibiotic-selected SCVs may revert to wild-type phenotype *in vivo* following cessation of treatment, this has not been reported for triclosan-selected SCVs (Brouillette*, et al.*, 2004). The present study therefore investigates the growth and virulence of an SCV-like *S. aureus* strain, created by

serial exposure to sub-inhibitory concentrations of triclosan. The potential reversion of this strain following subsequent repeated passage on triclosan-free medium was also studied.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth media

Staphylococcus aureus ATCC 6538 was acquired from the American Type Culture Collection and cultured on Mueller-Hinton agar (MHA), Colombia blood agar (CBA) or Mannitol salt agar (MSA, Oxoid, UK) aerobically at 37˚C for 18 h. For supplementation experiments, haemin (1, 10 or 100 μM, final concentrations), menadione (0.4, 4 or 40 μM, final concentrations) or thymidine (0.62, 6.2 or 62 μM final concentrations) were added to MHA. Cryogenic stocks of the bacteria were archived at -80˚C.

4.2.2 Selection of isolates with reduced susceptibility to triclosan

Reproducible concentration gradients of triclosan were created on Mueller-Hinton Agar by depositing stock solutions of triclosan (1 μg/ml, 10 μg/ml, 100 μg/ml or 1 mg/ml) with a Wasp II spiral plater (Don Whitley, Shipley, UK). Plates were dried for 1 h at room temperature prior to radial deposition of an overnight suspension of *S. aureus* and incubated for 4 d aerobically at 37˚C. Growth observed at the highest triclosan concentration was aseptically removed and used to inoculate further gradient plates. This process was repeated for ten passages. A further ten passages were performed on triclosan-free MHA. Wild-type bacteria (P0), those passaged ten times on triclosan (P10) and those passaged a further ten times on triclosan-free

Muller-Hinton Agar (X10) were archived at -80˚C for subsequent MIC and MBC determination.

4.2.3 Determination of bacterial minimum inhibitory concentrations (MIC)

Overnight cultures of *S. aureus* were grown to an approximate OD_{600} of 0.8 and were then diluted 1:100 in sterile Mueller-Hinton Broth (MHB) and aliquots (120 µl) were delivered to the wells of polystyrene 96-well plates (Corning Ltd, Corning, USA). Stock solutions (1.16 mg ml^{-1}) of triclosan were prepared in 25% ethanol, filter sterilised and stored at –80˚C. Doubling dilutions of triclosan were added to the diluted overnight cultures (final concentrations 232 µg ml^{-1} to 0.23 µg ml^{-1}). Sterile and triclosan-free controls were also included. Plates were incubated for 24 h aerobically at 37˚C with shaking at 100 rpm. MICs were determined as the lowest concentration of triclosan showing no turbidity in comparison to a sterile negative control. Three technical and two biological replicates were conducted. Previous validation studies with staphylococci and other genera showed that the triclosan solvent has no effect on the outcome of MIC or MBC determinations (Ledder*, et al.*, 2006).

4.2.4 Determination of bacterial minimum bactericidal concentrations (MBC)

Aliquots (10 µl) taken from MIC plates (above) were spot-plated onto MHA plates in triplicate and incubated aerobically at 37˚C. MBCs were determined as the lowest concentration of triclosan at which no growth was observed after 4 d of incubation.

4.2.5 Crystal violet biofilm assay

Overnight cultures of test bacteria were grown to an approximate OD_{600} of 0.8 and were then diluted 1:100 into sterile Muller-Hinton Broth to use as a bacterial inoculum for the assay. 150 µl of inoculum was delivered to each test well of a 96-well microtiter plate. Plates were incubated for 48 h at 37^{o}C and 20 rpm to promote biofilm growth. After incubation wells were washed twice with 200 µl of sterile PBS. After washing, 200 µl of 1 % crystal violet solution was added to the test wells and plates were incubated for 30 min at room temperature. Wells were washed twice with 250 µl of PBS and left to dry at room temperature for 1 h. Attached crystal violet was solubilised within the well by adding 250 µl of 70 % ethanol and plates were agitated at room temperature at 20 rpm for 1 h. After solubilisation, biofilm growth was viewed as change in OD_{600} relative to a sterile negative control.

4.2.6 Planktonic growth rate measurement

Overnight suspensions of *S. aureus* P0, P10 and X10 were grown to an approximate OD_{600} of 0.8, were diluted 1:100 in 30 ml of Muller-Hinton Broth in a sterile conical flask and incubated at 37^{o}C with shaking at 150 rpm. 1ml samples were removed at regular intervals and diluted as appropriate. Cultures were transferred into 1ml semi-micro cuvettes and optical density was measured at 600 nm using a Helios spectrophotometer (Pye Unicam Ltd., Cambridge, UK). Sterile controls were included. Growth rate was plotted as OD_{600} over time (h).

4.2.7 Determination of biofilm growth rate

Overnight suspensions of *S. aureus* were diluted 1:40 in Muller-Hinton Broth and aliquots (200µl) were delivered to the wells of a 96-well microtiter plate. Plates were incubated statically aerobically at 37˚C in humid containers to minimise evaporation for up to 74 h. At regular time points 1 ml samples of planktonic suspensions were transferred into semi-micro cuvettes and cell density was measured spectrophotometrically at 600 nm. The remaining biofilm was stained for 60 seconds with 1 % (w/v) crystal violet (200 µl), which was aspirated and wells were washed three times with PBS before allowing plates to air-dry. Biofilm-bound CV was eluted in 70 % ethanol (200 µl) and absorbance was measured spectrophotometrically at 630 nm. Biofilm units' (arbitrary units) were calculated by dividing biofilm absorbance by the corresponding planktonic OD. Sterile controls were also included.

4.2.8 Visualisation and quantification of biofilm architecture and viability

Overnight suspensions of *S. aureus* were diluted 1:40 in Muller-Hinton Broth (40 ml) in a Duran bottle containing a partially submerged sterile glass microscope slide. Bottles were incubated at 37˚C statically for 24, 48 or 72 h before gentle rinsing in 50 ml of sterile PBS. Biofilm viability and structure were visualised by fluorescence microscopy. Briefly, a working solution of BacLight LIVE/DEAD stain (Invitrogen Ltd, Paisley, UK) was prepared by adding 1 µl each of SYTO 9 (component A) and propidium iodide (component B) to 98 µl distilled water. This solution (20 µl) was applied directly to the biofilm and covered with a glass cover slip. Slides were incubated at room temperature in the dark for 15 min, according to the standard BacLight staining protocol. Biofilms were visualised with an Axioskop 2 fluorescence microscope with a x 10 objective lens (Carl Zeiss Ltd, Rugby, UK) and

images captured using a digital microscope eyepiece (Cosmos Biomedical, Derbyshire, UK) and exported as JPEG files. Bacterial cells incubated in the presence of both stains fluoresce either green (viable) or red (dead). The excitation and emission maxima for these dyes are 480 and 500 nm for SYTO 9 and 490 and 635 nm for propidium iodide. The percentage viable biomass was determined by calculating the proportion of red fluorescence as a percentage of total fluorescence. Biofilm three-dimensional structure was visualised with an LSM Confocor 2 confocal microscope with a x 10 objective lens (Carl Zeiss Ltd, Rugby, UK). Images were processed, surface-rendered and quantified using Imaris software (Bitplane AG, Zurich, Switzerland).

4.2.9 Determination of bacterial haemolysin activity

The method used for determination of haemolysin activity was adapted from that of Hathaway and Marshall (Hathaway & Marshall, 1980). Overnight suspensions of *S. aureus* were diluted 1:50 in Muller-Hinton Broth (50 ml) in a conical flask and incubated at 37˚C and 100 rpm till they reached an OD_{600} of 0.3. Whole defibrinated horse blood (Oxoid, UK) was added to a final concentration of 5% (v/v) before incubating for a further 3 h at 37^{o}C and 100 rpm. After incubation 1ml Aliquots were centrifuged at 16,000-x g (1-14 microfuge, Sigma, Dorset, UK) for 4 min at room temperature and the absorbance of the supernatant was measured spectrophotometrically at 540 nm. Colony counts were performed by plating appropriate dilutions of bacterial culture onto Mannitol salt agar in triplicate. To avoid variation caused by differences in cell number, specific haemolysis was calculated as ΔA_{540}/cfu. Negative and positive controls (sterile Muller-Hinton Broth and distilled water, respectively) were included.

4.2.10 Determination of bacterial DNase activity

This method was adapted from the Health Protection Agency National Standard Thermonuclease Test Method (2010). Overnight suspensions of bacteria were diluted 1:50 in Muller-Hinton Broth (50 ml) in a conical flask and incubated at 37˚C and 100 rpm to an OD_{600} of 0.3. Aliquots (1ml) were incubated at 100˚C for 15 min, centrifuged at 16,000-x g for 5 min and the supernatant was retained. Wells were cut into plates containing DNase test agar, using a sterile cork borer (40 mm diameter) (Oxoid, UK) and filled with supernatant (40 µl). Plates were incubated overnight at 37˚C, before being overlaid with 1M HCl. Polymerised DNA caused the agar to appear opaque, and clear zones surrounding the wells indicated levels of DNase activity. Zone diameters were recorded and colony counts were performed by plating appropriate dilutions of unheated cultures on Mannitol-salt agar to account for variations caused by differences in cell number.

4.2.11 Coagulase assay

Suspensions of bacteria in Mueller-Hinton Broth (1 ml, OD_{600} = 0.4) were added to 3 ml rabbit plasma with EDTA (Bactident coagulase, Merck, Darmstadt, Germany), in triplicate, and incubated at 37˚C in a water bath. Tubes were monitored for signs of coagulation over 3 h and scored on a five-point scale according to manufacturers' instructions.

4.2.12 Galleria mellonella pathogenesis assay

The pathogenesis model was adapted from Peleg et al. (Peleg*, et al.*, 2009). Final larval-stage *G. mellonella* (Live Foods Direct, Sheffield, UK) were stored in the dark

at 4˚C for less than 7 days before randomly assigning 16 to each treatment group and incubating at 37˚C for 30 min prior to use. Overnight suspensions of *S. aureus* strains P0, P10 and X10 were washed twice in PBS and diluted appropriately to achieve an optical density at 600 nm of 0.1 (5-8 x 10^5 cfu/ml, as confirmed by colony counts on Mannitol-salt agar) Aliquots of each suspension (5 µl) were injected into the hemocoel of each larva via the last left proleg using a sterile Hamilton syringe (2.5-4 x10^3 cfu per individual). Larvae were incubated in plastic petri dishes at 37˚C and the number of surviving individuals was recorded daily (dead larvae were unresponsive to touch and appeared black). An untreated group and a group injected with sterile PBS were used as negative controls. The experiments were terminated when at least 2 individuals in a control group had died. Three independent replicates were performed and significance was calculated using the Log-Rank test. Data were plotted as survival curves and representative data are presented. Dead individuals were homogenised in sterile PBS, diluted appropriately and aliquots were spread on Mannitol-salt agar to calculate bacterial load per individual at death.

4.2.13 Competitive fitness assay

Single colonies of P0 or P10 *S. aureus* were added to 10 ml of sterile Muller-Hinton Broth and incubated for 18 h at 37˚C and 100 rpm. Cultures were diluted 1:10 and adjusted to an OD_{600} of 1.5. Conical flasks containing 250 ml of sterile Muller-Hinton Broth were inoculated with either 500 µl of adjusted P0 or P10 inoculum, or a combination of 250 µl of both P0 and P10. Immediately after inoculation, 1 ml of culture was removed from each flask, diluted to 10^{-6} and 100 µl (10^{-2} to 10^{-6}) was plated onto Muller-Hinton Agar and Muller-Hinton Agar containing 1µg ml^{-1} triclosan (MHA_{TCS}) plates, in triplicate. Flasks were incubated at 37˚C and 100 rpm

for 24 h. After incubation, dilutions from 10^{-4} to 10^{-6} were plated onto MHA and MHA_{TCS} plates in triplicate and incubated at 37˚C for 18 h. Bacterial viable counts were determined after 18 h of incubation, and relative fitness was assessed, for bacteria grown independently and in combination, using the equation;

W= ln (RF/RI)/ ln (SF/SI)

Where W is relative fitness, RI and SI refer to the number of resistant and susceptible cells at the start point, respectively, and RF and SF to the number of resistant and susceptible cells at endpoint.

4.2.14 Proteomic analysis of the triclosan adapted SCV phenotype in S. aureus by 2D electrophoresis

4.2.14.1 Protein Extraction

To extract protein from strains of interest, 10 ml bacterial cultures were grown in Muller-Hinton Broth at 37˚C and 100 rpm for 18 h. Cultures were then diluted 1:100 into 200 ml of sterile Muller-Hinton Broth in a conical flask and incubated at 37˚C and 100 rpm until approximate mid-log phase of growth was reached (OD_{600} of 0.4). The culture was spun at 12,000 x g using a Denley DL136 bench centrifuge (DJB Labcare LTD, Buckinghamshire, UK) to pellet the bacterial cells. The cells were washed in three times in 3 ml of ice cold PBS before being resuspended in 1 ml of PBS. To extract protein from cultures of *S. aureus,* 50 μg/ml of lysostaphin was added to the resuspension mixture, before being incubated for 15 min on ice prior to sonication. Resuspended cells were disrupted via sonication using the MSE Ultrasonic disintegrator Soniprep 150 (Fisher Scientific Ltd, Loughborough, UK) at an amplitude of 10 microns in 6 x 30 second bursts, followed by 30 seconds of

cooling on ice at each interval. After sonication, protein was precipitated by mixing a 1:1:8 solution of cell lysate with trichloroacetic acid and ice-cold acetone. The protein solution was left to precipitate at -20˚C for 1 h. Protein was pelleted by centrifugation at 16,000 x g using a Sigma 1-14 microcentrifuge (Poole, UK) before being washed in three times in 1 ml of ice-cold acetone. The protein pellet was dissolved in 2 ml of rehydration buffer (9M urea, 2% CHAPS, 1% DTT, 2% Carrier ampholytes, 0.5% protease inhibitor, 0.001% bromophenol blue) via incubating for 1 h at room temperature and vortexing for 30 seconds every 15 min. Soluble protein concentration was quantified using the Bradford assay (Sigma, Poole, UK). In brief, 20 µl of rehydrated protein solution was added to 1 ml of Bradford's reagent and was incubated for 4 min at room temperature before OD_{595} was measured. Protein concentration was determined using a standard curve, produced using bovine serum albumin standards of known concentrations.

4.2.14.2 Protein loading and isoelectric focusing

Protein concentration was adjusted using rehydration buffer to the required level. Between 250 µg and 500 µg of protein per 200 µl total volume of buffer was loaded into an 11cm ReadyStrip^{TM} immobilised protein gradient (IPG) strip (Bio-Rad, Hertfordshire, UK). Each sample of rehydrated protein solution was pipetted along an individual lane in the PROTEAN IEF Focusing Tray, avoiding bubbles (Bio-Rad, Hertfordshire, UK). A single IPG strip was laid gel side down into the tray to allow contact with the rehydrated protein solution. Strips were then covered in 2 ml of sterile mineral oil and rehydrated under active conditions overnight using a PROTEAN IEF cell (Bio-Rad, Hertfordshire, UK). After strip rehydration, paper wicks dampened with 10 µl of deionised water were placed underneath the strips to

cover the electrodes. IPG strips were covered with 2 ml of sterile mineral oil before isoelectric focusing was conducted as follows; Conditioning step S1, 250 V for 15 min; Voltage ramping S2, voltage increases linearly until 8000 V is reached; finally a 500 V is maintained until the run is stopped. In the first dimension of electrophoresis, the proteins were separated along an IPG strip depending upon their isoelectric points.

4.2.14.3 Equilibration and second dimension electrophoresis

After the first dimension of focusing, IPG strips were removed from the PROTEAN IEF Focusing Tray and placed on dampened filter paper for 1 min to remove any excess oil from the gel side of the strip. At this point strips could be wrapped in cling film and stored at -80˚C until required for use. To equilibrate, strips were placed gel side up in a disposable plastic tray and 5 ml of equilibration buffer 1 (6M urea, 2 % SDS, 50 mM Tris-HCL pH 8.8, 2 % glycerol and 1 % DTT) was added, immersing the strip. Trays were agitated at 20 rpm at room temperature for 20 min before buffer was decanted and the process was repeated with 5 ml of equilibration buffer 2 (6M urea, 2 % SDS, 50 mM Tris-HCL pH 8.8, 2 % glycerol and 2.5 % iodoacetamide). In the second dimension run, proteins were separated by size, via SDS-denaturing electrophoresis. Polyacrylamide casting gels (34 ml distilled water, 25 ml 1.5 M Tris-HCL pH 8.8, 0.5 ml of 20 % SDS and 40 ml of 30 % bis-acrylamide) were polymerised by the addition of 0.5 ml of 10 % Ammonium persulphate and 100 µl of TEMED. The gel was constructed as follows; two glass plates separated by a 1 mm thick plastic spacer were assembled in a Bio-Rad gel caster (Bio-Rad, Hertferdshire, UK), approximately 50 ml of gel solution was poured between the plates immediately after addition of the APS/TEMED and the gels were left at room

temperature for 1 h to polymerise. After polymerisation, approximately 15 ml of stacking gel solution (34 ml distilled water, 6.25 ml of 1 M Tris-HCL, 0.25 ml of 20 % SDS, 8.5 ml of 30 % bis-acrylamide, 0.25 ml APS and 50 µl TEMED) was poured above the set casting gel. A few drops of isopropanol were added to the top of the gel to obtain a bubble free surface layer. IPG strips were loaded above the stacking gel with the gel side of the strip contacting the larger glass plate. 10 µl of Bio-Rad broad range protein marker was added to a paper wick, which was inserted to the top left corner of the stacking gel to act as a molecular weight ladder. Approximately 3 ml of overlay agarose was poured over the IPG strip and wick to obtain a smooth top layer. Gels were run at 20 V for 1-2 h until the bromophenol blue dye front could be seen to have loaded onto the gel. Gels were then run at 55 V for 15-18 h until the dye front had migrated approximately 80 % through the length of the gel.

4.2.14.4 Gel staining

Gels were fixed for 8 h in fixing solution (500 ml ethanol, 400 ml water and 100 ml acetic acid) at room temperature. Fixing solution was decanted and replaced by 500 ml of coomassie blue stain (0.8g coomassie blue R350, 400 ml of 40 % methanol and 400 ml 20 % acetic acid) and left for 18 h at room temperature and 20 rpm. After staining, the coomassie blue stain was decanted and gels were destained using a destain solution (500 ml methanol, 400 ml water and 100 ml acetic acid), which was continuously replaced until the gel appeared clear and the blue spots could easily be visualised. Gel spots of interest were excised and proteins were identified using tandem mass spectrometry, performed at The Biomolecular Analysis Facility within The University of Manchester. Gels were stored in a storage solution (25 ml acetic acid and 500 ml water) for up to 1 month.

4.3 Results

4.3.1 Triclosan exposure selected for isolates with reduced triclosan susceptibility

Initially, the minimum inhibitory concentration (MIC) of triclosan to S*taphylococcus aureus* (P0) was 0.23 µg ml^{-1}. After 5 passages (P5), this increased to 9.67 µg ml^{-1} and after 10 exposures (P10), to 29 µg ml^{-1} (a 126-fold increase compared to the parent strain (Figure 4.1).

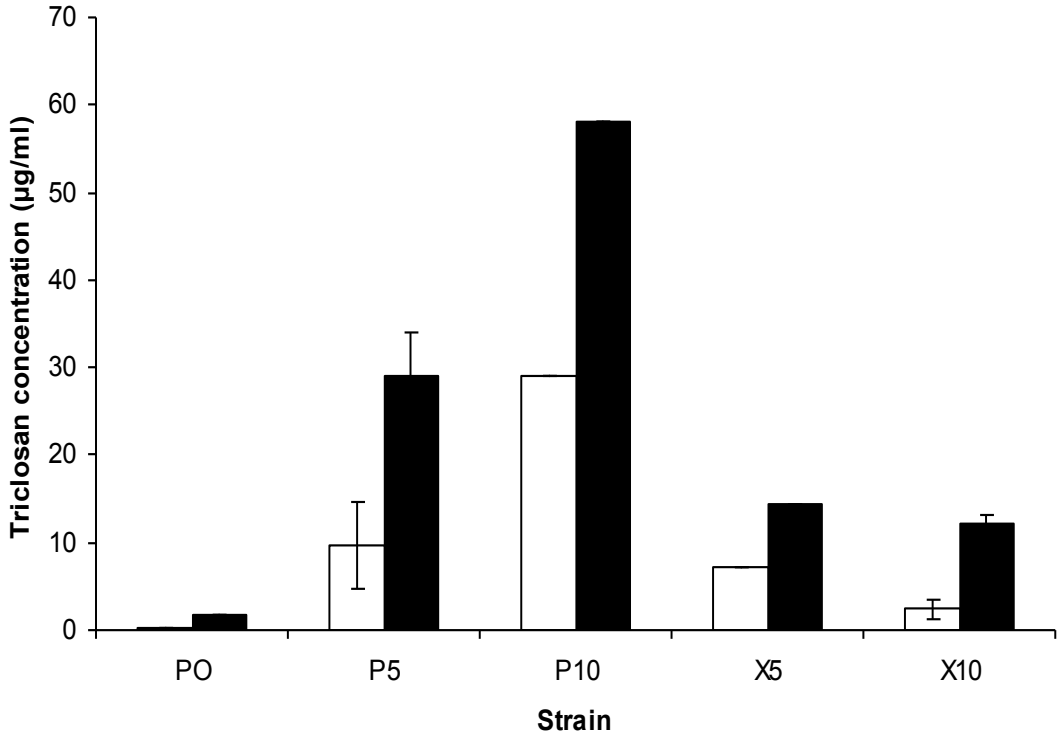

Figure 4.1. Minimum inhibitory concentrations (MICs, white bars) and minimum bactericidal concentrations (MBCs, black bars) of triclosan to *S. aureus* before exposure (P0), after 5 or 10 passages in the presence of triclosan (P5 and P10, respectively) and after 5 or 10 subsequent passages on triclosan-free medium (X5 and X10 respectively). Error bars show standard deviation (n=6).

Colonies of P10 were markedly smaller than those of their parent strain (Figure 4.2). Further passaging on triclosan-free medium resulted in a partial reversion of triclosan susceptibility and colony size; following 5 passages on MHA, the MIC was 7.3 µg

ml^{-1} and after 10 passages it was 2.4 μg ml^{-1}. Minimum bactericidal concentration (MBC) data showed a similar trend (Figure 4.1)

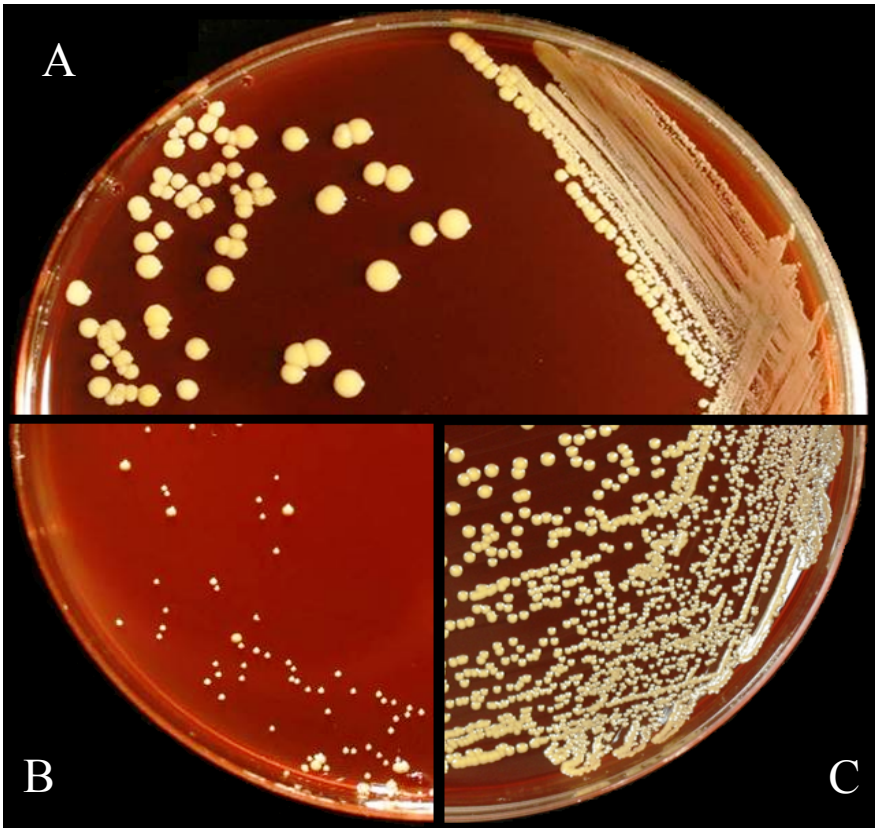

Figure 4.2. Colonies formed on Colombia blood agar by *S. aureus* parent strain (P0, A), triclosan-adapted strain (P10, B) and one passaged a further ten times on triclosan-free medium (X10, C) after aerobic incubation at 37˚C for 48 h. Colonies formed by the triclosan-adapted strain were markedly smaller and more heterogeneous than those formed by the parent strain.

4.3.2 Triclosan-adapted S. aureus grows more slowly than its parent strain in planktonic and biofilm modes

The growth kinetics of triclosan-adapted *S. aureus* (P10), its parent strain (P0) and the strain further passaged on triclosan-free medium (X10) were compared in shaken broth cultures (planktonic growth). As shown in Figure 4.3, P10 underwent an extended lag phase and entered stationary phase later than P0 and X10. The planktonic growth kinetics of P0 and X10 were not significantly different. The

exponential phase growth rate of P10 (0.19/h) was less than half that of P0 (0.43/h) and X10 (0.42/h)

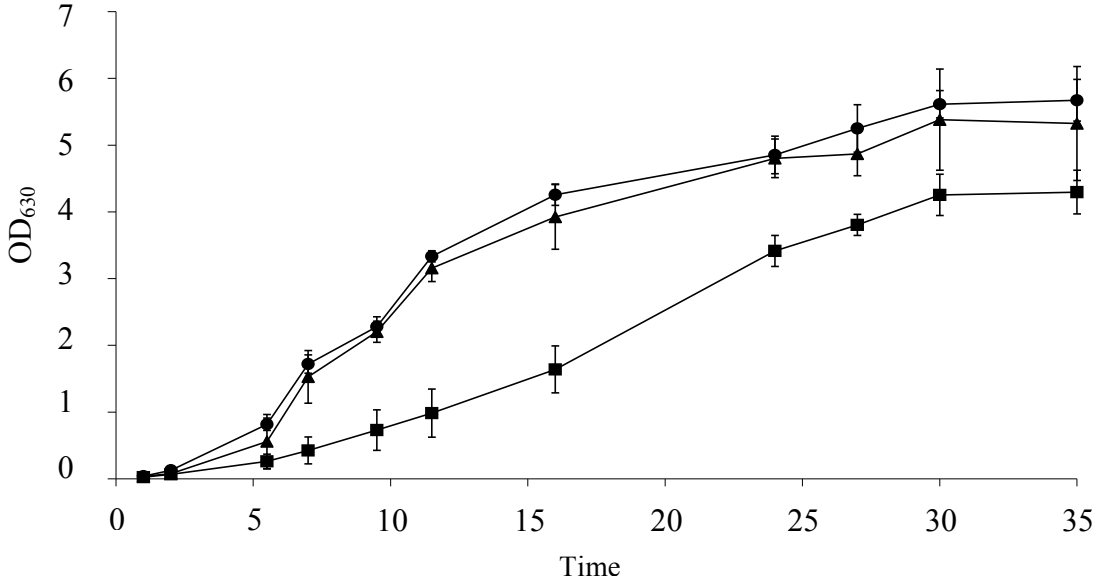

Figure 4.3. Planktonic growth of *S. aureus* parent strain (P0, triangles), triclosan-exposed strain (P10, squares) and one passaged a further ten times on triclosan-free medium (X10, circles). P10 exhibited an extended lag phase, slower growth and a delayed stationary phase when compared to P0. Error bars show standard deviation (n=3).

Biofilm growth was measured over 50 h using the well-characterised microplate assay (O'Toole & Kolter, 1998). Formation of biofilm by P10 and X10, relative to planktonic growth, was significantly lower than that of P0 (Figure 4.4). Strain X10 formed less biofilm mass than P10 for the first 15 h of growth, after which levels were not significantly different.

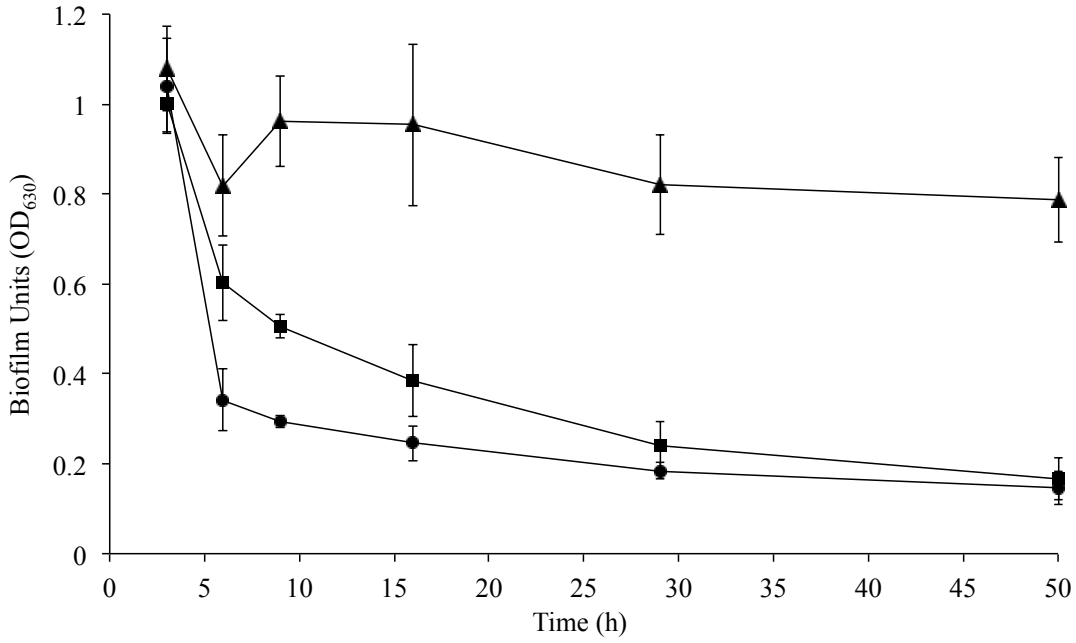

Figure 4.4. Biofilm growth of *S. aureus* parent strain (P0, triangles), triclosan-exposed strain (P10, squares) and one passaged a further ten times on triclosan-free medium (X10, circles) on polystyrene. Data are shown as 'biofilm units'. A biofilm unit is defined as the absorbance of the biofilm-bound crystal violet divided by the corresponding planktonic OD_{630} and corrects the data to give a representation of biofilm formation irrespective of planktonic mass. Biofilm formation by P10 was markedly lower than P0. This difference was significant at all time points from 6 h ($p<0.001$, $n=12$).

Biofilms formed on glass slides by P0, P10 and X10 differed markedly with respect to aerial coverage and structure (Figure 4.5). P0 was the only strain to indicate the formation of globular aggregates, whilst P10 and X10 demonstrated some level of bacterial attachment to the glass, the biofilm appeared to lack any mature structure.

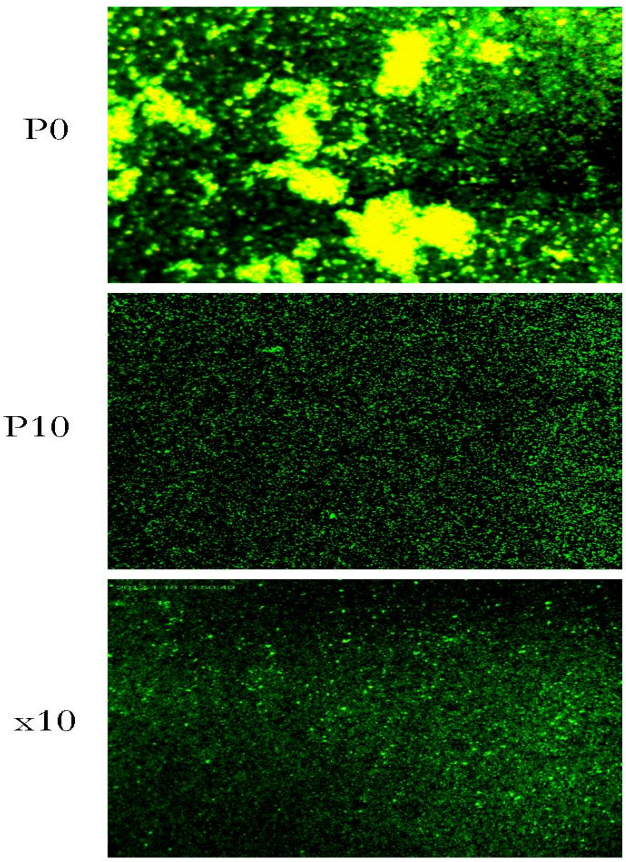

Figure 4.5. Biofilm growth on glass of *S. aureus* parent strain (P0), triclosan-exposed strain (P10) and one passaged a further ten times on triclosan-free medium (X10) as viewed by epifluorescence microscopy. Green fluorescence represents viable cells and red fluorescence represents non-viable cells. Representative fields of view at 24 h are shown.

Epifluorescence and confocal microscopy showed that P0 formed relatively thick three-dimensional microcolony structures, whereas strains P10 and X10 formed thinner, less structurally complex biofilms (Figure 4.6 A). Analysis of representative images indicated that the density of biomass in P0 biofilms was markedly higher than in those of P10 or X10 over 72 h (Figure 4.6 B).

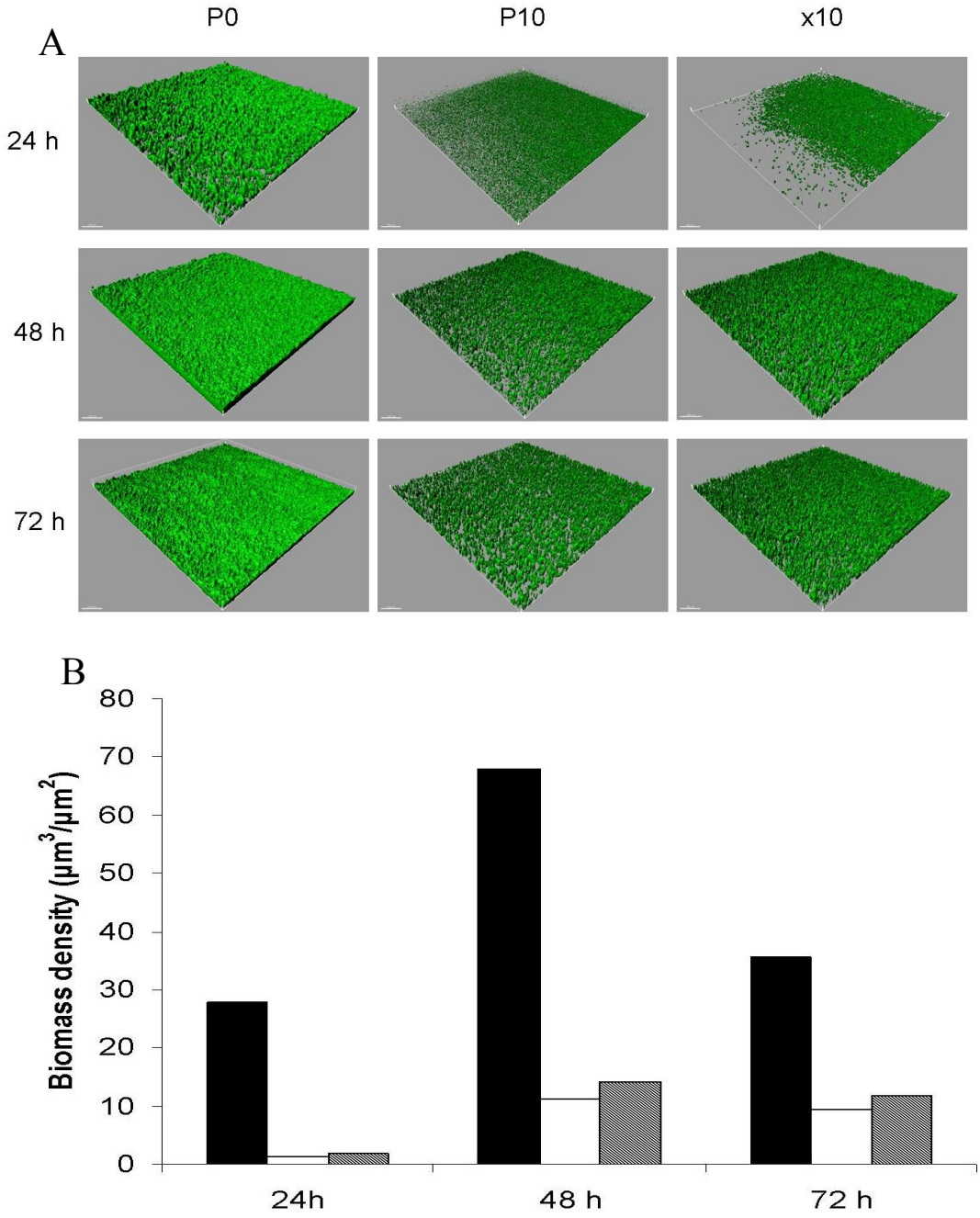

Figure 4.6. Biofilm growth on glass of *S. aureus* parent strain (P0, black bar), triclosan-exposed strain (P10, white bar) and one passaged a further ten times on triclosan-free medium (X10, grey bar) as viewed by confocal microscopy. Specialist software was used to surface-render the images, allowing comparison of relative density and structure (A), and measurement of biomass density (B). Representative images and data shown.

4.3.3 The in vitro haemolytic activity of triclosan-adapted S. aureus is lower than that of the parent strain

The ability of planktonic P0, P10 and X10 populations to lyse erythrocytes was investigated. Higher absorbance readings are indicative of lysis and release of haemoglobin into the supernatant (Figure 4.7).

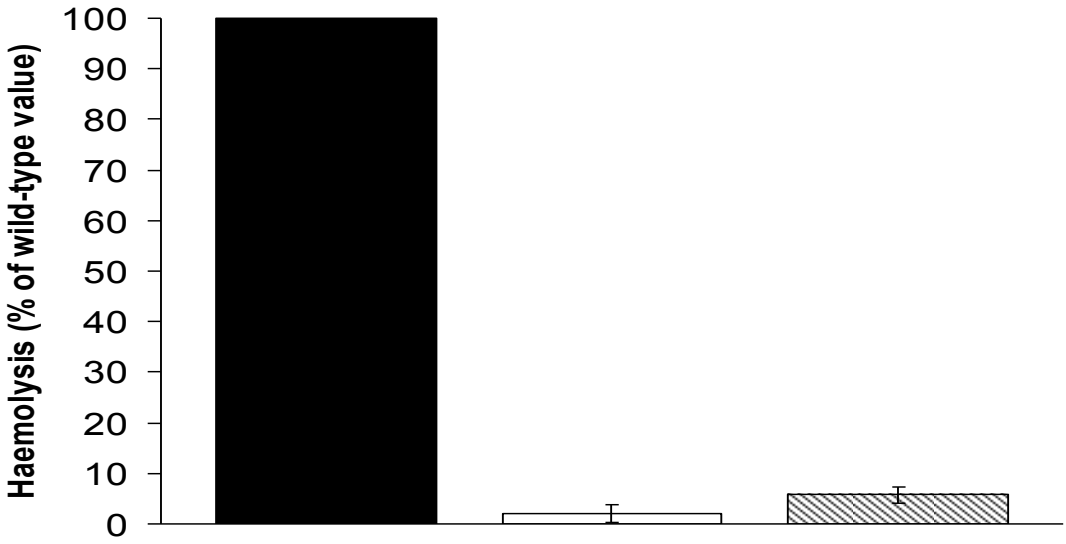

Figure 4.7. Haemolytic activity of *S. aureus* parent strain (P0, black bar), triclosan-exposed strain (P10, white bar) and one passaged a further ten times on triclosan-free medium (X10, striped bar). Whole blood (final concentration 5% v/v) was added to pre-stationary phase cultures and free haemoglobin from lysed erythrocytes was measured spectrophotometrically at 450 nm. Data are expressed as mean percentages of the mean P0 value. Error bars show standard deviation (n=3).

Haemolysis by P10 was, on average, 2% of the P0 value, a statistically significant difference (Figure 4.7, $p<0.01$). Although X10 showed a partial reversion of haemolytic activity, it was still significantly lower than the parent strain (5% of the P0 value).

4.3.4 The in vitro coagulase activity of triclosan-adapted S. aureus is lower than that of the parent strain

The levels of coagulase produced by planktonic P0, P10 and X10 populations were investigated using a standard tube coagulase test (Table 4.2).

Table 4.2. Coagulase activity of *S. aureus* parent strain (P0), triclosan-adapted strain (P10) and one passaged a further ten times on triclosan-free medium (X10). Tubes were monitored for signs of coagulation over 3 h and scored on a five-point scale according to manufacturers' instructions.

	Strain		
	P0	P10	X10
0.5 h	++++	-	-
1 h	++++	-	+
2 h	++++	+++	+++
3 h	++++	++++	++++

Negative symbol: no coagulase detected; +: small separate clots (negative); ++: small joined clots (negative); +++: extensively coagulated clots (positive); ++++: complete coagulation (positive).

Coagulase activity was observed in all strains. Although strain P0 displayed a rapid positive result after 30 min, coagulation was delayed in strains P10 and X10, which both took 2 h to show a positive result (Table 4.2).

4.3.5 The in vitro DNase activity of triclosan-adapted S. aureus is lower than that of the parent strain

The level of thermostable DNase produced by planktonic P0, P10 and X10 populations was investigated with an agar diffusion test. Zones of clearing after addition of heated culture supernatant indicate deoxyribonuclease activity. Activity (mean zone diameter 9.47 ± 0.53 cm) was only observed surrounding wells filled with culture supernatant from the P0 strain. No DNase activity was observed surrounding wells filled with culture supernatants from the P10 or X10 strains (Table 4.3). Cell density in the P0 cultures (4.6 x 10^8 cfu/ml) was not significantly different from those in the P10 or X10 populations (p=0.2).

Table 4.3. Summary indicating presence (+) or absence (-) of tested characteristics in strains P0, P10 and X10.

	Strain		
	P0	P10	X10
MIC to triclosan (μg/ml)	0.23	29	1.1
Slow planktonic growth	-	+	-
Impaired biofilm growth	-	+	+
Impaired DNase activity	-	+	+
Impaired haemolytic activity	-	+	+
Delayed coagulase activity	-	+	+
Impaired virulence	-	+	+
Reversal by hemin supplementation	-	-	-
Reversal by menadione supplementation	-	-	-
Reversal by thymidine supplementation	-	-	-

4.3.6 Supplementation with haemin, menadione or thymidine does not restore a non-SCV phenotype

To ascertain whether the SCV phenotype was caused by mutations affecting biosynthesis of haemin, menadione or thymidine, MHA specifically supplemented with these compounds was inoculated with P10, to see if there was any reversion in the SCV phenotype. The concentrations used were based on previous reports that maximal restoration of normal phenotypes occurred with 1.0 μM haemin, 0.375 μM menadione or 6.2 μM of thymidine (Webster*, et al.*, 1994, Boucher*, et al.*, 2010). Further plates were inoculated with P0 and X10 to control any growth-inhibitory effects of medium supplementation. No supplementation affected the colony morphologies of any strain (Table 4.3), suggesting that the phenotype of P10 is not attributable to defects in haemin, menadione or thymidine biosynthesis.

4.3.7 Triclosan-adapted S. aureus is less virulent in an invertebrate model

The *Galleria mellonella* pathogenesis assay was used to assess the relative virulence of the test strains *in vivo*. Groups of larvae were injected with P0, P10 or X10 and incubated at 37˚C. Dead individuals were recorded daily and data were plotted as survival curves (Figure 4.8A).

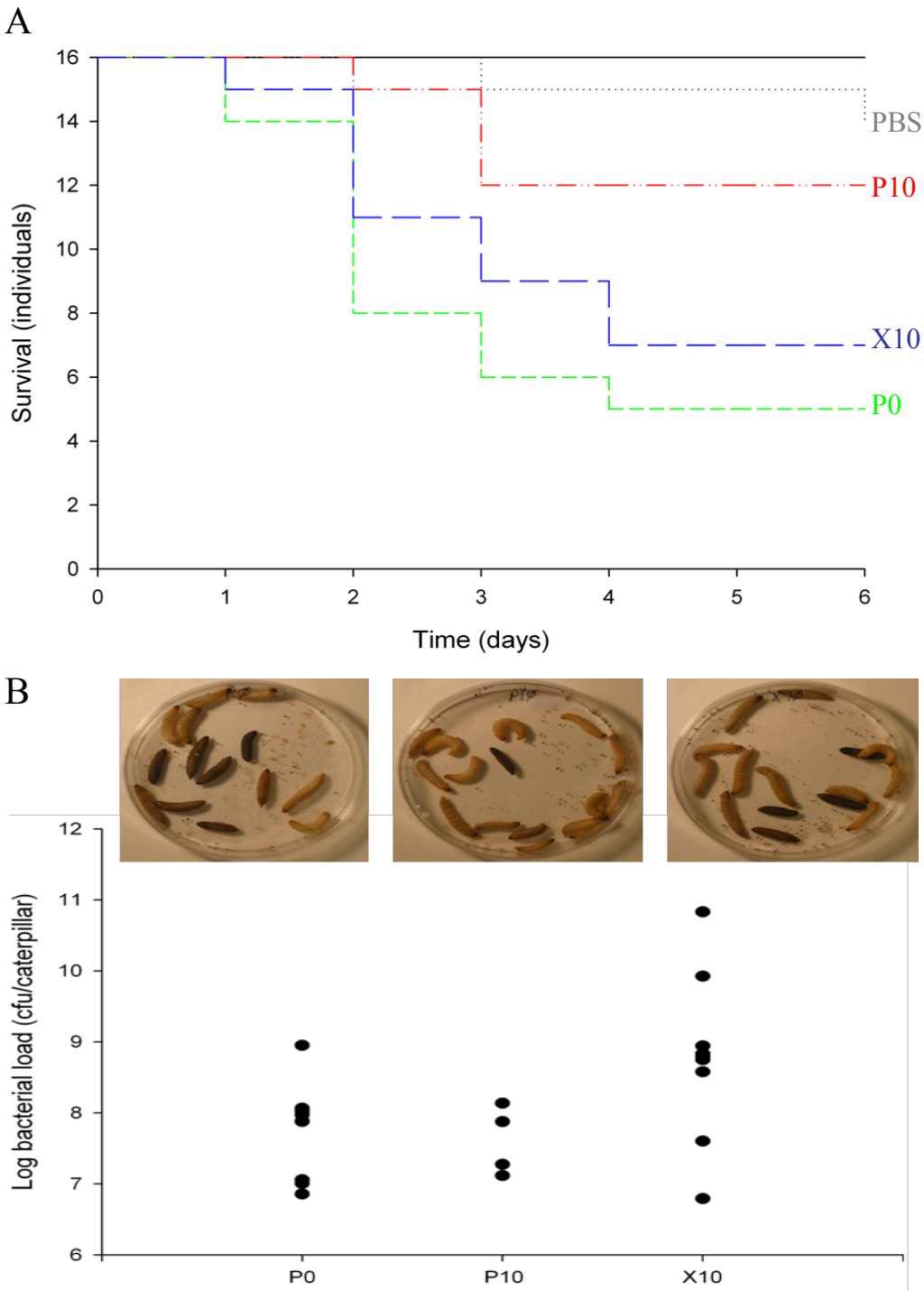

Figure 4.8. A) Example survival curve showing virulence of *S. aureus* parent strain (P0, green fine-dash curve), triclosan-exposed strain (P10, red dot-dash curve) and one passaged a further ten times on triclosan-free medium (X10, blue coarse-dash curve) in a *G. mellonella* survival assay (A, n=16), and B) bacterial load at death (each point corresponds to the bacterial load in an individual animal). P0 caused significantly higher death rates than both other strains, whereas the virulence of X10 was not fully restored. Untreated and PBS-treated control data are shown. Although the bacterial load recovered from dead larva varied, there was no significant difference between treatment groups. Example images of treatment groups are also show.

Over 6 days of incubation, the P10 treatment group suffered the fewest deaths whereas survival in the group inoculated with the parent strain P0 fell rapidly, levelling out at 5 individuals at day 4, in the example shown. In comparison, X10 displayed moderate virulence, suggesting a partial reversion in phenotype. A Log Rank test including all replicates showed that all three-treatment groups were significantly different (n=48). Within 24 h of death, the bacterial load of each individual was measured. Although numbers varied markedly between individuals (10^7 – 10^{11}), there was no significant difference between treatment groups (Figure 4.8 B). Colony morphologies of bacteria recovered from dead larvae were similar to the inoculum in all groups, suggesting that the SCV-like strain did not revert to wild-type, or vice versa, during infection. Results have been further summarized in Table 4.3.

4.3.8 Triclosan adapted S. aureus SCVs show abnormal cell morphology

The cellular morphology of *S. aureus* parent strain (P0), triclosan insusceptible P10 strain, and recovered X10 strain was viewed using TEM (Figure 4.9).

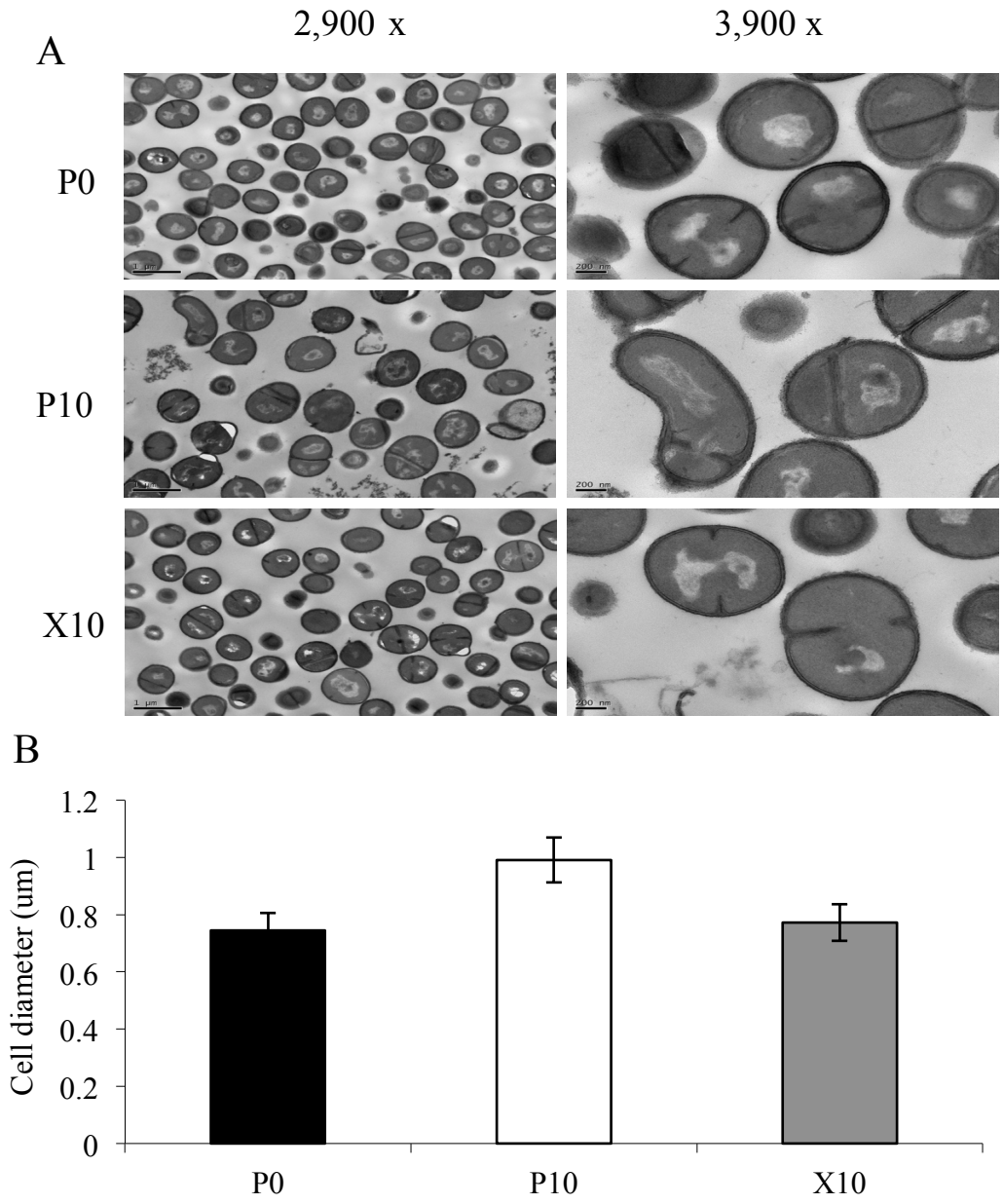

Figure 4.9. A) Cellular morphology of *S. aureus* parent strain P0, P10 and X10 strains visualised by TEM. B) Mean diameter of the fixed cells, P10 (white) cells were shown to be 32.8 and 28.3 % greater than those of P0 (black) or X10 (grey), respectively ($p<0.0001$). There was no difference between the diameters of P0 and X10.

High-resolution micrographs revealed that the P10 population exhibited a higher frequency of irregular-shaped or abnormally dividing cells due to the formation of off-centre septum. The mean diameter of the P10 cells were on average 32.8 % and

28.3 % greater than those of P0 or X10 respectively ($p<0.0001$). There was no significant difference between the diameters of P0 and X10.

4.3.9 S. aureus triclosan adapted SCV show impaired fitness compared to the parent strain

The relative Darwinian fitness (W) of P0 and P10 was compared when grown independently and when in competition with each other (Figure 4.10).

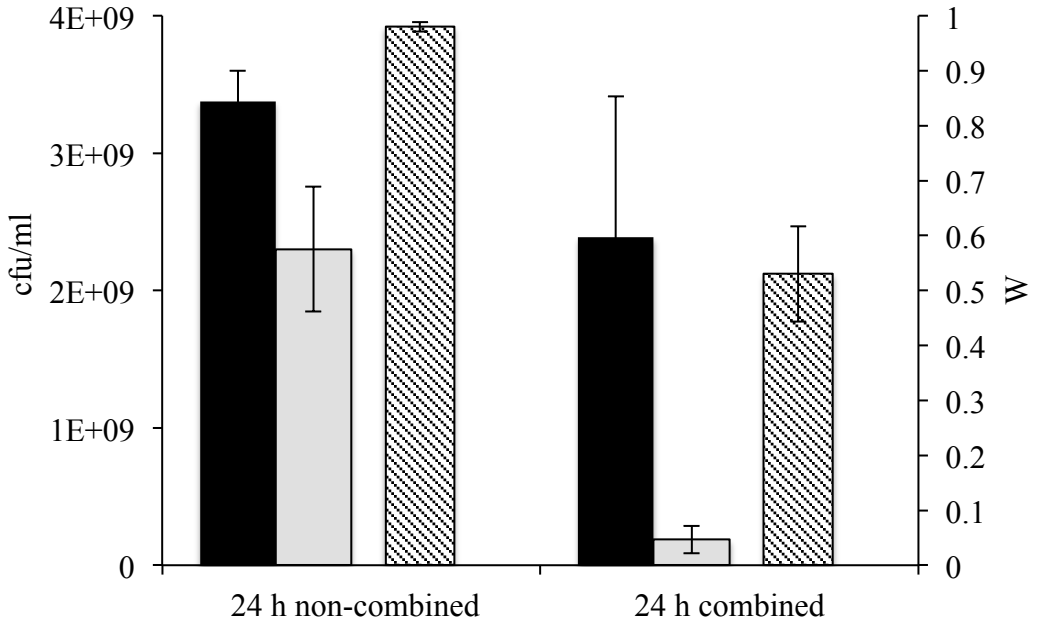

Figure 4.10. Competitive fitness assay comparing cfu ml^{-1} of P0 (black) and P10 (grey) strains after 24 h of growth, either separately or in combination. Relative fitness (W) of P10 compared to P0 is displayed when grown independent of each other and when in competition (patterned). Error bars show standard deviation (n=6).

By definition a relative fitness of 1 indicates no fitness effect between strains, a value of below 1 implies an impaired fitness, and above 1 an enhanced fitness. The relative fitness (W) of P10 to P0 was significantly lower ($P<0.001$) when the bacteria were

grown in combination than when grown separately. The relative fitness of P10 to P0 during independent growth was 0.98, compared to 0.53 during competition. Therefore, in a non-competitive environment P10 grew 2% slower than P0, whereas when in competition P10 grew 47% slower than P0.

4.3.10 Triclosan Adapted S. aureus SCV shows Alterations in Protein Expression

Two-dimensional (2D) gel electrophoresis of parent and triclosan insusceptible strains of *S. aureus* revealed differences in protein expression, which are displayed in Figure 4.11 A and B. Protein spots of interest are indicated in Figure 4.11 B (spots A-C), these were identified using tandem mass spectrometry (MS-MS) after electrospray ionisation, identification of proteins are shown in Table 4.4.

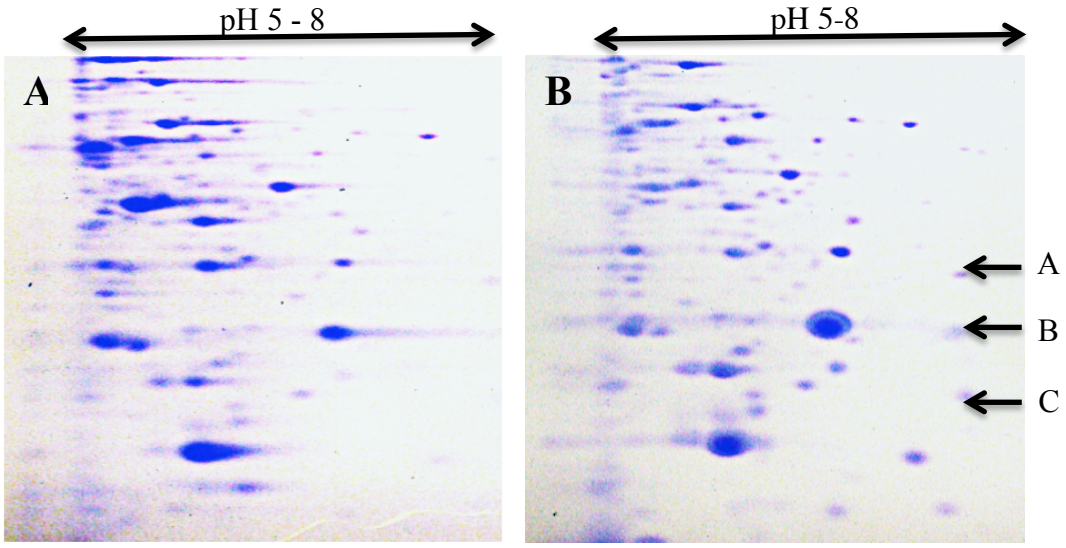

Figure 4.11. 2D gels showing protein expression profiles in A) P0 and B) P10 strains of *S. aureus*. Gels show 450 µg total protein concentration, IPG strip pH 5-8. Proteins of interest were excised and identified using esi MS-MS.

Upregulation of triclosan target enzyme FabI was observed in the P10 strain (protein B). There was also an increase in peptide deformalase (Def) production after

triclosan exposure (protein A), an enzyme that participates in protein synthesis. Identification of protein C revealed a possible increase in transglycosylase IsaA expression, an autolysin involved in cell wall cleavage during replication. The number of peptide fragments detected for each protein, during MS-MS, are summarised in Table 4.4.

Table 4.4. Upregulated proteins in *S. aureus* P10 strain after triclosan exposure. Proteins were separated using 2D electrophoresis and identified using ESI-MS-MS.

Sample	Identified proteins	Number of matched fragments
A	Peptide deformylase Def	11
B	Enoyl-[acyl-carrier-protein] reductase FabI	7
C	Probable transglycosylase IsaA	2

Peptide fragments were deemed to be 1-possible protein match, 2-probable match and 3 + -confirmed match.

4.4 Discussion

S. aureus is known to generate phenotypically distinct sub-populations termed small colony variants (SCVs), which have occasionally been isolated from patients with chronic and relapsing infections (Kahl*, et al.*, 1998, Rolauffs, 2002). Clinically occurring SCVs may result from haemin, menadione or thymidine auxotrophy, but may also result from mutations in other genes (Schaaff*, et al.*, 2003). SCV-like variants can arise in response to sub-lethal exposure to aminoglycoside antibiotics (Clements*, et al.*, 1999) and the biocide triclosan (Bayston*, et al.*, 2007). Concerns have been expressed therefore that exposure of *S. aureus* to triclosan might lead to

the emergence of *S. aureus* SCVs with reduced susceptibility to antimicrobials. However, care must be taken when extrapolating clinical relevance from *in vitro* data. The fact that triclosan is normally applied topically at concentrations considerably higher than those used to generate SCVs should be taken into account (Seaman*, et al.*, 2007), as should the fact that although elevated (0.0029% w/v), the MICs for triclosan in the adapted strains described in the current study remained orders of magnitude lower than the triclosan concentrations in washes used to control MRSA in hospitals (0.3 – 1.0 % w/v) (Zafar*, et al.*, 1995). Little is known about the physiology and pathogenicity of triclosan-induced SCVs and doubts have been raised concerning their ability to initiate infection in healthy individuals (Seaman*, et al.*, 2007).

There have been conflicting reports concerning the virulence of SCVs, whether of clinical origin or laboratory created. For example, a *hemB* mutant was more virulent than its parent strain in a murine septic arthritis model (Jonsson*, et al.*, 2003), but demonstrated no significant difference in virulence in a rabbit endocarditis model (Bayston*, et al.*, 2007). Another report suggested that *hemB* and *menD* mutants and SCVs of clinical origin were reduced in virulence (Sifri*, et al.*, 2006). Clinically-isolated SCVs may, however, be physiologically distinct from *hem* and *men* mutants generated in the laboratory and in this respect, recent proteomic analyses revealed significant differences in protein expression between a *hemB* mutant, a gentamycin-induced SCV and a clinical SCV isolate (Kriegeskorte*, et al.*, 2011). Markedly, *S. aureus hemB* SCVs showed an overexpression of proteins involved in glycolytic and related pathways (glyceraldehyde-3-phosphate dehydrogenase, enolase and phosphoglycerate kinase) and fermentative pathways (lactate dehydrogenase,

pyruvate formate lyase and alcohol dehydrogenase), possibly suggesting that this mutant generates ATP from glucose or fructose by substrate phosphorylation. It was also shown that *hemB* mutants have reduced expression of various virulence factors, with the exception of proteases and adhesins (Kriegeskorte*, et al.*, 2011). However, there has previously been little information available to indicate whether strains selected by triclosan exposure vary in expression of virulence determinants compared to the progenitor strains.

The current chapter has demonstrated that repeated exposure of *S. aureus* to triclosan can result in a phenotype with marked similarities to SCVs, but that are attenuated in biofilm forming capacity, haemolysis, DNase, coagulase activities and pathogenesis in an invertebrate model (Table 4.3). Additionally, triclosan insusceptible strains display abnormal cell morphology, demonstrate difficulty during cell division and have an impaired fitness, when compared to the parent strain. This suggests that whilst repeated exposure to triclosan may result in an SCV-like phenotype, this is not necessarily associated with increases in pathogenicity.

4.4.1 Effect of long-term triclosan exposure on virulence in S. aureus

Serial exposure of bacteria to concentration gradients of antimicrobials in agar is a highly selective procedure that has been validated as an effective method for selecting isolates with decreased susceptibility (McBain*, et al.*, 2004). The *G. mellonella* infection model has been previously used to study bacterial pathogenesis (Jander*, et al.*, 2000, Barbolt, 2002, Mylonakis*, et al.*, 2005), including antimicrobial insusceptibility in *S. aureus* (Peleg*, et al.*, 2009). Data generated with this model in

the current study showed a clear distinction between the virulence of strains P0, P10 and X10. Benefits of *G. mellonella* over other non-mammalian systems, such as *C. elegans,* include the fact that individual animals may be injected with a defined inoculum and can be maintained at human body temperature. Additionally, the system has been shown to broadly reflect virulence patterns seen in mammalian systems, due to the presence of a similar innate immune response, however, they are not capable of generating immunological memory (Jander*, et al.*, 2000).

With respect to specific virulence factors, DNase activity was markedly reduced following triclosan exposure, as was haemolysin and coagulase activity. It has been suggested that staphylococcal DNase interferes with the antimicrobial activity of neutrophil-produced extracellular traps (NETs) through the break down of the chromatin backbone (Altincicek*, et al.*, 2008). DNase-mediated NET degradation has been demonstrated for *S. aureus* in a murine model, in which mice infected with wild-type *S. aureus* exhibited a significantly higher mortality rate than those infected with the nuclease-deficient mutant (Besier*, et al.*, 2007). Host extracellular DNA in *G. mellonella* reportedly plays a similar role in the immune response as it does in humans (Aperis*, et al.*, 2007), suggesting that a reduction in DNase activity might partially account for the reduced virulence of the *S. aureus* SCV observed in our *G.mellonella* assay.

Haemolytic and coagulase activity was also down regulated in the P10 strain. A decrease in expression of bacterial toxin, alpha-haemolysin, has previously been shown in *hemB* SCV mutants (Bates*, et al.*, 2003). Alpha-toxin, a member of the beta-barrel toxin family, is thought to be a major cytotoxic agent in *S. aureus* and is

believed to form pores in cellular membranes. Therefore, downregulation of haemolytic agents, such as alpha-toxin, in our selected SCV strains would provide a plausible explanation for this reduction in haemolytic ability. Coagulase is a protein involved in the conversion of fibrinogen to fibrin when in contact with the blood. *S. aureus* is proposed to coat its surface with fibrin allowing it to resist phagocytosis, thus increasing its potential virulence (von Eiff*, et al.*, 1997). A decrease in expression of both haemolysin and coagulase activity may further explain the pathogenic defects observed in the P10 generated strain, especially the reduced virulence observed in the *G.mellonella* assay.

4.4.2 Biofilm forming capability of the small colony variant phenotype in S. aureus

Importantly, *S. aureus* became markedly defective in its ability to form biofilms following triclosan adaptation. Strains P10 and X10 formed biofilm less readily than the parent strain on polystyrene over 50 h incubations and biofilms grown on glass were less dense, exhibiting simpler architecture than the parent strain (Figures. 4.4-4.6). To our knowledge, there are currently no data in the literature concerning the ability of triclosan-selected SCVs to form biofilms, and data on other SCVs otherwise generated have not been conclusive; for example, *S. aureus* SCVs created by exposure to amikacin formed greater biofilm mass on polystyrene (Singh*, et al.*, 2009) but not on glass (Singh*, et al.*, 2010) and a *Staphylococcus epidermidis* SCV formed by a mutation in *hemB* was almost entirely biofilm-defective at 24 h but, contrary to findings presented in the current study, reverted to levels approximating the wild-type at 48 h (Altincicek*, et al.*, 2008). However, since the authors did not measure biofilm relative to planktonic growth, this may be a reflection of the

inherently slow growth of the SCV phenotype, rather than a specific measure of biofilm-forming capability.

In the current study, biofilm formation on polystyrene was measured quantitatively over 50 h and these data were then converted to relative biofilm-forming units (absorbance of the biofilm-bound crystal violet divided by the corresponding planktonic OD) and therefore reflect biofilm formation, irrespective of planktonic bacterial productivity. Since initial attachment does not appear to be impaired and biofilm deficiency is not due solely due to reduced growth rate, strains P10 and X10 appear to be specifically deficient in biofilm maturation. This may potentially be a consequence of repression of the intercellular polysaccharides or proteins PIA or Asap, respectively, which have previously been shown to be involved in biofilm development (Al Laham*, et al.*, 2007). The staphylococcal accessory regulator gene *sarA*, which controls several virulence determinants, including biofilm formation, haemolysins and DNase, may also be implicated (von Eiff*, et al.*, 2006). Furthermore, recent analysis has shown that an increase in DNase production, resulting from a mutation in the stress response factor *sigB,* inhibited biofilm growth by breaking down extracellular DNA (eDNA) (Kiedrowski*, et al.,* 2011). Although this appears to be in contrast to data presented in the current investigation, there is much to learn about the role of eDNA in such a multifactorial process as biofilm formation.

Since *S. aureus* infections related to medical devices are strongly associated with biofilm formation (Gilbert, 2003), the selection for biofilm-deficient strains may be clinically advantageous. This is because biofilms are markedly less susceptible to antimicrobial therapy than their planktonic counterparts (Nickel*, et al.*, 1985, Lewis, 2001), a phenomenon that is due to biofilm-specific physiology as well as diffusion-resistance (Lewis, 2007). Therefore, the inability to form a biofilm could potentially outweigh changes in susceptibility related to the SCV phenotype. Furthermore, despite a raised MIC to a single biocide, an inability to form biofilm may leave such SCVs vulnerable to a wide range of antimicrobial and immunological challenges.

4.4.3 Reversion of the S. aureus small colony variant phenotype in the absence of triclosan

Although the phenotype of the triclosan-adapted strain (P10) was practically identical to previously described SCVs in terms of colony size and planktonic growth, initial supplementation data indicated that P10 was not due to mutation in menadione, haemin or a consequence of thymidine auxotrophy. It is, however, possible that mechanisms other than auxotrophy to haemin, menadione or thymidine were responsible for the induction of an SCV-like response in this strain.

S. aureus generated from 10 further passages on triclosan-free medium (X10) showed partial reversion in planktonic growth rate and, to a lesser extent, in colony diameter, cell diameter and triclosan susceptibility. Only minor reversions in haemolytic activity and biofilm formation were observed and, like P10, no DNase

activity was detected. Strain X10 was significantly less pathogenic than the parent strain in the *G. mellonella* model and exhibited a marked decrease in triclosan susceptibility. Furthermore, P10 did not revert to normal colony size *in vivo* during the course of the infection model (data not shown).

4.4.4 Alterations in protein expression and cell morphology after exposure to triclosan

Proteomic analysis of the triclosan insusceptible (P10) and parent strain (P0) of *S. aureus* revealed changes in protein expression, notably an upregulation of triclosan target enzyme FabI in the P10 strain, which may explain the observed elevations in MIC and MBC, observed after triclosan exposure (McMurry & McMurry, 1998, Webber*, et al.*, 2008). An increase in expression of peptide deformylase, Def, a metalloenzyme involved in protein synthesis, may indicate an overall elevation in protein synthesis in P10 (Margolis, 2000). Additionally, a potential increase in IsaA, a lytic transglycosylase, was also observed in P10, in response to triclosan. IsaA is believed to hydrolyse bonds within peptidoglycan, assisting the expansion of the cell wall, thus allowing cell turnover and growth (Stapleton, 2007). TEM analysis of cell morphology revealed that the P10 strain showed a high proportion of cells with an abnormal shape and impaired division, resulting in significantly larger cells than the parent (P0) and recovered (X10) strains. It is therefore possible, that the over-expression of IsaA may be in response to this morphological defect, in an attempt to compensate for the lack of cell division observed in the SCV strain.

Variations in cell diameter between the strains were observed using TEM. The average P10 cell diameter was significantly larger than that of P0, however, this cell

enlargement appeared to reverse after recovery in a triclosan free medium, as X10 and P0 cells were not significantly different in size. Both thymidine and haemin auxotrophic SCVs have previously presented as enlarged cocci with multiple cross walls, when viewed using scanning electron microscopy, which is consistent with impaired cell separation (Kahl*, et al.*, 2003). This defective cell division may further help account for the slow growth rate and small colony size associated with the SCV phenotype.

4.4.5 Competitive fitness of triclosan insusceptible small colony variants compared to the wild-type strain in S. aureus

When comparing the relative fitness of the strains, P10 was shown to grow at a 2 % slower rate than P0 when grown independently, and a 47% slower rate when growing in competition, highlighting the competitive advantage of P0. The impaired ability of the P10 strain to undergo cell division may, in part, help account for this reduced relative fitness. Many studies have demonstrated that antimicrobial resistance is often linked to a fitness cost (Rozen*, et al.*, 2007, Kunz*, et al.*, 2012). Although adaption via natural selection does require beneficial mutations, most mutations are neutral or have a deleterious impact on the organism. The fitness of a particular bacterium is theorised to be directly proportional to its rate of transmission and its ability to compete with other strains within the host, and is inversely proportional to its rate of clearance from the host (Guo*, et al.*, 2012). Therefore, the clinical significance of the emergence of a bacterial strain with reduced antimicrobial susceptibility, must take into account its fitness. For instance, if bacteria acquire a mutation that provides antimicrobial resistance but results in a high burden to its fitness, then the mutated strain may never become fully established within a

population, due to its slow growth rate. Alternatively, it is argued that the mutated strain may be able persist at very low levels for a prolonged period, increasing the risk of transmission. Equally, if the insusceptible strain cannot compete with other strains *in vivo* e.g. within a mixed infection, then its pathogenic capability may be limited (Rozen*, et al.*, 2007). For example, when grown in competition P10 is readily outcompeted by P0, which may indicate a lack of ability of the SCV to persist during a mixed infection with different strains, however, this is an i*n vitro* estimate and does not account for rates of infection and rates of clearance within the host environment.

4.5 Conclusion

In conclusion, it has been shown that repeated exposure to triclosan can select for a triclosan insusceptible small colony variant phenotype in *S. aureus* ATCC 6538. This decrease in triclosan susceptibility may in part be attributed to an overexpression of target enzyme FabI. However, in adapting to this antimicrobial challenge the bacteria appears to have become significantly impaired in virulence and fitness, which may be due to a loss in activity of virulence factors such as DNase, coagulase and haemolysins, or because of a reduced growth rate and an inability to form biofilms. The SCV strain appears less virulent than the wild-type when compared in a *G. mellonella* model and was readily out competed when undergoing competition *in vitro,* demonstrating the impaired pathogenic capability of the triclosan selected SCV in *S. aureus.*

Chapter 5

Breadth of Antimicrobial Activity, Cytotoxicity and Mode of Action of Apolipoprotein E Derived Peptides

Abstract

The formation of bacterial biofilms on implanted medical devices often leads to chronic infection that cannot be resolved by antimicrobial therapy and immune clearance. Anti-infective device coatings, designed to inhibit bacterial colonisation, whilst exhibiting relatively low cytotoxicity and good stability are vital to ensure long-term device function. A common issue with such antimicrobial coatings is their biocompatibility within the human host. A tryptophan-rich peptide derivative of human apolipoprotein E receptor binding region (apoEdpL-W) has been previously developed that demonstrates strong antimicrobial activity. This chapter aimed to compare the antibacterial activity, anti-biofilm efficacy and cytotoxicity of apoEdpL-W, to that of biocides polyhexamethylene biguanide, chlorhexidine and triclosan, before and after incorporation into a range of polymers, often used in the manufacture of biomedical devices. Furthermore, underlying modes of action were investigated by studying the interactions of the antimicrobials with preformed lipid bilayers. The apoEdpL-W peptide displayed a broad-spectrum of antibacterial activity, both in the presence and absence of serum, against a panel of microorganisms often associated with device infections. ApoEdpL-W showed good retention into pHEMA hydrogels, whilst maintaining antibacterial activity, producing a controlled antimicrobial release system to prevent bacterial growth and subsequent biofilm formation on the hydrogel surface. From a mechanistic perspective, the antimicrobial activity of apoEdpL-W may, in part, be accredited to the peptides ability to readily bind and insert into phospholipid bilayers, as determined using the quartz crystal microbalance device and via dual-polarization interferometry. Biocompatibility index values were calculated for all test antimicrobials, against *E. coli* and *S. aureus*, indicating that apoEdpL-W had the highest BI value against both

bacteria, demonstrating its high antiseptic potential. The high antibacterial and anti-biofilm activity of apoEdpL-W, in addition to its low cytotoxicity, suggests it to be a candidate coating for the prevention of device-associated infections.

5.1 Introduction

Microbial contamination of implanted medical devices, such as catheters, stents and artificial joints, as well as wound dressings and gauzes, can lead to persistent bacterial infections (Stickler, 1996, Issam, 1998, Bjarnsholt*, et al.*, 2008). It is estimated that out of the two million health care associated infections (HCAIs) that occur in the US each year, half can be attributed to contaminated indwelling medical devices (Darouiche, 2004). Once established, such infections are difficult to treat due to the formation of bacterial biofilms, which are recalcitrant to many antimicrobial therapies and are rarely resolved by immune defence mechanisms (Campanac*, et al.*, 2002). It has been suggested that 60 % of all human infections are due to biofilm formation, in which the bacteria are shielded within a matrix of extra polymeric substance, protecting them for the potential impact of antiseptics and antibiotics, as well as components of the host immune system (Bagge*, et al.*, 2004, Leid, 2005, Bjarnsholt*, et al.*, 2007). Furthermore, a decrease in bacterial growth rate and the induction of stress response associated defences may further decrease the biofilms antimicrobial susceptibility. (Mah & O'Toole, 2001, Lewis, 2005).

Biofilms have been linked to chronic infections, due to their formation on the surface of in-dwelling devices, and significantly have been implicated in suture related infections (Katz, 1981, Stickler, 1996). Device-associated infections often lead to repeated surgeries, require lengthy periods of antibiotic treatment and ultimately

show a considerable level of morbidity (Darouiche, 2004). To reduce the incidence of implant infection, there has been significant research into the production of biomedical device surfaces that incorporate antimicrobial agents, in order to inhibit bacterial colonisation and subsequent biofilm formation (Maki*, et al.*, 1997, Gaonkar*, et al.*, 2003, Monteiro*, et al.*, 2009). Current approaches include (i) surfaces coated in an anti-adhesive material, such as polyethylene glycol (PEG) (Harris*, et al.*, 2004) (ii) surfaces containing covalently bound antimicrobials (Gottenbos*, et al.*, 2002, Hume*, et al.*, 2004) and (iii) surfaces containing antimicrobials that may be leached into the surrounding environment (Zhang*, et al.*, 1994). Examples of antiseptics commonly used in device coatings include cationic antimicrobials, such as polyhexamethylene biguanide (PHMB) or quaternary ammonium compounds (QACs) (Murata*, et al.*, 2007), as well as triclosan (Zhang*, et al.*, 2006). The use of biocides over antibiotics, as coating agents, is believed to be advantageous due to their multiple site-targeted mode of action, meaning the selection of resistant microorganisms is rare. Concerns have been raised, however, about the potential for biocides to select for bacteria with reduced susceptibility towards the primary agent, as well as towards third party agents such as antibiotics or other biocides. This may occur due to the presence of shared target sites or through modifications in the bacterial physiology, making the bacteria less susceptible (Chuanchuen*, et al.*, 2001). Therefore, it is of considerable interest to find alternative antibacterial coating strategies that can avoid the use of antibiotics, show a broad-range of activity and display long-term efficacy.

Cationic antimicrobial peptides (CAMPs) are emerging as candidate compounds for use in the development of antimicrobial device coatings, with host anti-infective proteins providing a vital sequence resource for CAMP design (Kelly, 2007). Peptide

mimics of host antimicrobial peptides (AMPs) have been artificially synthesised with high activity, high stability and low cytotoxicity (Statz*, et al.*, 2008). Many synthetic CAMPs show considerable chemical diversity, resistance to protease degradation, have been shown to inhibit biofilm formation and present a low risk of the development of reduced bacterial susceptibility, which makes them attractive coatings for biomedical devices (Kazemzadeh-Narbat*, et al.*, Willcox*, et al.*, 2008). Biomaterial surface coatings containing covalently bound antimicrobial peptides, or those that are gradually released into the surrounding environment, have previously demonstrated marked antibacterial activities (Willcox, 2008, Shukla, 2010). In particular, hydrogels have proven useful in providing systematic drug delivery systems, however, their effects are often short lived due to the inevitable leaching of the active agent into the surrounding environment (Kazmierska*, et al.*).

Antimicrobial immobilisation strategies currently involve the physical attachment (e.g. via adsorption or layer-by-layer assembly) and chemical attachment (e.g. via covalent bonding) of an AMP to a surface. The layer-by–layer assembly of peptide containing coatings often involves sandwiching the peptides between two polyionic films (Etienne, 2004), this allows the construction of multiple layers enabling a high amount of peptide to be loaded into a surface coating. There is, however, some question as to whether the peptide within the lower layers will be able to undergo any direct contact with the surrounding environment, thus potentially limiting its antibacterial potential. The antimicrobial activity of such coatings may largely be controlled by the peptides ability to diffuse though the layers and out of the matrix surface, which would be affected by the thickness of the assembly matrix and by any peptide-polymer interactions (Etienne, 2004, Sukhishvili, 2005). Furthermore,

biofilm formation on top of the layers may also limit the surface diffusion of the embedded peptide. Due to the continuous release of the active agent, the long term stability of such coatings is not yet fully characterised (Etienne, 2004).

Peptides may chemically interact with a surface forming a covalent attachment, alternatively, non-reactive surfaces can be modified via the addition of reactive functional groups such as thiols, aldehydes and carboxylic acids to optimise peptide binding, as well as to control binding orientation to maximise peptide activity (Jonkheijm, 2008). The number and spacing of the reactive groups on the surface has been shown to greatly influence AMP coating activity (Chen, 2009). Another common approach to covalently immobilise peptides to a surface, is to use functionalised polymer resins such as polyethylene glycol (PEG) brushes, which act as spacers to which the peptides can readily adhere and interact freely with the bacteria due to the flexibility of the brush (Chen, 2009). Unfortunately, the antimicrobial activity of bound peptides, in general, is lower than when in its free state (Gabriel, 2006). Additionally, variations in pH and ionic strength of the surrounding medium have been shown to be further influence peptide activity (Goldman, 1997). Therefore, an understanding of bound peptide mode of action, as well as knowledge of the external factors of the implants intended environment, is crucial in developing effective, long-term stable coatings.

The human Apolipoprotein E (apoE) genotype has previously been shown to influence the outcome of infection (Itzhaki*, et al.*, 1997, Itzhaki*, et al.*, 2004, Burgos*, et al.*, 2005). The receptor-binding region of apoE has demonstrated direct anti-bacterial activity (Dobson*, et al.*, 2006). A series of stabilised octadecamer tandem

repeat peptides, based around apoE141-149, referred to as the apoEdp peptides, have been used to investigate the biological activity and anti-infective activity of the apoE binding region in more detail (Kelly, 2007). ApoEdp peptides have previously demonstrated marked antibacterial action against *P. aeruginosa* and *S. aureus* (Dobson*, et al.*, 2006). Since apoEdp has anti-infective properties and shows low haemolytic activity, it is possible that it could be modified to obtain a safe and effective device coating against bacterial pathogens (Kelly, 2007).

A simple dip coating technique, involving the antimicrobial coating of polymers frequently used in device manufacture, has been used to produce an effective, locally acting antimicrobial delivery system, enabling an extended duration of antimicrobial release from a surface. The antibacterial efficacy of dip-coated surfaces depends largely on the physicochemical properties of the antimicrobial, which influence its uptake, surface interaction, activity once immobilised and release over time (Besier*, et al.*, 2007).

Whilst there have been multiple evaluations of the activity of various biocide-coated surfaces (Gilbert & McBain, 2002, Balazs*, et al.*, 2004, Turner*, et al.*, 2009), there is no systematic comparison of a wide range of biocides and antimicrobial peptides incorporated onto different polymers, in terms of their spectrum of antibacterial activity, anti-biofilm efficacy and their relative cytotoxicity. Therefore, this chapter aims to evaluate the antimicrobial and anti-biofilm activity of the unmodified apoEdp peptide and a tryptophan substituted form of the peptide apoEdpL-W, in comparison to the commonly used antimicrobials chlorhexidine, polyhexamethylene biguanide (PHMB) and triclosan. The bactericidal activity of each antimicrobial was

determined before and after incorporation into Poly (2-hydroxyethyl methacrylate) (pHEMA), polyethylene glycol (PEG), polyurethane (PU) or Polydimethylsiloxane (PDMS) discs. We examined the cytotoxicity and calculated resulting biocompatibility index values for each test compound, in order to to evaluate their antiseptic potential. Additionally, we studied the interactions of the antimicrobials with a preformed phospholipid bilayer, to gain insight into their possible modes of action.

5.2 Materials and Methods

5.2.1 Bacterial strains

Pseudomonas aeruginosa ATCC 9027 and *Staphylococcus aureus* ATCC 6538 were obtained from Oxoid (Basingstoke, UK). *Burkholderia cepacia* ATCC BAA-245*, Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13883 were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Staphylococcus caprae* MRBG 9.3, *Staphylococcus warneri* MRBG 9.27*, Staphylococcus epidermidis* MRBG 9.33 and *Staphylococcus haemolyticus* MRBG 9.35 were obtained from the axillae of three male volunteers, ranging from 25 to 30 years old. Species were identified using 16s rRNA sequencing prior to use.

5.2.2 Chemicals and bacterial growth media

Triclosan and chlorhexidine were obtained from Sigma-Aldrich (Dorset, UK). Vantocil a 20 % aqueous solution of PHMB was obtained from Arch Chemicals Inc. (Manchester, UK). Peptides were purchased from Alta Bioscience (Birmingham, UK)

having been synthesized using 9-fluorenylmethyl carbamate chemistry and purified by High Performance Liquid Chromatography. Bacteriological media was purchased from Oxoid (Basingstoke, UK). All other chemical reagents were purchased from Sigma-Aldrich unless otherwise stated.

5.2.3 Bacterial culture growth and cryopreservation

Bacteria were cultured onto Muller-Hinton Agar (Oxoid, UK) and incubated aerobically at 37˚C for 18 h unless stated otherwise. Bacterial cryogenic stocks were produced and archived at -80˚C.

5.2.4 Cell culture maintenance

A L929 murine fibroblast cell line was cultured at 37˚C/ 5% CO_2 in Eagle's minimal essential medium (EMEM) supplemented with Earle's salts, L-glutamine and 10% foetal bovine serum. Cells were grown to 80-100 % confluency within a T-175 flask, in which the media was changed every 3-4 d. Once confluent, cells were washed twice in 20 ml of warm phosphate buffered saline (PBS) before inducing cell detachment via the addition of 5 ml of 0.25% trypsin. Cells were incubated in the presence of trypsin at 37°C/ 5% CO_2 for 15 min and detachment was verified using a Nikon Eclipse TS100 inverted microscope at x 20 objective (Nikon UK Limited, Surrey, UK). After detachment, 15 ml of fresh EMEM was added to the flasks and the cell suspension solution was decanted into a sterile universal, before being centrifuged at 1600 x g, using a Hettich 320R system (DJB Labcare Ltd, Buckinghamshire, UK), for 10 min to pellet cells. After centrifugation media was decanted and cells were resuspended in 20 ml of warm EMEM. 5 ml of cell

suspension was used to seed a fresh T-175 flask and total volume for each flask was made up to 20 ml with sterile EMEM before incubation at 37˚C/ 5% CO_2.

5.2.5 Determination of bacterial minimum inhibitory concentrations (MICs)

Overnight cultures of bacteria were grown to an approximate OD_{600} of 0.8, were diluted 1:100 in sterile Mueller-Hinton Broth (MHB) and aliquots (120 µl) were delivered to wells of polystyrene 96-well plates (Corning Ltd, Corning, USA). Doubling dilutions of antimicrobials were added to the diluted overnight cultures. Sterile and antimicrobial-free controls were also included. Plates were incubated for 24 h aerobically at 37˚C with shaking at 100 rpm. MICs were determined as the lowest concentration of antimicrobial showing no turbidity in comparison to a sterile negative control. Three technical and two biological replicates were conducted.

5.2.6 Determination of bacterial minimum bactericidal concentrations (MBC)

To determine MBCs, 10 µl aliquots were taken from each well of the previously performed MIC plates and spot plated onto Muller-Hinton Agar plates in triplicate. Plates were incubated at 37˚C. MBCs were determined as the lowest concentration of biocide at which no growth occurred after 4 d of incubation.

5.2.7 Interactions of antimicrobials with biomaterials

5.2.7.1 Preparation of polymer discs

Polyurethane (PU) was purchased from American polyfilm inc (Connecticut, USA) and discs were cut to the required size prior to use. Polydimethylsiloxane (PDMS)

was produced using the Sylgard 184 Elastomer kit (Dow Corning, Michigan, USA) according to manufacturers instructions. Polyethylene glycol diacrylate (PEG) was prepared using 6.57 ml PBS, 1.43 ml Polyethylene glycol and 48 µl 2-hydroxy-2-methyl-propiophenone (Darocur 1173). The reagents were combined in a Petri dish (90 mm x 15 mm) and placed under a UV lamp for 90 seconds to induce polymerisation. Both PDMS and PEG discs were cut to appropriate size using a cork borer (80 mm diameter) autoclaved and stored for later use. pHEMA gels containing 2% methacrylic acid were prepared using 9.78 ml 2-hydroxyethymethacrylate (HEMA), 19 µl ethylene glycol dimethacrylate (EGDMA), 50 µl Daracur 1173 and 0.2 ml methacrylic acid. The mixture was aliquoted into the wells of a glass coated 96-well microtiter plate (CoStar 3631, Sigma, Poole, Dorset, UK). Polymerisation was carried out under a UV lamp for 30 seconds. Plates were submerged in 70% ethanol for 48 h followed by 30% ethanol for 48 h. Gels were continuously washed in water for 24 h until transparent, autoclaved and stored in water.

5.2.7.2 Attachment of ApoEdp peptides to discs

Biomaterial discs were soaked for 18 h in 200 µl of 250 mg ml^{-1} fluorescein labelled apoEdp or apoEdpL-W. Discs were rinsed in three times in 1 ml of PBS for 1 min. Fluorescence was measured at 566 nm using the FLUOstar OPTIMER fluorescence plate reader (BMG LABTECH, Aylesbury, Bucks, UK). Peptide retained on the discs after each wash step was calculated as;

Total available peptide - peptide lost in each wash step.

5.2.7.3 Visualisation of peptide absorption using confocal scanning laser microscopy

The attachment and migration of fluorescein labelled apoEdp peptides into 2% methacrylic acid pHEMA hydrogels was monitored using a Nikon upright C1 confocal microscope with a x 20 objective lens (Nikon UK limited, Surrey, UK). A commercial contact lens (ACUVUE® *2*™) was used as the test material, to ensure consistency of material thickness between experiments. To enable visualisation of the lens boundaries, they were soaked in 1 ml of 16.7 μm propidium iodide for 18 h at room temperature, before addition of the fluorescent peptide. The inner surface of the lens was exposed to 50 μl of 250 mg ml^{-1} apoEdp or apoEdpL-W (1% fluorescein tagged peptide) for 30 min, 3 h or 6 h. The excitation and emission maxima for these dyes are 490 and 635nm for propidium iodide and 494 and 521 nm for fluorescein. Z-stack images were produced and processed using Imaris software (Bitplane AG, Zurich, Switzerland).

5.2.7.4 Antimicrobial activity of treated discs

Polymer discs were soaked for 18 h in 200 μl of 250 μg ml^{-1} of apoEdp, apoEdpL-W, chlorhexidine, PHMB or triclosan. Discs were rinsed in twice in 1 ml of PBS for 1 min before being challenged with 400μl of 1:100 diluted overnight culture of *Staphylococcus aureus* or *Pseudomonas aeruginosa,* within the well of a 24-well microtiter plate. Overnight suspensions were diluted in PBS to an OD_{600} of 0.1 prior to well inoculation. This was representative of approximately 2.4 x 10^6 to 3.2 x 10^6 cfu ml^{-1} of *S. aureus* and 1 x 10^6 to 1.2 x 10^6 cfu ml^{-1} of *P. aeruginosa,* as confirmed by colony counts on MHA. Plates were incubated for 18 h at 37˚C and 100 rpm. Bacterial viability was determined before and after exposure by plating 0.1 ml of test culture onto Muller-Hinton Agar plates in triplicate. The percentage reduction in

bacterial viability (cfu/ml) was calculated for each treatment, relative to a control treatment of PBS.

5.2.7.5 Determination of anti-biofilm activity of treated hydrogels

In order to assess the anti-biofilm potency of a treated hydrogels, pHEMA based contact lenses (ACUVUE® *2*™) were conditioned with 200 µl of 250 µg ml^{-1} of an antimicrobial for 3, 8 or 24 h at room temperature before being exposed to 1 x 10^5 cfu ml^{-1} of bacteria (1 ml) for 48 h at 37˚C to promote biofilm formation. BacLight LIVE/DEAD staining (Invitrogen Ltd, Paisley, UK) was used to visualise biofilm viability via fluorescence microscopy. In brief, 10 µl of propidium iodide and SYTO 9 were added to 980 µl of distilled water. Contact lenses were removed from the bacterial inoculum and rinsed in twice in 1 ml of PBS, before being stained with 50 µl of LIVE/DEAD working solution. Lenses were incubated at room temperature in the dark for 15 min, according to the standard BacLight staining protocol. After incubation, lenses were placed on a glass microscope slide and were flattened by making 4 radial incisions from the centre of the lens to the edge and placing a cover slip over the top. Biofilms were visualised with a Nikon upright C1 confocal microscope using a x63 objective lens, under oil immersion (Nikon UK limited, Surrey, UK). Bacterial cells incubated in the presence of LIVE/DEAD stained either green (viable) or red (dead). The excitation and emission maxima for SYTO 9 are 480 and 500 nm respectively. Images were processed and quantified using Imaris software (Bitplane AG, Zurich, Switzerland).

5.2.8 Determining antimicrobial/lipid interactions

5.2.8.1 Preparation of small unilamellar liposome vesicles

Phospholipid 1,2-dioleoyl-sn-glycero-phosphocholin (DOPC) was obtained from Avanti Polar Lipids Inc (Alabama, USA). Lipids were dissolved in chloroform and dried overnight under nitrogen in a glass tube to form a thin film. Lipids were hydrated to 1mg ml^{-1} in 0.02 M HEPES buffer (150 mM NaCl, 2 mM $CaCl_2$, pH 7.4) to obtain multilamellar vesicles. The lipid solution was then passed through a 0.05 μm pore membrane filter (Whatman, Kent, UK) using the Avanti Mini-Extruder system to obtain large unilamellar vesicles (Avanti Polar Lipid, Alabama, USA). The vesicle size distribution was characterized using dynamic light scattering, determined by the Zetasizer Nano S (Malvern Instruments Ltd, Worcestershire, UK).

5.2.8.2 Monitoring of frequency and energy dissipation shifts across a lipid bilayer using the quartz crystal microbalance device

A background of the quartz crystal microbalance device (QCMD) equipment and methods have been detailed by Keller and Kasemo (Keller & Kasemo, 1998). In brief, QCMD chips were sonicated for 20 min in 20 % sodium dodecyl sulphate before being rinsed with 1 ml of water then 1 ml of ethanol, they were dried under a stream of nitrogen and subjected to 20 min in a UV/Ozone cleaning chamber (UV/Ozone ProCleaner, BioForce Nanosciences Inc, Iowa, USA). The rinse and cleaning steps were repeated before use. The QCMD system (Q-sense E1, BiolinScientific/ Q-Sense, Sweden) was run in 0.02 M HEPES buffer for 20 min to reduce drift and obtain a stable baseline reading. Liposomes were diluted 1:10 into HEPES buffer and were absorbed onto the surface of a SiO_2 coated piezoelectric quartz crystal oscillator sensor chip at 20˚C, to form a lipid bilayer. Frequency and

dissipation shifts after 10 min of exposure of the bilayer to each antimicrobial (20 μm) were recorded where (n= 3, 5, 7 and 9 corresponds to an overtone of the fundamental frequency, 5MHz). Data was recorded using Q-soft and analysed using Q-Tools software (Q-sense E1, BiolinScientific/ Q-Sense, Sweden).

5.2.8.3 Dual-polarisation interferometry

Dual polarisation interferometry (DPI) can be used to determine real time measurements of surface interactions across a lipid bilayer (Mashaghi, 2008). The Analight® Bio200 dual-polarization interferometer (Farfield Sensors Ltd, Salford, UK), comprises of a helium-neon laser (emission 623.8 nm), which selects plane-polarized light to be confined within a sensor, made up of two waveguides stacked on top of each other. On the addition of an adhering reagent to the sensor, for example by adding DOPC, which forms a phospholipid bilayer on the top waveguide, the speed of light within the top waveguide changes. Changes in transverse magnetic (TM) and transverse electric (TE) polarization are calculated from the distance of the interference fringe movements, which are visualised using an element-imaging device in the far-field, the output of which passes to a digital processing unit. TM and TE respond to the addition of certain molecules to the sensor surface allowing the thickness and density of the forming bilayer to be determined using optical theory.

Liposomes were prepared as described previously. A Farfield unmodified sensor chip was cleaned using 1 ml of water followed by 1ml of ethanol, sensors were dried using a stream of N_2 and subjected to 20 min cleaning in an UV/Ozone chamber before use. After insertion into the Analight® Bio200 dual-polarization

interferometer, the sensor was calibrated in 80 % ethanol and water, before 0.02 M HEPES buffer was run over the sensor at a speed of 50 µl min^{-1} for 1 h until the fringes had stabilised. 1 ml of 1 mg ml^{-1} DOPC was run over the sensor until a stable lipid bilayer formed, after which 1 ml of 20 µm antimicrobial solution was flowed across the bilayer surface. Changes in TE and TM before and after bilayer formation and antimicrobial addition were monitored, from which the resulting thickness of the layer, mass uptake and change in birefringence throughout the bilayer could be calculated.

5.2.9 Haemolysis assay of antimicrobials

Freshly washed sheep RBC were added to peptide and biocide dilutions, in a 96-well microtiter plate (2 x 10^7 RBC per well) and incubated for 2 h at 37˚C. Plates were centrifuged at 3000 x g for 15 min, then 180 µl of supernatant was transferred to a fresh 96-well plate containing Triton X-100. Absorbance was measured at 540 nm to determine the concentration of peptide at which 5% haemolysis (EC_5) had occurred. 100 % haemolysis was taken as the average absorbance for RBC in just Triton X-100.

5.2.10 Biocompatibility indexes

The Biocompatibility index is described in detail by Müller and colleagues (Müller & Kramer, 2008). BI testing involves the parallel assessment of the *in vitro* cytotoxicity and the antibacterial activity of a test agent. This is achieved by determining the IC50, via neutral red and MTT assays, and the RF value, the lowest concentration of an antimicrobial to achieve a $3Log_{10}$ reduction in bacterial cfu ml^{-1}. Essentially, BI is defined as IC50/RF.

5.2.10.1 Cytotoxicity assays

The neutral red (NR) assay and the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] test have been standardized for L929 cells and described in detail (Müller & Kramer, 2008). For the NR assay, 0.1 ml of an L929 cell suspension was seeded into a 96-well cell culture treated microtiter plate at a density of 1 x 10^6 cells per well, in order to reach 80 % confluence after 24 h incubation at 37˚C and 5 % CO_2. After confluency was achieved, 0.1 ml of medium including the appropriate antimicrobial concentration was added to the plate wells and cells were then incubated for 30 min at 37˚C and 5% CO_2. Cells were washed twice in 0.2 ml of EMEM and were incubated for a further 3 h in 0.2 ml of media containing 50 mg L^{-1} of NR dye. After incubation, media was removed and cells were washed with 0.25 ml of PBS for 3 min. Following washing, cells were exposed to 0.2 ml of 1 % (v/v) acetic acid/ 50% (v/v) ethanol to extract the NR dye. For the MTT assay, after reaching confluency, cells were washed as described previously and incubated with 0.1 ml of EMEM containing 0.5 mg of MTT and incubated for 4 h at 37˚C and 5% CO_2. After incubation media was aspirated and the blue formazan product was solubilised in 0.2 ml of 0.04 M HCL in 2-propanol. NR and MTT plates were placed on a Flowgen 2D bench rocker (Scientific Laboratory Supplies, East Riding of Yorkshire, UK) for 1 h before being measured spectrophotometrically at a 540 nm test wavelength and 655 nm reference wavelength. A log dose-response curve was produced from average percentage cell viabilities, determined from the NR and MTT assays, and resulting IC_{50}s were determined graphically. Each test was repeated in experimental replicates of 6 and three biological replicates.

5.2.10.2 Inactivation combinations

Test compounds were inactivated via incubation at 37˚C for 30 min with TSHC 3% (w/v) Tween 80, 0.1% (w/v) histidine, 0.1% (w/v) cysteine and 3% (w/v) saponin.

5.2.10.3 Quantitative suspension test of antimicrobial activity

Bacterial inoculum were prepared in Muller-Hinton Broth containing 1 x 10^8 cfu ml^{-1} of bacteria as judged by separate experiments (data not shown). Inoculum was diluted 1:10 into EMEM cell culture media containing graded concentrations of test antimicrobials and incubated for 30 min at 37˚C. 1 ml of solution was then transferred to 9 ml of TSHC and incubated at 37˚C for 30 min to neutralize the antimicrobial. Serial dilutions were prepared in Muller-Hinton Broth and 0.1 ml of solution was plated in triplicate onto Muller-Hinton Agar plates. Resulting cfu ml^{-1} of test organisms were calculated compared to a control that had not undergone any antimicrobial exposure. The $\log_{10}$ reduction factor (RF) for each antimicrobial concentration was calculated according to:

$RF= \log_{10}nc-\log_{10}nd$

Where nc is the number of cells in the initial inoculum (cfu ml^{-1}) and nd is the number of cells after contact with the antimicrobial. Experiments were repeated in triplicate.

5.2.11 Agar overlay assay of antimicrobial cytotoxicity

To determine the direct cytotoxicity of antimicrobial impregnated polymers we performed an agar overlay assay using an L929 cell line according to ISO standards (2010). In brief, 2.4 x 10^6 cells in 10 ml of EMEM were seeded into 60 mm diameter cell culture plates. Cells were incubated for 48 h at 37ºC and 5% CO_2 to

form a monolayer on the base of the dish. After incubation media was removed by aspiration and cells were washed twice in 10 ml of PBS. 10 ml of EMEM containing 1% agar was added to each dish and was allowed to solidify at room temperature. After the agar set, 10 ml of a 0.1% neutral red solution was added to the centre of each plate, which was then rotated to evenly distribute the dye, left for 15 min, and excess solution was removed by aspiration. Polymer discs were soaked in 200 μl of 250 mg ml^{-1} of respective antimicrobials for 18 h at room temperature and rinsed three times in 1 ml of PBS for 1 min before use. After rinsing, each disc was placed in the centre of an individual cell culture dish. Plates were incubated for 24 h at 37˚C and 5% CO_2 before being checked for cell lysis. Disc toxicity was characterised by a white colourless zone of dead cells around the implanted region. Both zone lysis (size of lysis zone) and lysis index (percentage of lysed cells within the zone) were assessed on a scale of 1-5, 1 being low lysis and 5 being complete lysis. Response index was determined as zone lysis/ lysis index and correlated to a level of cytotoxicity from none to high. Untreated discs acted as positive controls.

5.3 Results

5.3.1 Interactions of apoEdp and apoEdpL-W with coating polymers

The interactions of apoE-derived peptides with material surfaces have not previously been studied. In this chapter, the attachment and retention of such CAMPs in four polymers frequently used in the manufacture of biomedical devices was evaluated. We selected a peptide comprising of the naturally occurring apolipoprotein-E receptor-binding region sequence, presented as a tandem repeat (apoEdp). We compared the behaviour of apoEdp with a tryptophan-rich derivative of this peptide

(apoEdpL-W), which contains a higher proportion of bulkier, hydrophobic amino acid residues.

When observing the absorption of peptides to test polymers, the levels retained on the pHEMA and PEG hydrogels were high (before subsequent washing), with over 95% of available peptide associating with the materials, for both apoEdp and apoEdpL-W (Figure 5.1). After further washes, peptide was eluted from both hydrogels; approximately 44-47% of apoEdp was lost, compared to 19-21% of apoEdpL-W (Figure 5.1). Conversely, peptide absorption to the impermeable PDMS and PU was low. Levels of apoEdp taken up after initial treatment of PDMS were lower than levels of apoEdpL-W (at around 15% and 8% respectively). The amount of peptide retained on PU was similar for both peptides, at 7% for apoEdp and 8% apoEdpL-W. After washing, the level of peptide remaining on PDMS and PU showed no significant difference in value, for either peptide ($P>0.05$).

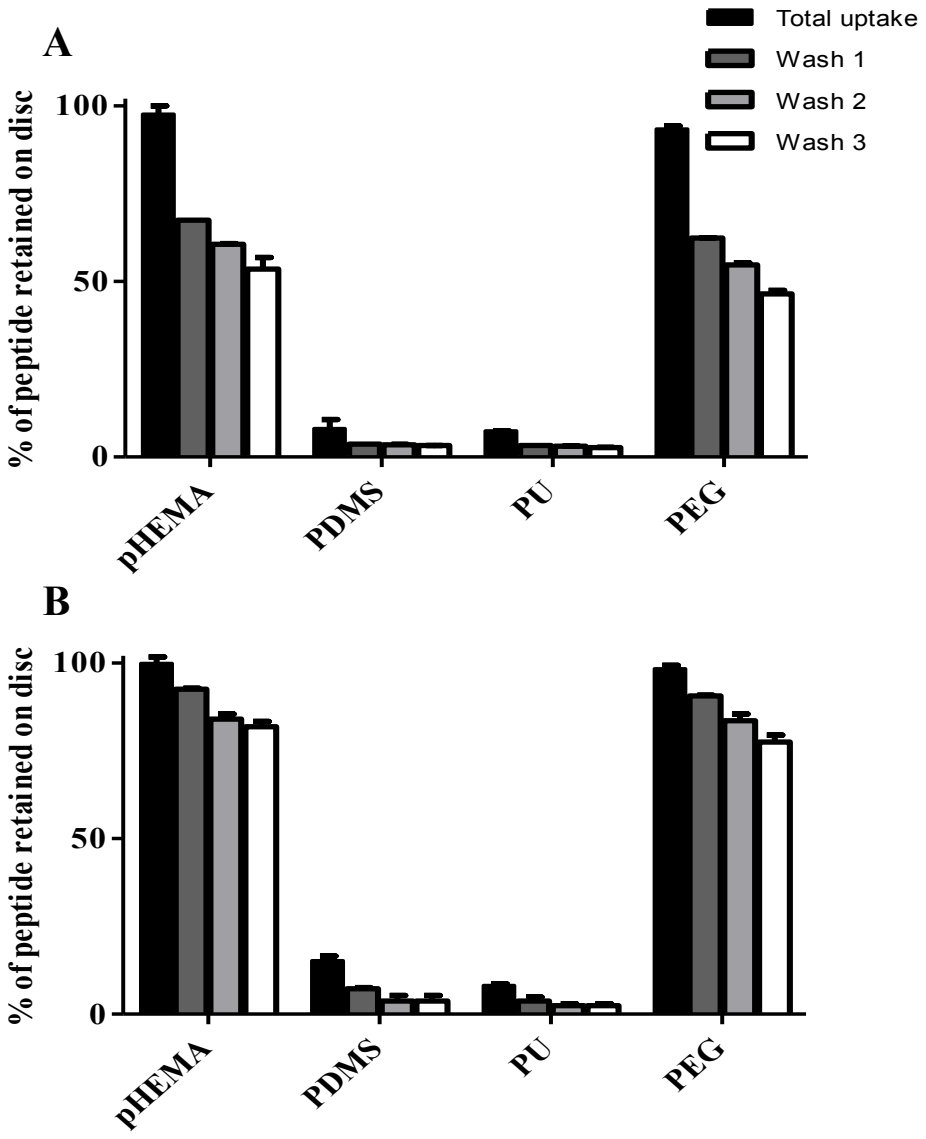

Figure 5.1. Adherence of apoE-derived AMPs to pHEMA, PDMS, PU and PEG biomaterial discs. Discs were soaked for 18 h in 250 μg ml^{-1} of (A) apoEdp or (B) apoEdpL-W, before being washed in three times in 1 ml of PBS. Quantity of peptide retained on the material after each wash is shown (expressed as a percentage of total available peptide). Data represents duplicate experiments repeated in triplicate.

The high peptide levels associated with both hydrogels likely reflects peptide absorption into the material; this was confirmed using CLSM imaging (Figure 5.2). ApoEdp moved rapidly into a pHEMA hydrogel (moving through approximately 84 μm of material in around 6 h), however, the rate of movement of apoEdpL-W was

less rapid (requiring around 8 h to move through the same thickness of pHEMA). Additionally, the rate of entry appeared to reduce for apoEdp the further into the material the peptide penetrated (for example it required 30 min to move halfway through the hydrogel and subsequently 6 h to cross the entire thickness) (Figure 5.2). In comparison the rate of apoEdpL-W uptake was fairly constant.

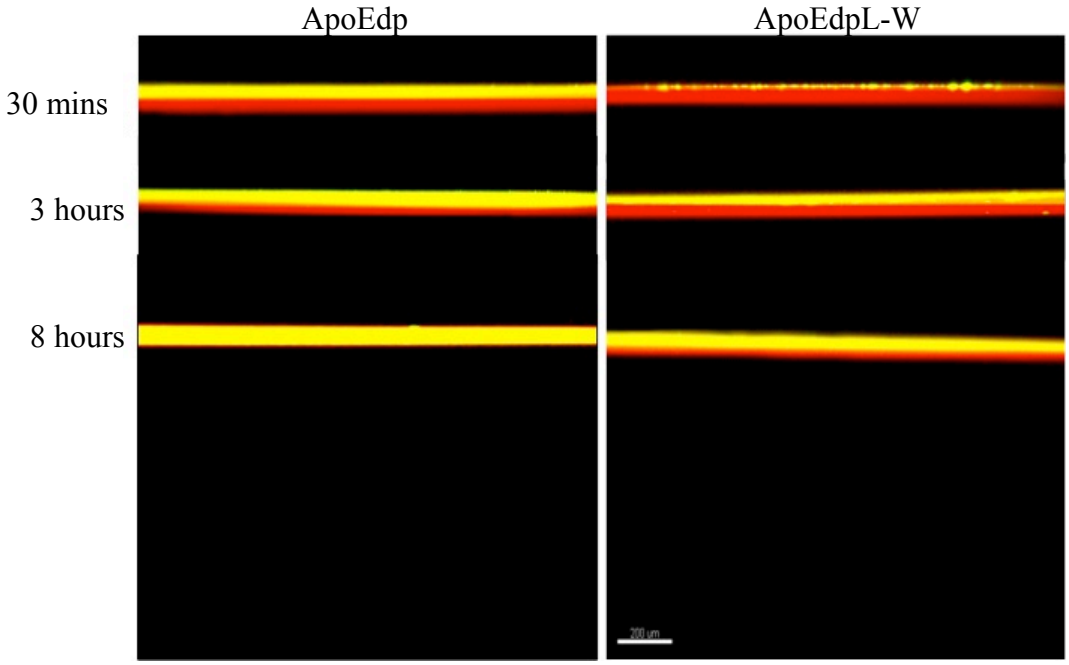

Figure 5.2. Entry of fluorescein-tagged peptides into propidium iodide stained pHEMA hydrogels, imaged by CSLM. Images represent hydrogel cross-sections after treatment with apoEdp or apoEdpL-W.

5.3.2 Relative antimicrobial activity of CAMP-treated and biocide-treated polymers

To evaluate the relative antimicrobial potential of the treated biomaterials, the antibacterial activities of PDMS, PU, PEG, and pHEMA discs were compared after pre-treatment with apoEdp, apoEdpL-W, chlorhexidine, PHMB or triclosan. Antimicrobial treated polymer discs were exposed to either *S. aureus* or *P. aeruginosa* in planktonic culture. After 18h incubation, the resulting percentage reduction in bacterial viability was measured (Figures 5.3 and 5.4).

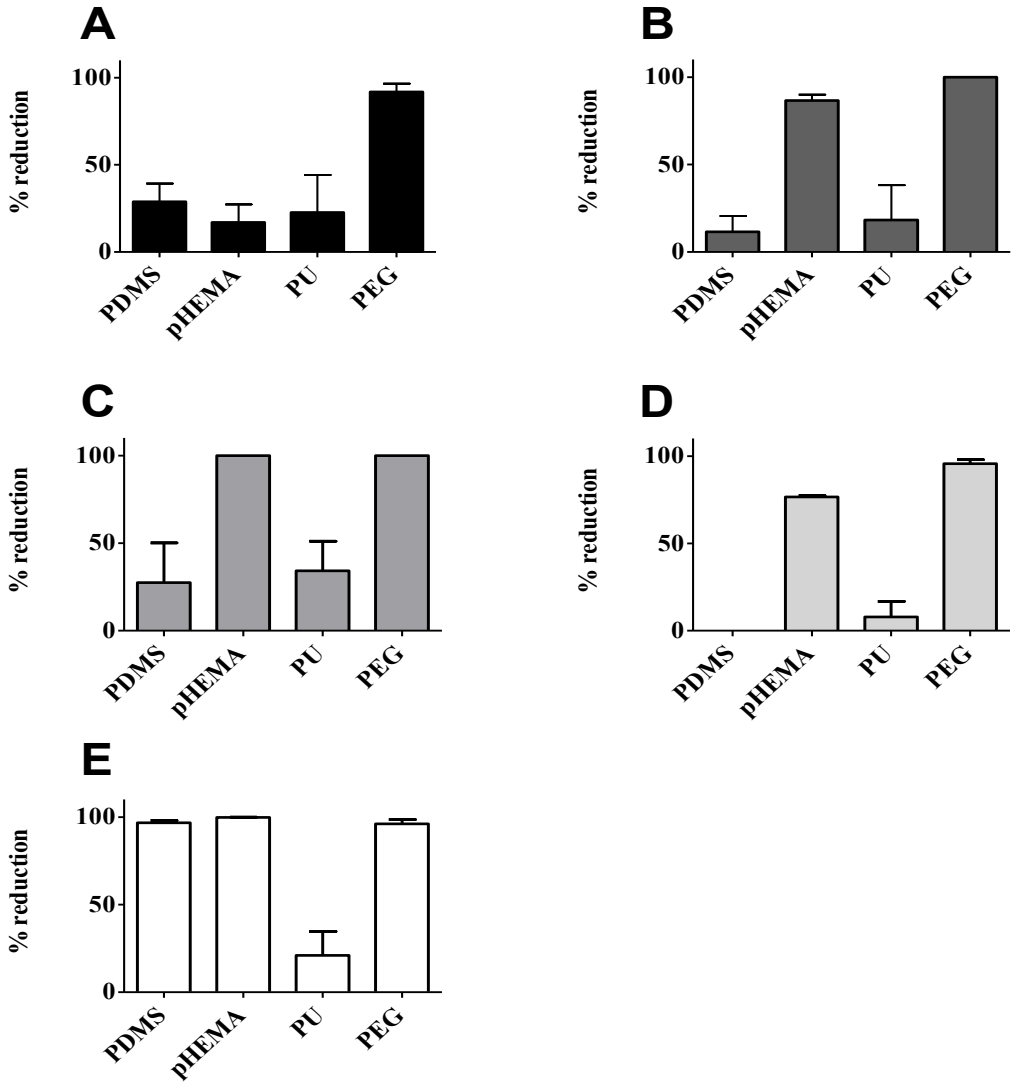

Figure 5.3. Reduction in viable *Staphylococcus aureus* after exposure to materials previously treated with A) ApoEdp, B) ApoEdpL-W, C) chlorhexidine, D) PHMB or D) triclosan. Activity is expressed as % reduction in viable counts relative to an untreated control. (Error bars represent standard deviation, n=6).

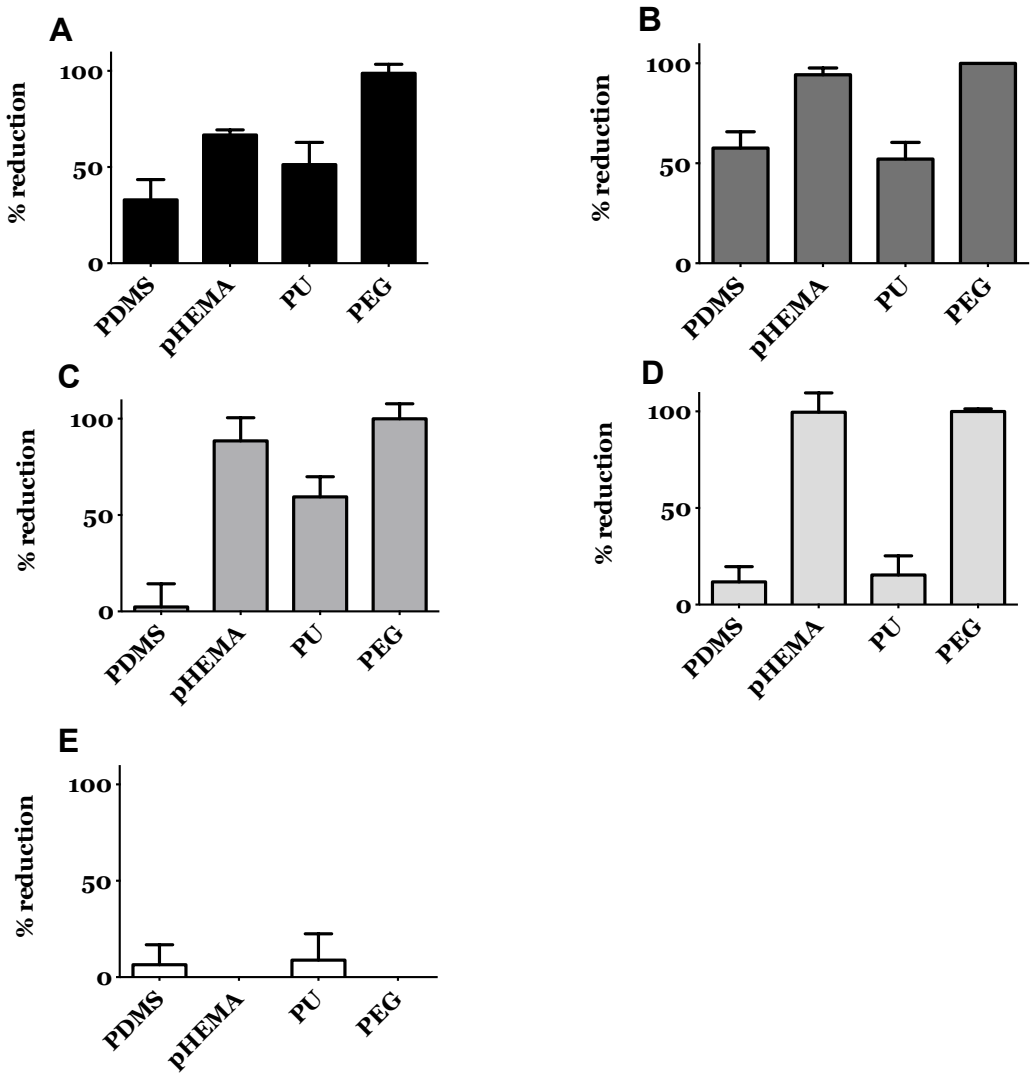

Figure 5.4. Reduction in viable *Pseudomonas aeruginosa* after exposure to materials previously treated with A) ApoEdp, B) ApoEdpL-W, C) chlorhexidine, D) PHMB or D) triclosan. Activity is expressed as a % reduction in viable counts relative to untreated control. (Error bars represent standard deviation, n=6).

Across the test panel of biomaterials, the antimicrobial treated PEG and pHEMA discs resulted in the greatest decrease in viable *S. aureus* and *P. aeruginosa* after exposure, perhaps reflecting the ability of these materials to readily internalise CAMPs and biocides, without loss of antibacterial activity (Figure 5.3 and 5.4). Triclosan-treated materials showed strongest activity against *S. aureus* (with the exception of PU), however, these also had the least activity against *P. aeruginosa.*

ApoEdpL-W treated-materials performed very similarly to those treated with PHMB and chlorhexidine against *S. aureus* and were the most effective against *P. aeruginosa*. ApoEdp-materials were arguably the least effective against *S. aureus* and the second most effective against *P. aeruginosa* (Figures 5.3 and 5.4).

5.3.3 Anti-biofilm potency of antimicrobial treated polymers

Since staphylococcal biofilms are one of the most frequent causes of device associated infections, we examined the impact of pre-treating pHEMA hydrogels with apoE peptides on *S. aureus* biofilm growth and viability. Biofilm viability was assessed using the BacLight LIVE/DEAD staining kit and imaged using CSLM (Figure 5.5).

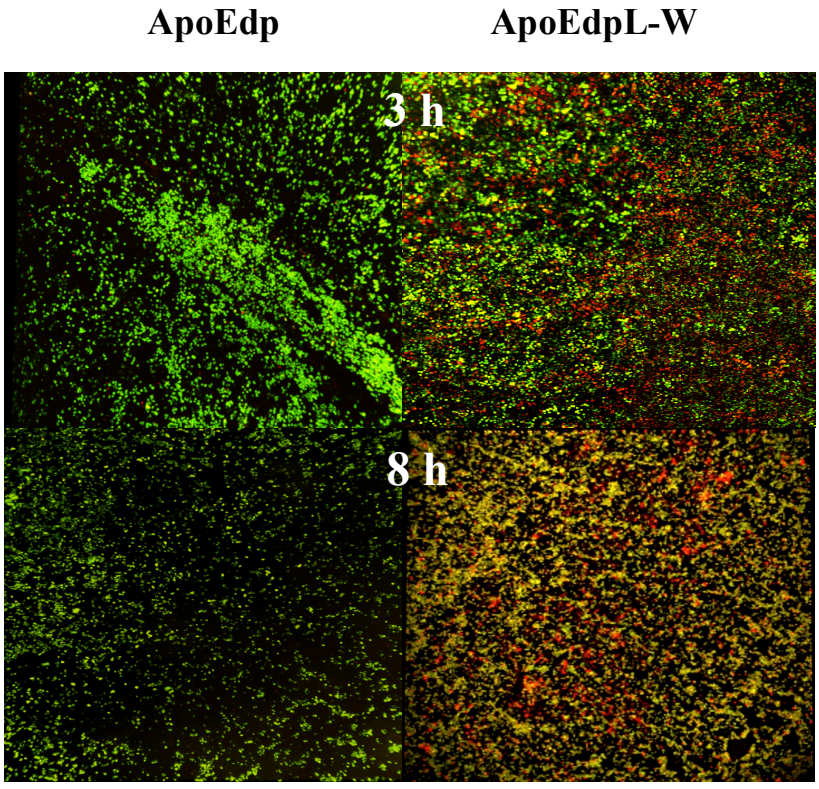

Figure 5.5. Growth of an *S. aureus* biofilm on a treated pHEMA hydrogel surface. Hydrogels were soaked in apoEdp or apoEdpL-W for 3 or 8 h before inoculation with *S. aureus*. Biofilms were visualised using CSLM.

Hydrogels treated with apoEdpL-W showed a clear reduction in biofilm viability, which became more pronounced as the length of exposure time increased. The apoEdp surfaces, however, showed limited anti-biofilm efficacy (relative to an untreated control).

5.3.4 Binding dynamics of antimicrobials to a model membrane system

The antibacterial mechanism of action of both CAMPs and some of the test biocides likely involve interactions with a phospholipid bilayer, potentially leading to disruption of the bacterial cytoplasmic membrane. To gain further insight into these mechanisms, we compared the interactions of apoE peptides and biocides with a preformed DOPC phospholipid bilayer, to understand whether they showed similarities in their interactions. By using two biomolecular analysis systems, the quartz crystal microbalance device (QCMD) and the AnaLight 4D Workstation, a dual polarisation interferometer, we compared real time interactions of the antimicrobials with a lipid bilayer. This assessment involved comparing antimicrobial mass uptake onto the bilayer, energy dissipation, changes in bilayer thickness and alterations in birefringence, when exposed to the test agents.

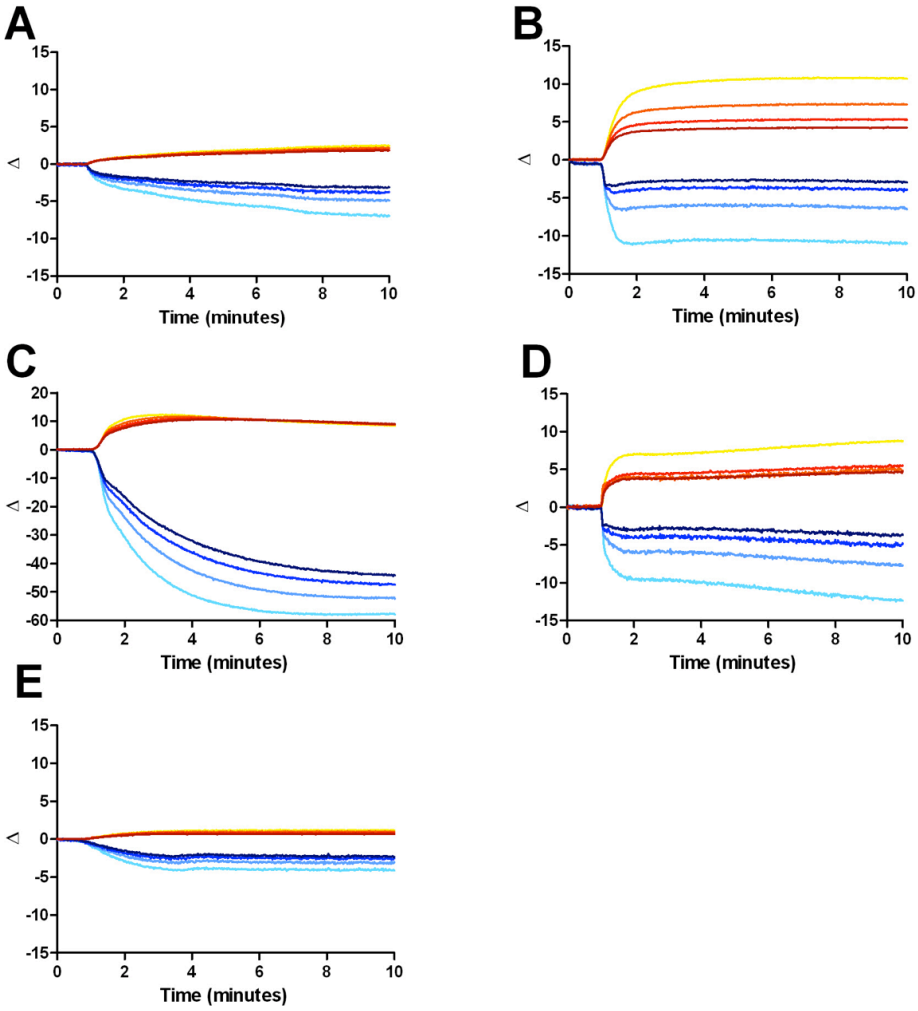

Figure 5.6. QCMD analysis showing changes in frequency and energy dissipation across a DOPC lipid bilayer after the initial 10 min of exposure to A) ApoEdp, B) ApoEdpL-W, C) chlorhexidine, D) PHMB and E) triclosan. Lines indicate different overtones of the fundamental frequency (5 MHz). Blue tones indicate changes in frequency and red tones indicate changes in energy dissipation. T=0 is taken as point of antimicrobial addition.

Observed decreases in resonance frequency, after exposure to the antimicrobials, were associated with total mass uptake on the bilayer, however, this includes a certain amount of water due the coupling of interacting agents with water molecules. To further verify antimicrobial/lipid interaction, the change in energy dissipation from the surface was also monitored. Energy dissipation can be used to determine the viscoelasticity of the bilayer, which may be impacted by any adhering agent. For

instance, if a molecule attaches flat to the bilayer surface, little water couples to the bilayer giving a low dissipation value. However, if a molecule attaches standing upright more water will couple to the film making it highly dissipative (Figure 5.6). Shifts in energy dissipation may be attributed to changes at (i) the lipid bilayer and chip interface, (ii) the lipid bilayer-liquid interface or (iii) within the bilayer itself.

When analysing a rigid bilayer, the Saubrey equation can be used to directly determine mass uptake from frequency change (Vogt, 2004). We assessed the impact of a 10-min antimicrobial exposure on a preformed DOPC bilayer; data represents frequency overtones 3, 5, 7 and 9, of the fundamental frequency 5 mHz, with a higher numerical overtone representing changes detected deeper within the layer.

Triclosan addition resulted in the smallest changes in frequency and energy dissipation across the bilayer, out of all the antimicrobials tested, resulting in a mass uptake of approximately 0.72 ng/mm^2, perhaps reflecting the relatively small role direct phospholipid interaction plays in its mode of action (Figure 5.2). Both PHMB and apoEdpL-W showed a similar level of bi-layer deposition, at 2.43 ng/mm^2 and 2.29 ng/mm^2 respectively. Comparatively, ApoEdp addition resulted in a smaller reduction frequency than apoEdpL-W, indicating approximately 1.26 ng/mm^2 of bilayer adherence. The extremely high level of deposition observed for chlorhexidine may suggest the biocide is aggregating either before or after bilayer interaction (Figure 5.6).

Bilayer exposure to PHMB and ApoEdpL-W resulted in a ΔD shift up to 10, which was highest for ApoEdpL-W, suggesting a large increase in bilayer viscosity after

antimicrobial treatment. Addition of triclosan and apoEdp resulted in very small dissipation shifts, which may be due to weaker molecule adherence or the flatter orientation of the agents when associating with the layer, causing less entrapment of water, resulting in a more rigid film (Figure 5.6).

Figure 5.7 shows the absorption kinetics of the antimicrobials to the bilayer. At first, both apoEdp and triclosan show the shallowest gradients of all the test actives, suggesting a higher level of dissipation per unit of mass uptake, than apoEdpL-W and PHMB. However, the addition of triclosan only resulted in an overall change in dissipation of 0.88 and frequency of 2.65, showing that whilst the biocide is able to cause a high level of phospholipid disruption once bound, its interaction is perhaps limited by an overall lack of uptake. Initially, PHMB addition resulted in a very steep gradient, indicating a low level of disruption per unit of mass uptake, however, after the first few seconds this rapidly changed and the gradient began to flatten out, indicating a progressively higher level of disruption per unit of mass uptake. Similarly, at first addition of ApoEdpL-W demonstrated a low level of bilayer disruption per unit of adhered mass, however, over time the gradient decreased. This may indicate that once a threshold concentration of bound apoEdpL-W is achieved, the activity exerted on the bilayer alters and disruption becomes more pronounced. It is possible that this may be due to a change in orientation of the bound peptide, altering the association between the molecule and the lipid bilayer. For instance, if the peptide re-orientated into a more perpendicular state, it would lead to the entrapment of a larger amount of water and increase the dissipation from the bilayer surface. The type of perpendicular arrangement may be indicative of a potential mode of action, e.g. the formation of pores within in membrane.

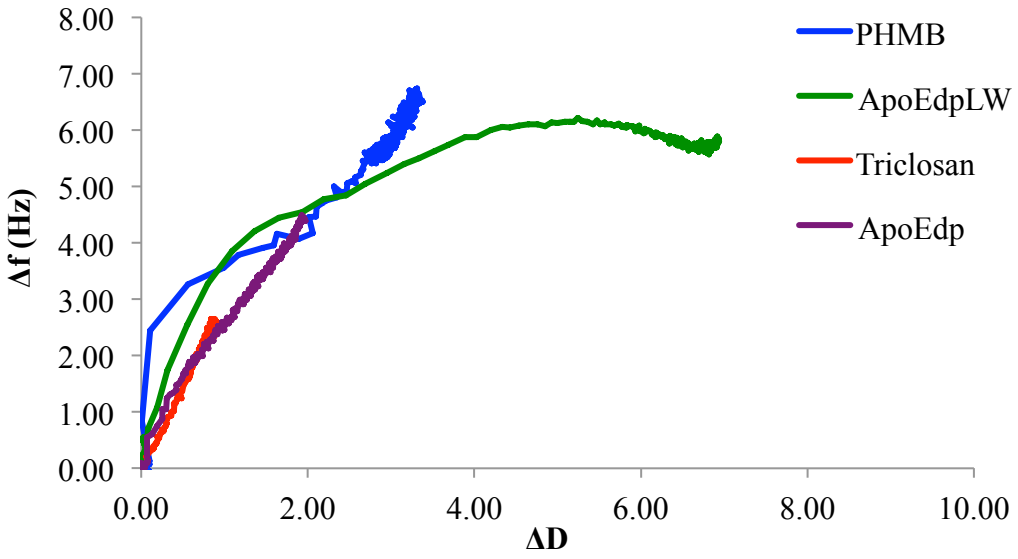

Figure 5.7. Adsorption kinetics of the interaction of apoEdp, (purple), apoEdpL-W (green), PHMB (blue) and triclosan (red) with a preformed DOPC lipid bilayer. Δf shows change in resonance frequency and ΔD shows shift in energy dissipation from time of antimicrobial addition.

Due to the similarities observed between apoEdpL-W and PHMB, when using the QCMD, we further investigated their impact on a lipid bilayer using the AnaLight 4D dual polarization interferometer (DPI). This allowed real time measurements of mass uptake, changes in bilayer thickness and alterations in birefringence (corresponding to the level of disorganization within the lipid bilayer). Changes in bilayer properties after exposure to the antimicrobials are documented in Table 5.1.

Table 5.1. Change in the thickness, mass and birefringence of a DOPC bilayer after exposure to apoEdpL-W and PHMB.

	ΔThickness (nm)	**Δ**Mass (ng/mm^2)	**Δ**Birefringence
ApoEdpL-W	0.549	0.407	-0.0070
PHMB	0.585	0.423	-0.0096

Changes in bilayer thickness and mass uptake after exposure to apoEdpL-W and PHMB, as determined via DPI, were very similar. As both antimicrobials absorb to the layer surface there is a drop in birefringence, this indicates that they are inducing disorder within the layer, likely due to insertion. PHMB exposure resulted in a very slightly higher drop in birefringence than ApoEdpL-W, suggesting it exerts a higher level of disruption to the model membrane system.

5.3.5 Haemolytic activity of ApoEdp derived peptides

Studies have implicated haemolytic activity to be a particular limitation for some CAMPs. In previous studies, it has been shown that apoE-derived peptides have very little such activity, which may relate to the distribution of charged residues along the peptides (Kelly, 2007). This hypothesis was tested by measuring the haemolytic activity for mutants of the apoEdpL-W sequence, in which a single residue was replaced either by AiB or alanine (which are uncharged) or by the corresponding D-amino acid (which is likely to effect the peptide conformation, but not alter charge distribution). The haemolytic activity of the peptides was compared to that of PHMB, chlorhexidine and triclosan.

Table 5.2: Effect of single amino acid substitutions within the apoEdpL-W sequence on haemolytic activity. Shading indicates peptides where a single charged residue was removed. Peptides showing measurable haemolytic activity are indicated in bold.

Residue substituted from apoEdpL-W (WRKWRKRWWWRKWRKRWW)	Haemolysis EC_5 μM		
	Substitution		
	AiB	Alanine	D-amino acid
W	>40	>40	>40
R	**17**	>40	>40
K	**10**	**30**	>40
W	>40	>40	nd
R	**15**	**11**	>40
K	**13**	**28**	>40
R	**19**	>40	>40
W	>40	>40	>40
W	>40	>40	>40
W	**20**	>40	>40
R	>40	**36**	>40
K	**11**	**17**	>40
W	>40	>40	>40
R	**11**	**22**	>40
K	**19**	>40	>40
R	>40	**36**	>40
W	>40	>40	>40
W	>40	>40	>40

Haemolysis data indicated that the substitution of charged arginine or lysine residues with the uncharged alanine or AiB residues resulted in increased haemolytic activity (Table 5.2). Substitutions with the corresponding D-amino acid, which would not affect charge, did not increase haemolytic activity (Table 5.2). These data suggest the pattern of charged residues in this sequence is optimal to avoid haemolytic activity. In addition, haemolytic activity of the CAMPS compared to the biocides was shown to be ordered apoEdpL-W>CHX>PHMB>apoEdp>TCS at concentrations of 60 μM and below (data not shown). This may, in part, be due to a weaker interaction of apoEdp and triclosan with the cell membrane compared to ApoEdpL-W and PHMB,

as potentially indicated by their lower level of adherence to a DOPC bi-layer (Figure 5.6). However, by 70 μm there was no significant difference between the haemolytic ability of ApoEdpL-W, chlorhexidine and PHMB, or between ApoEdp and triclosan.

5.3.6 Biocompatibility index testing of antimicrobials

We investigated the antiseptic potential of the antimicrobials by determining their biocompatibility index values, which compares the antimicrobial activity of a compound to its cytotoxicity, when tested under identical conditions. Table 5.3 shows the avergae IC_{50} values for the antimicrobials in comparison to RF values- the concentration of active agent required to give a $3\log_{10}$ reduction in bacterial cfu ml^{-1}. Average IC_{50} showed ApoEdpL-W to be less toxic than PHMB, chlorhexidine and triclosan after 24 h, with chlorhexidine achieving the highest toxicity. RF values for *E. coli* were equal to 50 μg ml^{-1} for apoEdpL-W, chlorhexidine and PHMB, and were 150 μg ml^{-1} for triclosan. Against *S. aureus,* the RF value for ApoEdpL-W was lower than the other antimicrobials tested at 6 μg ml^{-1} compared to 50 μg ml^{-1} for chlorhexidine and PHMB, and 100 μg ml^{-1} for triclosan (Table 5.3). The resulting BI values (IC_{50}/RF) were higher for ApoEdpL-W than the other test agents for both bacteria, giving it the highest antiseptic potential.

Table 5.3. Biocompatibility Index values. Ratio of average antimicrobial IC_{50} to RF value for *E. coli* and *S. aureus.*

Antimicrobial	MTT IC_{50} (μg/ml)	NR IC_{50} (μg/ml)	Mean IC_{50} (μg/ml)	rf *E. coli* μg/ml	BI *E .coli* (IC_{50}/rf)	rf *S. aureus* μg/ml	BI *S. aureus* (IC_{50}/rf)
ApoEdpL-W	170	140	155	50	3.1	6	25.8
Chlorhexidine	90	90	90	50	1.8	50	1.8
PHMB	140	140	140	50	2.8	50	2.8
Triclosan	100	130	115	150	0.8	100	1.2

5.3.7 Comparative efficacy window of apoEdpL-W and traditional biocides

To further understand the relative selectivity of action of apoEdpL-W towards bacterial cells, we compared the cytotoxicity (IC_{50}) of the test agents to their antibacterial activity (MIC), against a panel of organisms associated with medical device infection (Table 5.3, Figure 5.8 and 5.9). The relatively low antibacterial activity of apoEdp compared to apoEdpL-W has been reported previously (Kelly, 2007).

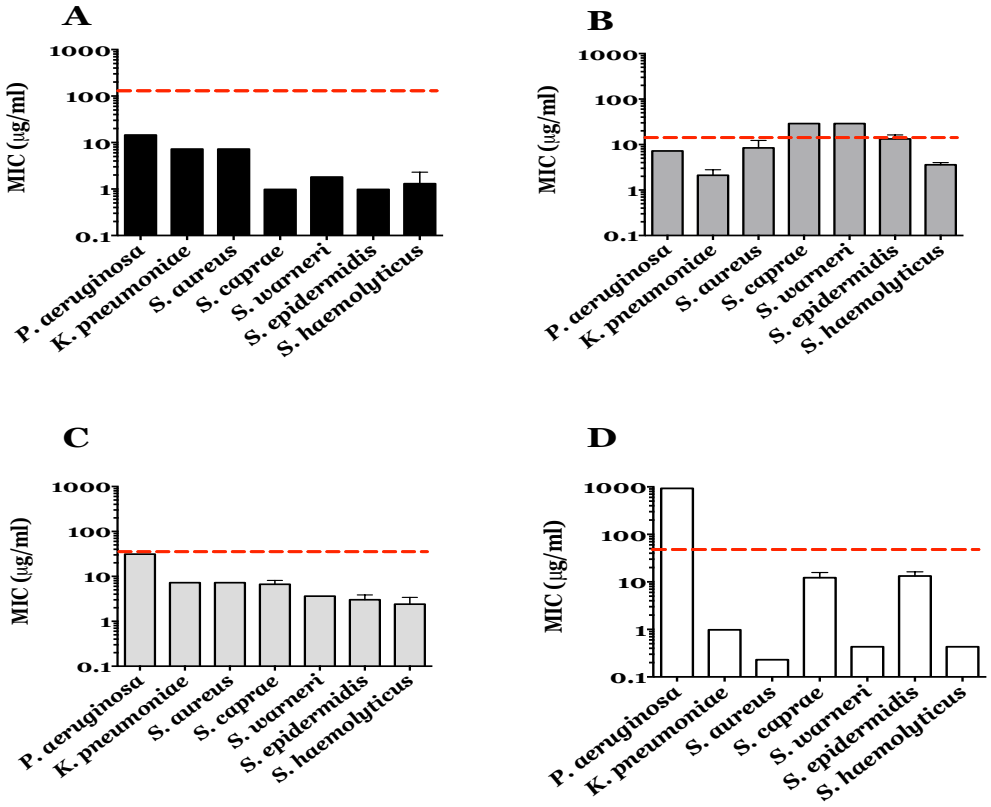

Figure 5.8. Minimum inhibitory concentrations (MIC) for apoEdpL-W (A), chlorhexidine (B), PHMB (C), and triclosan (D) against bacteria commonly associated with medical device infection. Testing was performed in Muller-Hinton Broth. Mammalian cell toxicity (IC50 for L929 cells) is indicated by dotted line. Error bars show standard deviation; n=6.

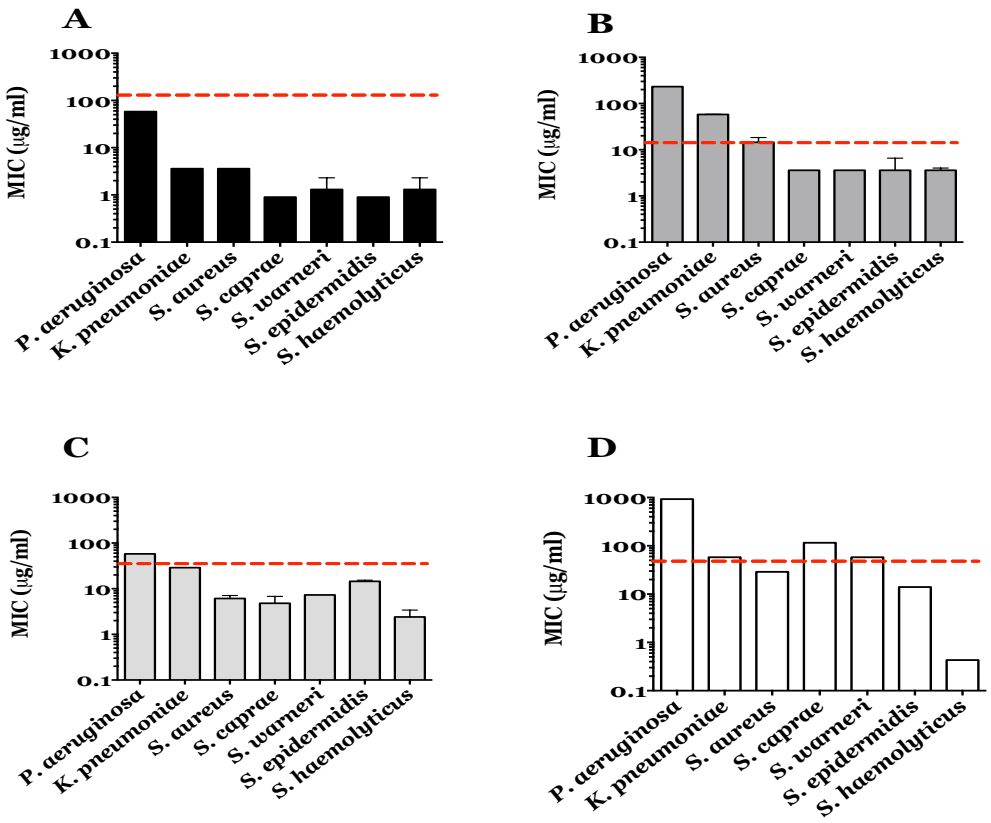

Figure 5.9. Effect of serum on minimum inhibitory concentrations (MIC) for apoEdpL-W (A), chlorhexidine (B), PHMB (C), and triclosan (D) against bacteria commonly associated with medical device infection. Testing was performed in Muller-Hinton Broth containing 10% foetal bovine serum. Mammalian cell toxicity (IC_{50} for L929 cells) is indicated by dotted line. Error bars show standard deviation; n=6).

Minimum inhibitory concentrations, displayed in Figure 5.8 and 5.9, demonstrate the bacteriostatic capabilities of the actives in the absence and presence of serum. For apoEdpL-W, MIC was achieved by concentrations of 15 µg ml^{-1} or less for all test bacteria in the absence of serum and all microorganisms apart from *P. aeruginosa* in the presence. Figure 5.9 represents MICs in the presence of 10 % FBS resulting in a reduction in efficacy for all antimicrobials with the exception of apoEdpL-W. This coupled with the high IC_{50} value observed against a L929 murine fibroblast cell line (Figure 5.8 and 5.9), suggests the window of activity for the peptide is much larger than that of traditional biocides, especially in the presence of serum.

5.3.8 Visualisation of cytotoxic and anti-biofilm effects of antimicrobials

We used CSLM to visualise the cytotoxic effects of antimicrobial treated pHEMA hydrogels on L929 cell vitality, in parallel to its anti-biofilm potency against *P. aeruginosa* (Figure 5.10). Propidium iodide staining of L929 cells, that had been exposed to antimicrobial treated pHEMA hydrogels, revealed a higher proportion of dead cells after exposure to triclosan and chlorhexidine, compared to those exposed to PHMB and apoEdpL-W. The effects of pre-treating pHEMA with antimicrobials before incubating within a planktonic culture of *P. aeruginosa*, showed apoEdpL-W and PHMB to be the most effective at preventing viable biofilm establishment on the gel surface (Figure 5.10).

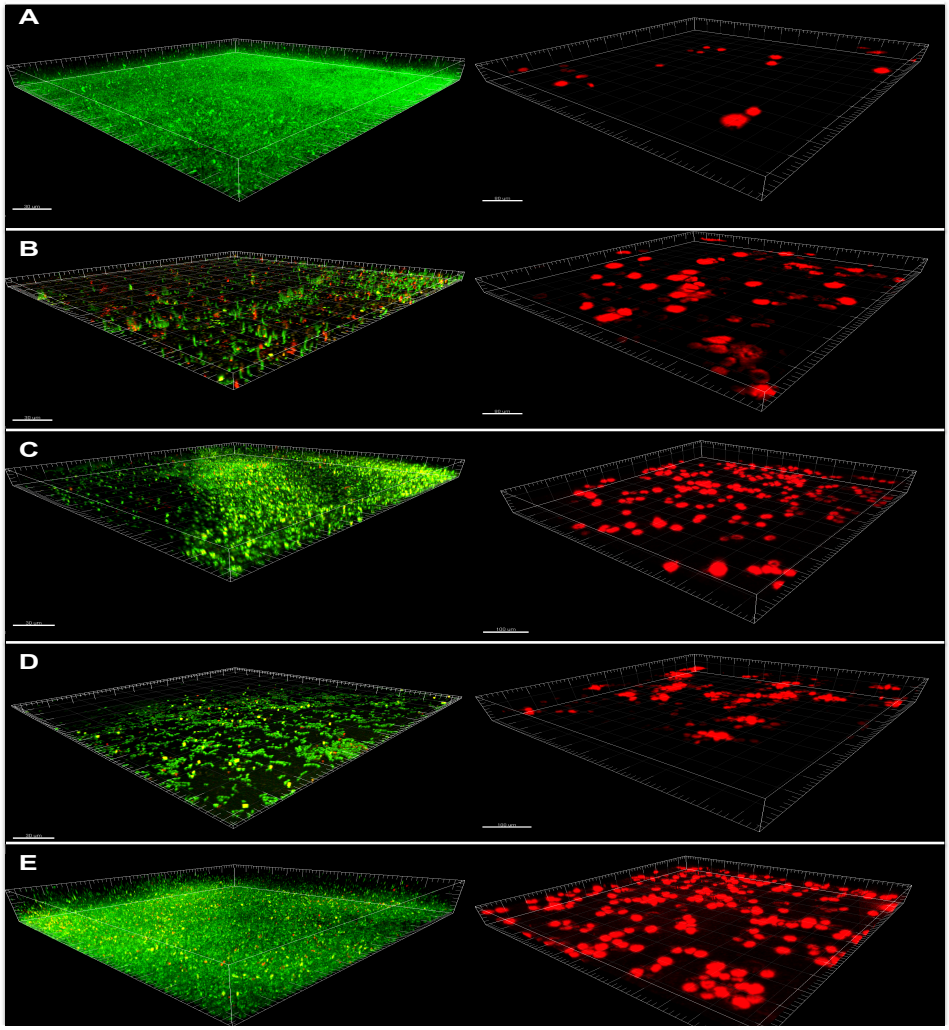

Figure 5.10. CSLM image showing (left) the growth of a *P. aeruginosa* biofilm on a pHEMA hydrogel in comparison to (right) a propidium iodide stained L929 cell line exposed to a pHEMA hydrogel that has been previously exposed to A) PBS, B) ApoEdpL-W, C) chlorhexidine, D) PHMB and E) triclosan.

In addition to the previous toxicity determinants, a separate agar overlay assay was performed comparing the cytotoxicity of all materials (pHEMA, PDMS, PU and PEG) treated with the peptides and biocides (with the addition of silver as a positive control). Results indicated that cytotoxicity was greatest for silver and lowest for apoEdpL-W (Silver nitrate> TCS> CHX> PHMB> ApoEdpL-W) when averaged across the test panel of biomaterials (Table 5.4).

Table 5.4. Agar overlay assay to determine cytotoxicity of antimicrobial treated polymers against an L929 mammalian cell line.

Antimicrobial	Biomaterial	Zone lysis	Lysis index	Response Index	Cytotoxicity
ApoEdpL-W	PDMS	0	0	0/0	None
	pHEMA	1	0	1/0	Mild
	PU	0	0	0/0	None
	PEG	1	0	1/0	Mild
CHX	PDMS	1	0	1/0	Mild
	pHEMA	2	3	2/3	Moderate
	PU	1	2	1/2	Mild
PHMB	PDMS	0	0	0/0	None
	pHEMA	1	0	1/0	Mild
	PU	1	0	1/0	Mild
	PEG	1	0	1/0	Mild
TCS	PDMS	2	3	2/3	Moderate
	pHEMA	3	3	3/3	Moderate
	PU	1	3	1/3	Mild
	PEG	2	3	2/3	Moderate
Silver nitrate	PDMS	2	2	2/2	Moderate
	pHEMA	3	4	3/4	Moderate
	PU	3	2	3/2	Moderate
	PEG	3	4	3/4	Moderate

5.4 Discussion

5.4.1 Activity of antimicrobial impregnated polymer coatings

It has been previously reported that the apoE receptor binding region has direct anti-bacterial properties (Kelly, 2007). In this chapter, two cationic peptide derivatives of apoE were compared to a panel of commonly used biocides in their potential as novel antiseptic coatings for biomedical devices. ApoEdpL-W, a tryptophan substituted version of apoEdp, showed a higher level of absorption and retention into four biomaterials frequently used in the manufacture of medical devices. The antibacterial activity of apoEdpL-W treated materials, against *S. aureus* and *P. aeruginosa,* compared favourably to those treated with apoEdp or commonly used biocides. Whilst the exact antibacterial mode of action of the peptides is unknown, their cationic nature makes disruption of the bacterial membrane likely. Proposed models include the carpet model, whereby the peptides cover the membrane surface

destroying it in a detergent like manner or the by the formation of toroidal pores (Sato & Sato, 2006) leading to leakage of cytoplasmic components, or allowing the cell entry of the peptides causing an interference in biosynthetic processes (Brogden, 2005). ApoEdpL-W treated biomaterials, in general, showed higher antibacterial activity than those treated with apoEdp, which may be due to the larger amount of the peptide that is retained on the materials after washing, or due to a higher antibacterial potency. This enhanced antimicrobial activity of apoEdpL-W may be due to differences in charge distribution, allowing it to more readily form into an α-helical structure which may aid its interaction with the bacterial cell membrane (Kelly, 2007).

5.4.2 Antimicrobial inhibition of S. aureus biofilm formation on a hydrogel surface

The anti-biofilm potency of apoEdpL-W was demonstrably higher than that of apoEdp against *S. aureus* biofilms grown on pHEMA hydrogels. This activity increased as exposure time of the hydrogel to the peptide increased, likely due to an increased level of uptake. Previous research has documented high CAMP tolerance in staphylococcal biofilms due to the presence of polysaccharide intracellular adhesion (PIA), a major biofilm exopolysaccharide which has been found to protect bacteria against CAMPs from skin and neutrophil granules, such as human β-defensins and LL-37 (Vuong*, et al.*, 2004). PIA is a positively charged exopolysaccharide that most likely repels the positively charged CAMP away from the bacterial cell. Since staphylococci are some of the most common pathogens linked to device associated infections, the ability of apoEdpL-W to effectively

disrupt staphylococcal biofilms is a significant attribute, when considering it as a medical device coating.

5.4.3 Antimicrobial disruption of a phospholipid bilayer

QCMD analysis further revealed differences in lipid bilayer interactions for the test compounds. ApoEdpL-W and PHMB showed an increased ability to interact with a phospholipid bilayer and induce disorder, when compared to apoEdp and triclosan. The result for chlorhexidine was deemed to be ambiguous, due to the likely aggregation of the antimicrobial resulting in a far higher absorbance than would be possible for a molecule of that size. The absorption kinetics of apoEdpL-W and the high shift in energy dissipation after phospholipid exposure, may suggest that the peptide adheres to the bilayer in a way that causes an increase in viscoelasticity, for instance by inserting in a perpendicular manner coupling large amounts of water to the layer. Comparatively, both triclosan and apoEdp showed an overall small shift in energy dissipation within the layer, likely due to low mass uptake.

The weaker adherence of apoEdp to the lipid bilayer may, in part, help explain its low antibacterial activity, compared to apoEdpL-W. Triclosan, which largely acts by inhibiting the FabI enzyme in bacteria (McMurry*, et al.*, 1998), also showed a weaker association with the bilayer than the other biocides. However, triclosan demonstrated marked efficacy against all test bacteria, with the exception of *P. aeruginosa,* which is know to have intrinsic resistance (Chuanchuen*, et al.*, 2003). ApoEdp and triclosan also showed the lowest haemolytic activity of all test compounds, which could be accredited to their impaired ability to interact with a

phospholipid bilayer, reducing their capability to disrupt the red blood cell membrane.

By using DPI, the similarity between PHMB and apoEdpL-W in the model membrane system was highlighted, in terms of mass uptake and disruption of the bilayer. Both compounds gave a resulting drop in birefringence during lipid interaction, which indicates an induced disordering within the layer and is likely caused by insertion of the compound. This may help explain the high antibacterial efficacy for both these compounds against the test organisms. However, it must be noted that the mode of action of many cationic antimicrobials involves an initial adherence to the bacterial cell, which is dependent on interactions with electronegative components of the cell envelope, such as LPS and lipoteichoic acids (Broxton*, et al.*, 1984, Ikeda, 1984, Majerle*, et al.*, 2003). Thus, the phospholipid bilayer disruption observed in this study is likely a small part of a much larger underlying mechanism, in terms of antibacterial activity.

5.4.4 Antiseptic evaluation of antimicrobials

A good antiseptic agent should demonstrate marked antibacterial activity at a concentration that is non-toxic to mammalian cells. The window of efficacy of apoEdpL-W was shown to be higher than other antimicrobials due to its relatively high IC_{50} value against a mammalian cell line, compared to its low MIC values, against a test panel of bacteria. In the presence of serum, the activity of the antimicrobials decreased, with the exception of apoEdpL-W. This may be due to an interaction between the cationic biocides and negative components of the serum

rendering them less active (Brinster*, et al.*, 2009). Triclosan has previously shown reduced efficacy against Gram-positive pathogens in the presence of serum, this is believed to be due to the bacteria's ability to gain an exogenous supply of fatty acids from the serum, thereby bypassing the inhibitory effects of the biocide (Brinster*, et al.*, 2009).

The biocompatibility index indicates the low cytotoxicity of apoEdpL-W in parallel with its potency against *S. aureus* and *E. coli*. ApoEdpL-W and PHMB showed the lowest cytotoxicity out of all antimicrobials tested. This may seem surprising due to their relatively high ability to disrupt a phospholipid bilayer, however, since mammalian cell membranes are relatively neutral in charge, this lack of toxicity may be due to the limited attraction of the cationic agent to the cell surface, rendering direct contact less likely (Javadpour*, et al.*, 1996) .

5.4.5 Conclusion

These data demonstrate the apoEdpL-W peptide to be a promising potential anti-infective coating for biomedical devices. The peptide shows an ability to adsorb and be retained in a variety of coating polymers without losing its antibacterial activity and demonstrates marked antibacterial efficacy against a wide range of bacteria often associated with device infections, both in the presence and absence of serum. It is considered that antibacterial mode of action may, in part, involve the direct associating of the peptide with a phospholipid bilayer, leading to insertion and bilayer disruption. However, since apoEdpL-W showed low levels of toxicity against a mammalian cell line, it is considered that the antibacterial mode of action may involve additional interactions of the peptide with the electronegative components of

the cell envelope increasing the likely hood of association with the bacterial cytoplasmic membrane. ApoEdpL-W showed the highest biocompatibility index value, against *E. coli* and *S. aureus* out of all the test actives, demonstrating its good potential as an antiseptic agent.

Chapter 6

The Effect of Apolipoprotein E Derived Antimicrobial Peptides on *in vitro* Dental Plaque Biofilms

Abstract

Dental plaque is a complex sessile microbial community that forms on the tooth surface and which plays an important role in oral health and disease. The control of dental plaque accumulation by the regular application of antimicrobials and the increasing prevalence of antimicrobial-coated dental implants, are believed to improve oral hygiene and reduce the incidence of oral disease (e.g. periodontitis and dental caries). In this chapter, a synthetic peptide analog of human apolipoprotein E (apoEdpL-W) was evaluated against established dental plaque communities *in vitro.* In a model system, 48 h dental plaques were formed on glass slides before being treated with varying concentrations of apoEdpL-W, as well as common oral therapeutic agents triclosan and chlorhexidine. Plaque viability post treatment was visualised using LIVE/DEAD staining/epifluorescence microscopy and quantified using a viability mapping technique. Plaques treated with triclosan were shown to be considerably less viable than those treated with apoEdpL-W or chlorhexidine, although viable regions within the plaque biofilm after triclosan exposure were still apparent. ApoEdpL-W caused a small reduction in biofilm viability, at a level that was not significantly different to chlorhexidine, at all test concentrations.

6.1 Introduction

The human oral microbiota consists of a consortium of microorganisms, that form predominantly on surfaces and are referred to as dental plaque (Marsh, 1994). The formation of plaque is believed to occur in an ordered sequence of events (Rosan, 2000) (Figure 6.1). Initially, the absorption of a conditioning film or 'pellicle', comprised of salivary proteins and glycoproteins, on the tooth surface, aids the reversible attachment of the bacteria via weak physiochemical interactions (Ruardy, 1997). This may subsequently lead to semi-permanent bacterial attachment due to interactions between adhesins on the bacterial cell surface and complementary receptors on the pellicle surface (Lamont, 2000). Initial colonization provides new attachment sites, such as lectins, for secondary colonizers, which may increase the microbial diversity of the plaque. The coadhesion of bacteria often leads to a functional organization within the biofilm, for example, associations between obligatory anaerobic bacteria to oxygen consuming species may aid their survival in the aerobic oral cavity, thus leading to clustering of species (Rickard, 2003). Bacterial multiplication leads to biofilm growth and increased exopolymeric substance production, forming a three-dimensional functionally organized structure.

Once established the plaque microflora is relatively stable, despite constant microbial immigration and nutrient variation (Marsh, 2003, Rasiah, 2005). However, variations in pH, fermentable sugars and poor oral hygiene, leading to plaque accumulation, may result in shifts in the balance of the microbiota (Marsh, 1994). Active detachment may occur due to the production of hydrolytic enzymes by sessile organisms that cleave the adhesion mediated attachments on the bacterial cell surface, or through shear forces exerted on the biofilm during cleaning (Cavedon, 1993).

Colonization resistance largely stems from the resident floras ability to outcompete any invading organisms, for nutrients and attachment sites, and by the production of inhibitory factors (Marsh, 1994).

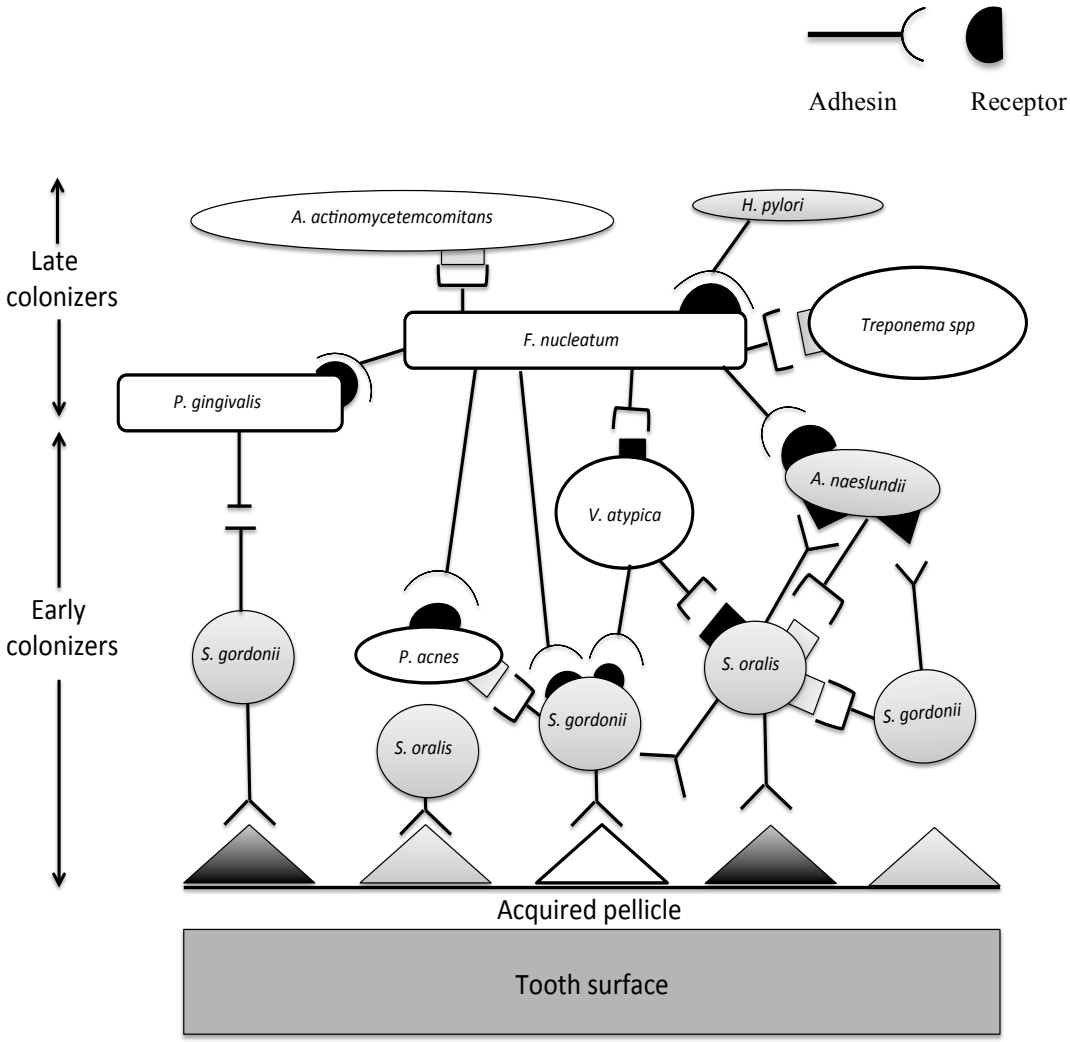

Figure 6.1. Diagrammatic representation of a multispecies dental plaque biofilm. Pellicle formation due to absorption of salivary proteins to the surface acts as a point of attachment to early colonizers, subsequent organisms adhere due to coaggregation and receptor mediated interaction between lectins on one organism and carbohydrate receptors on the other, adapted from Rickard, 2003.

The oral cavity is a very heterogeneous environment and the conditions under which oral biofilms develop are often linked to the overall health of the host (Vroom*, et al.*, 1999). In health, dental plaque plays a role in protecting the host against invading exogenous species, which may be capable of causing disease, such as periodontitis

and dental caries (Marsh, 1994). The development of molecular identification techniques such as 16S rRNA gene sequencing, has revealed that there are distinct bacterial flora associated with healthy compared to diseased oral cavities (Aas*, et al.*, 2005). Diseased cavities have previously been linked to an increase in *Porphymonas gingivalis*, *Streptococcus mutan*s and *Lactobacillus* spp, which are believed to rapidly metabolise dietary carbohydrates producing acids, which demineralize the tooth enamel, leading to the development of dental caries (Marsh, 2003). However, for the most part, the oral microbiota is in harmonious relationship with the host. The establishment of disease is often associated with disruptions in the habitat of the microflora from exogenous sources such as a high sugar based diet or following the use of antibiotics (Newbrun, 1982). The ecological plaque hypothesis suggests that plaque accumulation around the soft tissue of the gingival margin initiates host inflammatory response, leading to secretion of gingival fluid that provides nutrients to many oral pathogens (Marsh, 1994). Additionally, trauma to the cavity can allow bacteria to colonize areas that are not normally accessible to them, which may lead to invasion by opportunistic pathogens (Budtz Jörgensen, 1970).

Oral implants, such as artificial root devices, may lead to the development of chronic gum infections leading to a condition called peri-implantis. This results in a large amount of inflammation in the mucosa around the device, which can lead to bone damage and implant loss. There is considerable research into producing antimicrobial coatings for oral implants, to help inhibit bacterial colonisation, including antibiotics such as gentamicin, that have provided promising results (Lucke, 2003).

Antimicrobial agents, in general, have been widely used to control plaque growth and improve oral hygiene. Chlorhexidine is a cationic bisbiguanide with a broad-spectrum of antibacterial activity and low cytotoxicity, which targets the bacterial cytoplasmic membrane. The ability of chlorhexidine to bind to the tooth surface reducing pellicle and subsequent plaque formation has lead to the incorporation of the biocide in a range of dental products (McBain, 2003). As well as demonstrating good ability to reduce oral bacterial viability, chlorhexidine has also been shown to aid the prevention of gingivitis due to its inhibitory effects on the establishment of plaque (Hennessey, 1973).

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a chlorinated bisphenol, which has demonstrated activity against a range of Gram-positive and Gram-negative bacteria and is commonly incorporated into dental hygiene products due to its antiplaque efficacy (McBain, 2003). At high concentrations, triclosan is thought to disrupt the bacterial cytoplasmic membrane, whereas at lower levels, it is believed to inhibit the synthesis of fatty acids in bacteria by inhibiting a NADH-dependent enoyl-acyl carrier protein reductase, FabI (McMurry, 1998). In potential oral pathogens, such as *streptococcus mutans* which lack expression of target enzyme FabI, the biocide is proposed to prevent glycolysis via inhibiting cytoplasmic enzymes such as pyruvate kinase, lactic acid dehydrogenase and aldolase, as well as by targeting the phospho*enol*pyruvate:sugar phosphotransferase system (PTS), leading to a decrease in bacterial metabolism and reducing the resulting growth of plaque (Phan, 2006).

The use of cationic antimicrobial peptides as therapeutic antimicrobial agents has gained interest over the past decade due to their low cytotoxicity (Maher & McClean, 2006), their specificity towards bacterial cells (Matsuzaki*, et al.*, 1995) and their apparent low risk of selecting for insusceptible bacterial mutants (Ge, 1999). The oral bacterial flora is though to be regulated, in part, by the actions of antimicrobial proteins and peptides such as histatins and defensins, which are components of the innate immune defence system (Zasloff, 2002). Many antimicrobials peptides have demonstrated marked effectiveness against oral bacteria, including Streptococcus, Actinomyces, Fusobacterium, Prevotella and Vellonella species *in vitro* (Dale & Ouhara, 2005). Their broad-range activity makes such peptides promising agents in the treatment of bacteria found in the saliva and dental plaque.

In vitro dental plaque biofilm models can be used to assess antimicrobial treatments in terms of their ability to (i) prevent the formation of plaque and (ii) affect the viability of established mature dental plaques. *In vitro* modelling of plaques allows the parallel assessment of multiple antimicrobial actives, in a stable environment, with controlled nutrient availability and sheer forces (McBain, 2003). Technologies, such as confocal microscopy, have provided detailed insight into plaque architecture, with viability staining showing bacterial vitality varying throughout the biofilm. This revealed that the highest proportion of viable cells were in the central areas of the plaque (Auschill, 2001). Such techniques allow the evaluation of the effects of antimicrobial actives on plaque viability, throughout the different regions of the biofilm.

This current chapter compares the effects of a novel human apolipoprotein E derived antimicrobial peptide (apoEdpL-W) to commonly used oral antiseptics chlorhexidine and triclosan, in their ability to kill artificial dental plaque biofilms *in vitro*. Assessment of antimicrobial impact on plaque viability was performed using LIVE/DEAD staining and epifluorescent microscopy to visualise dead regions of the biofilm after antimicrobial exposure. This approach aims to evaluate the potential of apoEdpL-W as a dental health treatment.

6.2 Materials and Methods

6.2.1 Growth of in vitro dental plaques

Sterile glass slides were partially submerged in 50 ml Duran bottles containing 50 ml artificial saliva (g l^{-1} in distilled water): Mucin (type II, porcine, gastric), 2.5; bacteriological peptone, 2.0; tryptone, 2.0; yeast extract, 1.0; NaCl, 0.35; KCl, 0.2; CaCl2, 0.2; cysteine hydrochloride, 0.1; haemin, 0.001; vitamin K1, 0.0002 (Ledder*, et al.*, 2006). Bottles were inoculated with 1 ml of fresh human saliva, isolated from a 25-year-old healthy female volunteer, before being incubated at 37˚C statically for 48 h.

6.2.2 Antimicrobial treatment of plaque

Plaque coated glass slides were rinsed in twice in 10 ml PBS for 1 min and were then exposed to 100 µl of 500 µg ml^{-1}, 1mg ml^{-1} or 2 mg ml^{-1} of antimicrobial for 10 min using an adhesive GENE FRAME® (ABgene Ltd, Epsom, UK) to confine the antimicrobial solution to a defined area.

6.2.3 Visualization of plaque by epifluorescent microscopy

Biofilm viability and structure was visualised by fluorescence microscopy. Briefly, a working solution of BacLight LIVE/DEAD stain (Invitrogen Ltd, Paisley, UK) was prepared by adding 10 µl each of SYTO 9 (component A) and propidium iodide (component B) to 980 µl distilled water. This solution (50 µl) was applied directly to the biofilm and covered with a glass cover slip. Slides were incubated at room temperature in the dark for 15 min, according to the standard BacLight staining protocol. Biofilms were visualised with an Axioskop 2 fluorescence microscope with a 20 x objective lens (Carl Zeiss Ltd, Rugby, UK) and images captured using a digital microscope eyepiece (Cosmos Biomedical, Derbyshire, UK) and exported as JPEG files. Bacterial cells incubated in the presence of both stains fluoresce either green (viable) or red (dead). The excitation and emission maxima for these dyes are 480 and 500 nm for SYTO 9 and 490 and 635 nm for propidium iodide. The percentage viable biomass was determined by calculating the proportion of red fluorescence as a percentage of total fluorescence relative to an untreated control. Images were processed, surface-rendered and quantified using Imaris (Biplane AG, Zurich, Switzerland) and ImageJ (National Institutes of Health, Bathesda, Maryland).

6.3 Results and Discussion

This study compared the anti-plaque activity of apoEdpL-W to two antimicrobial agents commonly used in oral hygiene products. During this investigation 48 h old dental plaques were exposed to varying concentrations of apoEdpL-W, chlorhexidine or triclosan before viability was measured using the BacLight LIVE/DEAD staining kit and epifluorescence microscopy.

6.3.1 Antimicrobial efficacy against mature plaque biofilms

The visualisation and consequent viability mapping of antimicrobial exposed dental plaques showed triclosan to be the most effective treatment in terms of decreasing plaque viability (Figure 6.2 and 6.3). Triclosan exposed plaques were significantly less viable than those treated with either chlorhexidine or apoEdpL-W at all test concentrations (Figure 6.3). After triclosan exposure, plaque images revealed large regions of dead cells on the plaque surface with more viable underlying regions (Figure 6.2). The % viability of triclosan treated plaques was not significantly different when comparing those treated with 500, 1000 or 2000 μg ml^{-1} ($P>0.05$), however, viability did not reach below 15 % at all treatment concentrations, which may suggest a limitation in triclosans ability to penetrate into the deeper layers of the biofilm or the presence of triclosan insusceptible cells (Phan & Marquis, 2006) (Mah & O'Toole, 2001).

ApoEdpL-W and chlorhexidine treatments showed no significant difference in reduction of plaque viability at all concentrations tested ($P>0.05$) and resulted in fewer unviable plaque regions post-treatment than triclosan (Figure 6.2). The bactericidal action of either agent was limited to small regions, observed as red fluorescence, with large amounts of viable biofilm visible at treatments up to 2 mg ml^{-1} (Figure 6.2). Chlorhexidine and apoEdpL-W treated plaques did not reach below 25 % viability even at the highest test concentrations, which may be accredited to the limited diffusion of the antimicrobials into the plaque, or possibly due to the low antimicrobial susceptibility of the bacteria after the induction of various defence mechanisms, in response to antimicrobial exposure or nutrient limitations within the biofilm (Mah & O'Toole, 2001).

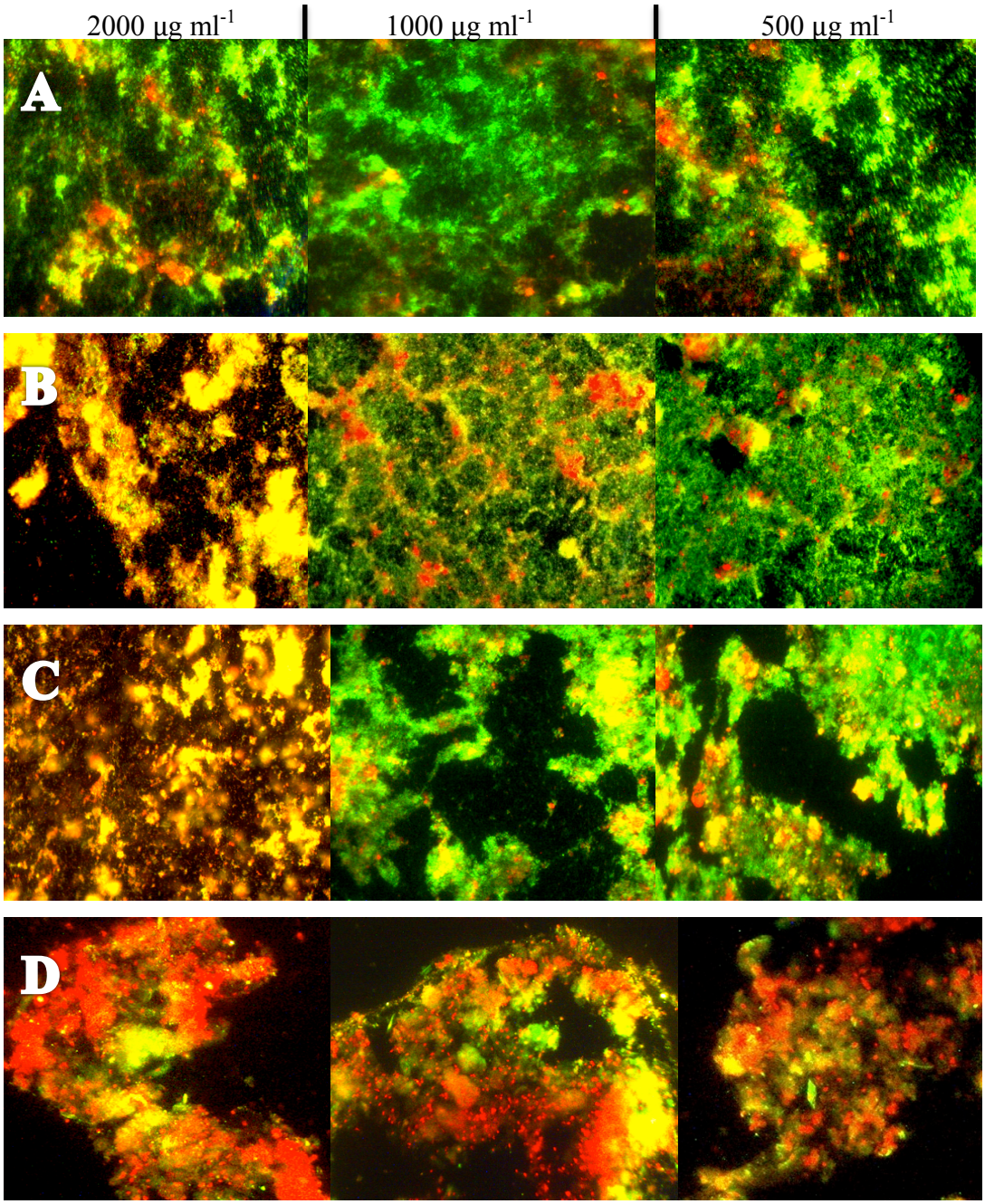

Figure 6.2. Dental plaque biofilms after exposure to A) PBS, B) ApoEdpL-W, C) chlorhexidine and D) triclosan. Plaques were as viewed by epifluorescence microscopy. Green fluorescence represents viable cells and red fluorescence represents non-viable cells.

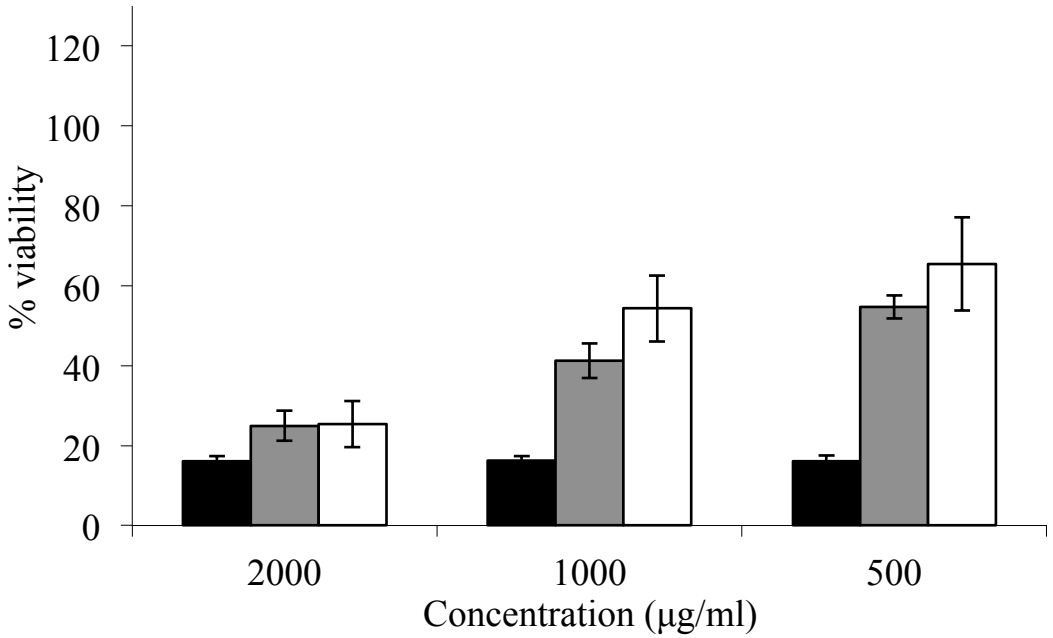

Figure 6.3. % Viability of plaque biofilms after antimicrobial exposure. Viability is determined relative to an untreated control. Black indicates triclosan, grey chlorhexidine and white apoEdpL-W. Error bars show standard deviation (n=5).

6.3.2 Triclosan activity against dental plaque

Triclosan has previously demonstrated high levels of antibacterial activity against oral microorganisms and has been shown to reduce plaque formation *in vitro* (Lindhe, 1993). It is often used in dentifrice and mouthwash formulations and is widely considered to be extremely effective against oral pathogens (Cullinan, 2003). Confocal analysis has revealed that the effect of antimicrobial treatment on already established plaques is often limited to the most superficial layers, due to limited diffusion of the active agent (Zaura-Arite*, et al.*, 2001). This demonstrates that antimicrobials are often better at preventing biofilm development than eradicating it once it is already established.

6.3.3 Chlorhexidine activity against dental plaque

The findings for chlorhexidine plaque treatment in this study are consistent with previous work documenting dental biofilm resistance to 0.2 % chlorhexidine *in vitro.* It has been suggested that cationic antimicrobials are bound and inactivated by plaque EPS reducing their efficacy (Zaura-Arite*, et al.*, 2001). Chlorhexidine is found in dental products at concentrations up to 0.2%, suggesting that in use concentrations may still not completely eradicate the plaque biofilm. It must, however, be considered that this method of *in vitro* plaque production does not account for the mechanical and immunological clearance that would occur *in vivo* and the level of plaque accumulation on the slides may be significantly denser than would be expected in the oral cavity. In comparison, other investigations have shown that 0.2 % chlorhexidine is able to rapidly penetrate artificially condensed plaque up to 2 mm (Melsen, 1983). This suggests a possible alternative mechanism of chlorhexidine insusceptibility within the plaque biofilm, such as an induced defence response, for example, the expression of efflux pumps or antimicrobial modifying enzymes (Marsh, 2004). Alternatively, nutrient depletion within an established plaque biofilm may produce slow growing cells that are less susceptible towards a particular antimicrobial agent, than their actively growing counterparts (Marsh, 2006).

6.3.4 ApoEdpL-W activity against dental plaque

The effect of apoEdpL-W exposure on mature dental plaques has not previously been investigated. Antimicrobial peptides found within the saliva are thought to help modulate oral microbial communities (Marsh, 2006). Whilst, the use of artificially

synthesised peptides as dental treatments are not currently commercially available, there have been promising experimental results, suggesting they may be of therapeutic value (Helmerhorst*, et al.*, 1999). Whilst apoEdpL-W did not appear to have a significant impact on plaque viability, until tested at high concentrations, its overall efficacy was comparable to that of chlorhexidine. Chlorhexidine is known to be an effective plaque inhibitor, thus analysis into the preventative action of apoEdpL-W on plaque formation would be an area of potential future interest.

The culturing of dental plaque allows an effective way to assess antimicrobial impact on plaque biofilm viability. Unfortunately, due to the density of the plaque and the limitations of the microscope system, we were unable to evaluate the deeper parts of the biofilm and any antimicrobial action that may have occurred there. The use of confocal microscopy would allow a more detailed analysis of the plaque architecture and any variation in viability throughout the depths of the biofilm.

6.3.5 Conclusion

In conclusion, the apoEdpL-W peptide displays *in vitro* anti-plaque efficacy on a similar level to chlorhexidine, a gold standard for oral care treatment products, and thus may have potential as an anti-plaque treatment or an anti-infective coating for dental implants. However, neither apoEdpL-W nor chlorhexidine show the same reduction in plaque viability as triclosan, highlighting the high level of activity of triclosan against oral microorganisms and the resilience of the established dental plaque towards antimicrobial therapy.

Chapter 7

General Discussion

7.1 Study Overview

The European Center for Disease Prevention and Control estimates that over four hundred thousand patients experience hospital acquired infections in European hospitals every year, which results in at least thirty thousands deaths. A high percentage of these infections are associated with biofilm formation on implanted medical devices, which frequently do not respond to antimicrobial therapy (Donlan & Costerton, 2002). Due to the severity of biofilm associated infections, there has been a constant effort to develop novel device coatings that are refractory to microbial adhesion, colonization and biofilm formation (Gottenbos*, et al.*, 2002, Turner*, et al.*, 2009). Biocides have previously been used to produce anti-infective coatings with significant results (Barbolt, 2002, Gaonkar*, et al.*, 2003). However, there is concern that extensive biocide use may lead to the selection of bacteria with reduced biocide susceptibility (Moore, 2008). Additionally, considerations of cytotoxicity and the potential to generate cross-resistance with third party agents, for example antibiotics, has further fuelled the need for new antimicrobial actives (Chuanchuen*, et al.*, 2001, Allmyr*, et al.*, 2006).

This thesis aimed to evaluate the potential of the apoEdpL-W peptide as a novel device coating agent by producing a comprehensive comparison of the peptide to commonly used biocides triclosan, PHMB and chlorhexidine, as well as to an unmodified version of the peptide (apoEdp). The test compounds were evaluated in their antimicrobial activity, anti-biofilm activity, cytotoxicity and their interactions with various biomaterial-coating polymers. Initially, the activity of the antimicrobials against a test panel of 18 bacteria in both planktonic and biofilm states, was compared by determining MICs, MBCs and MBECs. The generation of microbial

insusceptibility after long-term sub-lethal level antimicrobial exposure was also studied, using pure culture training experiments. Any alterations in biocide susceptibility and biofilm forming ability were monitored, along with any observed further phenotypic adaptions. An in depth analysis of *S. aureus* SCV formation after triclosan treatment was performed, including monitoring of any change in planktonic growth, biofilm growth, relative fitness and differences in expression of virulence factors. Proteomic analysis of *S. aureus* was performed before and after SCV formation and alterations in cell morphology were monitored using TEM.

The cytotoxicity of each antimicrobial was assessed in parallel to its antibacterial activity and resulting biocompatibility index values were generated, correlating to the test compounds antiseptic potential. The uptake and retention of the test agents onto different biomaterial polymers, often used in medical device manufacture, and the resulting antibacterial activities of said polymers were also evaluated. Furthermore, we compared the anti-biofilm potency of pre-treated hydrogels, relative to their toxicity against a mammalian cell line. To gain insight into the mode of action of apoEdpL-W and to understand its relative selectivity towards bacterial cells over mammalian ones, we studied antimicrobial mass uptake onto a phospholipid bilayer and the resulting disruption to the bilayer, determined using the QCMD and via DPI. Finally, it was determined whether apoEdpL-W showed any efficacy against mature dental plaque biofilms.

7.2 Intrinsic insusceptibility and phenotypic adaption towards antimicrobials

Initial biocide susceptibility testing (MIC/MBC) revealed the broad-spectrum of activity of apoEdpL-W, as well as that of PHMB, chlorhexidine, cetrimide and

triclosan. Bacteria varied considerably in their response to an antimicrobial and many demonstrated innate insusceptibility towards a particular agent. For instance in this study, *S. marcescens* was relatively insusceptible towards apoEdpL-W, cetrimide and triclosan, *B. cepacia* towards triclosan and PHMB and *P. aeruginosa* towards cetrimide and triclosan. Biocide insusceptibility can be attributed to multiple factors in bacterial physiology, leading to a change in (i) antimicrobial penetration of the bacterial cell (Frenzel, 2011), (ii) antimicrobial accumulation (Padilla*, et al.*, 2010), (iii) adaption of target sites due to modification or over-production (Yu*, et al.*, 2010) and (iv) changes in communication e.g. via quorum sensing or gene transfer (Kong, 2006). Previous studies have shown intrinsic insusceptibility to encompass factors such as spore formation (Bloomfield & Arthur, 1994), lack of porins hindering biocide diffusion into the cell (Frenzel, 2011), as well as the presence of active efflux systems (Ziha Zarifi, 1999) or antimicrobial degrading mechanisms (Sieprawska-Lupa*, et al.*, 2004).

The evolution of bacterial resistance to chemotherapeutic agents is of considerable concern to public health. The concept of drug resistance, such is as applied to antibiotics, often cannot be applied to biocides due to their multiple target site mode of action, meaning that a single mutation in unlikely to lead to treatment failure. However, alterations in biocide susceptibility have been noted, and if these provide a selective advantage to a pathogenic microorganism, it is an area that requires monitoring. The current investigation observed acquired insusceptibility in multiple organisms that were 'trained' to grow in increasing levels of antimicrobials. Frequently, however, these alterations were not stable and would revert when the bacteria were allowed to recover in a biocide free medium. This type of phenotypic

adaption has previously been attributed to changes in outer membrane proteins (Winder, 2000), mutations in specific target sites (Zhu*, et al.*), an increase in active efflux (Mima*, et al.*, 2007) and various stress induced responses (Guerin Mechin, 2000). It has been argued that this kind of temporary adaption is unlikely to play a significant role in determining the long-term survival of the bacteria and therefore its clinical significance in minimal (Russell, 2003). However, it is equally considered that the ability to persist at low antimicrobial concentrations then revert to unexposed form may in fact increase the likelihood of the transmission of infection (Proctor*, et al.*, 2006). The clinical relevance of this adaption may largely be determined by the virulence and fitness of the microorganism once it has recovered.

The acquired reduction in antimicrobial susceptibility in the 'trained' organisms occasionally remained lower than the unexposed strains, even after the selective pressure was removed, which may indicate the selection of an inherently stable mutation. This study generated the production of several microorganisms that maintained an increased level of insusceptibility (MIC and MBC) after antimicrobial exposure and recovery, compared to their unexposed counterparts. For apoEdpL-W this included *C. indologenes, E. faecalis, S. caprae, S. epidermidis, S. haemolyticus* and *S. warneri.* Cetrimide induced irreversible changes in bacterial susceptibility in *S. haemolyticus*, *S. lugdunensis* and *S. epidermidis,* whilst chlorhexidine caused permanent adaption in a wide variety of species including *K. pneumoniae, S. marcescens, S. maltophilia, B. cereus, B. cepacia* and *S. lugdunensis*. The only organism that demonstrated a non-reverting decrease in susceptibility towards PHMB was *C. indologenes.* Triclosan induced insusceptibility in *E. coli, S. aureus* and *E. faecalis* that also did not revert in the absence of the biocide. Whilst this type

of step-wise selection has yielded bacteria with increased MICs and in some cases MBCs, it is a very selective procedure that may not essentially be mimicked in a "real life" situation. In addition, it is important to note that these values, even though higher than would be expected in a normal strain, are still below in use concentrations for the antimicrobials and therefore would not lead to wide spread antimicrobial resistance.

Antimicrobial agents may select for mutations within their targets, some of which may be common to other agents. It is therefore of concern that the misuse of biocides could lead to the evolution and persistence of drug resistance in bacteria, consequently decreasing the effectiveness of available therapies (Chuanchuen*, et al.*, 2001). However, the acquisition of any new mutation, whilst providing advantageous to bacterial survival in the antimicrobials presence, may result in a cost to bacterial fitness and virulence, due to alterations in other physiological characteristics (von Eiff*, et al.*, 2000). During this study it was observed that several microorganisms demonstrated significant alterations in their ability to form biofilms after antimicrobial exposure. This may be due mutations affecting systems directly involved in bacterial adhesion and biofilm maturation (Adnan*, et al.*, Rice, 2005), or potentially due to the selection of mutants with altered fitness levels leading to abnormal growth rates, which may effect the development of the biofilm (Gilbert, 1990, Latimer*, et al.*, 2012). Since biofilm formation is often an important virulence determinant, this may indicate that the insusceptible organisms have altered pathogenicity, compared to the parent strains.

Whilst *in vitro* studies have highlighted the potential of bacteria to adapt in the presence of an antimicrobial (Moore, 2008), care must be taken when extrapolating the clinical relevance from susceptibility testing data. Studying change in MIC allows us to see the potential emergence of antimicrobial tolerance, however, MICs are generally tested under nutrient-rich conditions, during which the organic material in the test media may interact with the biocide lowering its activity. Often an organism that is capable of growing in nutrient-rich media at high biocide concentration may not survive disinfection at the same concentration (Russell, 2008).

7.3 Triclosan-Induced Insusceptibility in *S. aureus* SCVs

Cross-resistance between triclosan and certain antibiotics has previously been reported (Chuanchuen*, et al.*, 2001). This is suggested to be due to the induction of a broad substrate range efflux mechanism, able to elute both triclosan and the antibiotic from the bacterial cell (Chuanchuen*, et al.*, 2001, Braoudaki & Hilton, 2004). Triclosan is used in the treatment of Gram-positive skin infections and is an active component in skin washes used in hospitals to help prevent the transmission of MRSA (Seaman*, et al.*, 2007). It is therefore very worrisome that the use of the biocide has the potential to induce further antibiotic resistances.

After sub-lethal level triclosan exposure we saw the development of an insusceptible small colony variant phenotype in *S. aureus.* Small colony variants have been previously isolated from patients experiencing chronic relapsing infections (Seifert, 1999, Seifert*, et al.*, 2003). Their ability to survive low levels of antimicrobial treatment and persist within the host, potentially avoiding immune attack, has lead to

concern over the development of difficult to treat infections (von Eiff*, et al.*, 2000). However, many SCVs have previously shown defects in pathogenic determinants, such as haemolysins, coagulase and DNase activity (Bayston*, et al.*, 2007). Whilst there have been previous reports of triclosan selected SCVs there has been little in depth analysis of their pathogenic potential.

In the current study, *S. aureus* SCVs were selected for after sub-lethal level triclosan exposure. They displayed typical characteristics usually associated with SCVs, such as the formation of small pinpoint colonies that lack pigmentation (Kriegeskorte*, et al.*, 2011). The elevated MIC and MBC towards triclosan in the isolates may be due to an overexpression in target enzyme FabI, as determined using 2D gel electrophoresis. In adapting to the presence of triclosan, a reduction in planktonic growth rate and biofilm forming ability was observed, meaning that despite a raised MIC and MBC, *in vivo* the SCV may be vulnerable to a wide range of antimicrobial and immunological challenges. A loss in haemolysis, coagulase and DNase activity may have resulted in the reduced virulence observed in a *G. mellonella* pathogenicity assay, when compared to the parent strain. The SCV strain showed enlarged cocci with abnormally formed septum, when viewed under TEM. It is possible that an upregulation of autolysin, IsaA, may be being produced to compensate for the defects in cell division. Additionally, an increase in peptide deformylase, Def, expression may be indicative of an overall increase in protein synthesis in response to triclosan.

When the strains were grown in competition, P0 rapidly outcompeted P10, which may indicate a lack of ability of P10 to persist during a mixed infection. Since the SCV phenotype did not revert in the presence of hemin, menadione or thymidine, it seems unlikely that the SCVs selected for in this study are due to a deficiency in these compounds. It is plausible that the reason for the small colonies is due to defective cell division, indicated by formation of irregular septum, thus resulting in a slow growth rate. However, the underlying reason for this abnormal cell morphology is not yet known, and may be an area of potential future research.

The formation of small colony variants represents one of many phenotypic adaptions observed when bacteria encounter an antimicrobial stress. Although, in the case of triclosan adapted SCVs in *S. aureus,* it appears that in becoming less susceptible towards the biocide that this particular strain has lost certain pathogenic determinants. The lack of restoration of virulence factors once the bacteria had recovered in the absence of triclosan, supports the idea that the selection of triclosan insusceptible mutants may not necessarily be cause for concern, in terms of infection, due to the SCVs impaired pathogenic potential and lack of competitive fitness.

7.4 The Potential of Antimicrobial Coatings in Combating Device Associated Infection

The development of a medical device coating that displays long-lasting activity against a broad spectrum of bacteria without compromising device function would greatly benefit patient care. In addition to having a broad-spectrum of antimicrobial activity and not generating microbial insusceptibility, a successful anti-infective coating should display low cytotoxicity. Biomolecular analysis techniques allowed

us to study the interactions of the antimicrobials with preformed phospholipid bilayers, which gave insight into potential modes of action and allowed us to theorize about the reasons behind their relative selectivity towards bacterial cells over mammalian ones. The apoEdpL-W peptide displayed low toxicity against an L929 murine fibroblast cell line, as determined by the biocompatibility index and the agar overlay assay. ApoEdpL-W and PHMB showed an enhanced ability to interact with a phospholipid bilayer, compared to apoEdpL-W and triclosan. Interestingly, both apoEdpL-W and PHMB were the least toxic of all the test compounds, whilst maintaining marked antibacterial activity. This may tentatively suggest that interactions with other negative components of the bacterial cell envelope apart from the phospholipids, such as LPS and teichoic acids, are vital in the compounds antibacterial mode of action and without this strong electrostatic attraction, for instance when exposed to a mammalian cell membrane, that there is little interaction. This selectivity towards the bacterial cell compared to the mammalian cell gives apoEdpL-W a good window of activity in terms of its potential as an antiseptic agent.

The apoEdpL-W peptide demonstrated the ability to absorb and be retained onto a variety of coating polymers, without losing its antibacterial activity. It maintained good efficacy even when in the presence of serum, when the effects other antimicrobials such as triclosan became significantly lessened. Whilst the dip coating of materials may seem an inefficient method due to the inevitable leaching of the antimicrobial from the polymer surface, it provides a simple, cheap, less labour intensive way to produce an antimicrobial surface coating, allowing the rapid testing of multiple antimicrobials and biomaterials in parallel. Hydrogels, such as pHEMA and PEG, proved to be capable of absorbing large amounts of antimicrobial in a short

period of time, forming a controlled release system, able to effectively combat bacterial contamination. However, this type of antiseptic surface diffusion may only be suitable for applications where a short-term of antimicrobial release is required.

The pre-treating of pHEMA hydrogel surfaces with apoEdpL-W proved effective in reducing *S. aureus* and *P. aeruginosa* biofilm formation. Staphylococcal biofilms in particular are one of the most frequent causes of device related infections (Francolini & Donelli, 2010), therefore the ability to prevent *S. aureus* biofilm establishment is a promising attribute in a potential device coating. Critically, this form of 'batch' formation of a biofilm, whilst giving some insight into biofilm susceptibility, does not necessarily represent the response generated by biofilms *in vivo*. Biofilms formed, for instance, on implanted device surfaces are often generated in continuous systems, allowing the constant replacement of nutrients and removal of wastes. Therefore, further investigation into the efficacy of apoEdpL-W in biofilm prevention, in a continuous model system to simulate an *in vivo* infection, would be an area of future research.

The impact of apoEdpL-W treatment on dental plaque biofilms was analysed *in vitro*. Whilst the peptide did confer some anti-plaque efficacy, on a similar level to that of oral antiseptic chlorhexidine, this preliminary investigation would benefit from a more detailed analysis. Areas for future investigation could include the determination of the microbial composition of dental plaque after peptide exposure, as well as an in depth analysis of the plaque architecture and the distribution of dead cells within the biofilm after antimicrobial treatment, which could be obtained using confocal microscopy.

7.5 Future Work

Whilst the current study has provided insight into certain aspects of antimicrobial chemotherapy, there are many areas of potential future research generated from this work. More detailed insight into the impact of antimicrobial exposure on bacteria could be obtained through transcriptomics and proteomic analysis of the inherently stable mutants selected for during this study. This would give insight, not only of the mechanisms that render the bacteria less susceptible towards an antimicrobial agent, but also of any other induced changes in the bacteria's physiology, that may affect pathogenicity.

The generation of cross-resistance between coating compounds and third party agents such as antibiotics and AMPs of the innate immune system would be an important area of research, as the generation of bacteria that could resist the effects of the host immune system would be of significant consequence. One important aspect not considered in this study is immunological response towards the apoE peptides. Antimicrobial peptides have previously demonstrated innate immune modulatory capabilities, by recruiting lymphocytes, monocytes and polymorphonuclear leukocytes to the site of infection, enhancing bacterial clearance and limiting sepsis (Hancock, 2002). If the apoE-derived peptides were capable of provoking this type of innate response then this would greatly improve their *in vivo* antibacterial efficacy. Comparatively, it would be vital that they did not trigger any unwanted immune activation. For instance, if the peptides in fact generated a specific adaptive immune response, since it is derived from a host-protein, this could lead to an irrevocable autoimmune attack.

The *in vivo* efficacy of any potential treatment would need to be evaluated, both in animal models and clinical trials, if aiming to develop a clinically useful device coating. *In vitro* susceptibility testing, whilst giving a good indication of bacterial response, is highly influenced by experimental conditions and does not take into account the physiological and immunological changes experienced during infection. In addition, whilst susceptibility testing gives insight into the antimicrobial efficacy against individual microorganisms, infection is frequently associated with a mixed flora and may involve multiple interactions between microorganisms that cannot be accounted for in this type of assessment.

7.6 Conclusion

With an ageing population it is likely that the need for implanted biomedical devices will continue to rise, resulting in an increasing threat of difficult to treat device related infections to the populous. The severity of device associated infections and their high economic consequence highlight the need to develop device coatings that prevent microbial contamination without impairing device function. The long-term effects of active coatings, in terms of their impact on bacterial susceptibility, as well as their effect of healthy tissues and cells need to be carefully monitored. The findings from the thesis suggest that the apoEdpL-W peptide is a good candidate for further development as an antimicrobial coating for biomedical device surfaces, having the properties of broad-spectrum of antibacterial activity, biocompatibility and low level induction of bacterial insusceptibility. Although we have investigated certain criteria that are required of an effective anti-infective device coating, there are still areas that require further exploration.

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Chapter 8

Appendix

8.1 The Impact of Triclosan Exposure on Protein Expression in *E. coli*

This current investigation aimed to evaluate changes in protein expression in *E. coli* after long-term exposure to triclosan, using a pure culture passage based training regime *in vitro*.

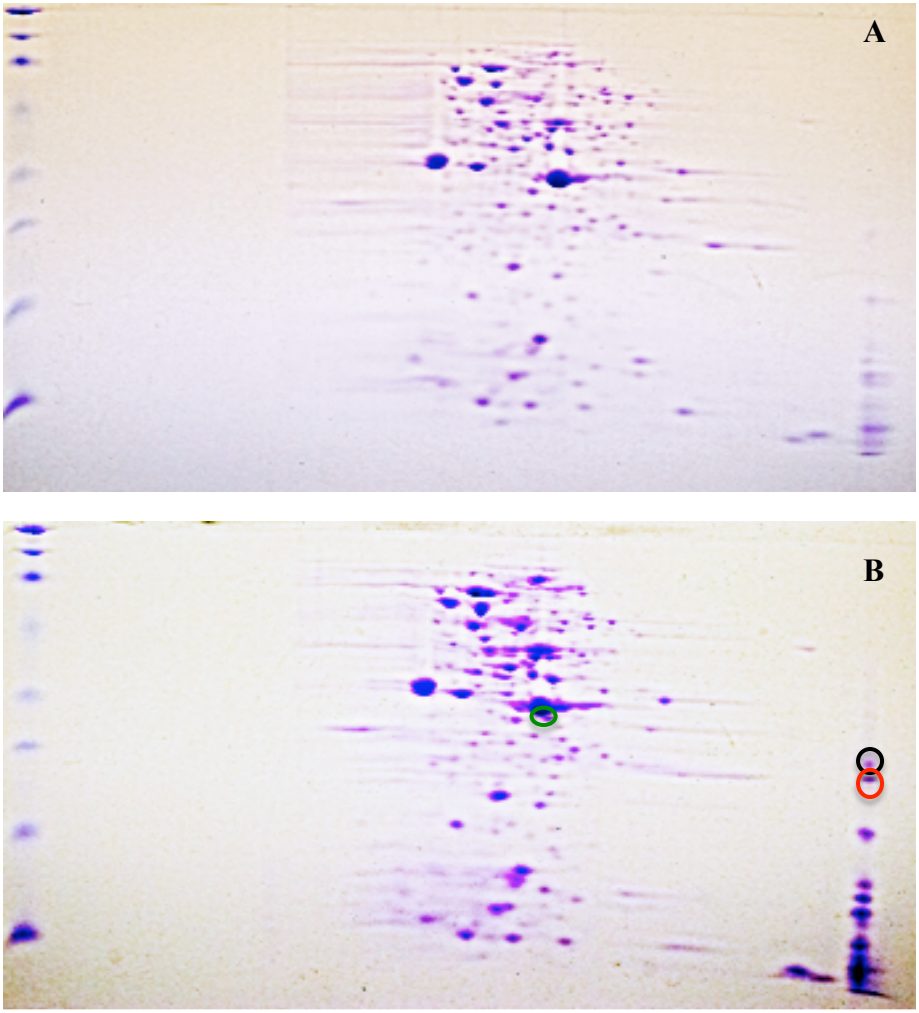

Figure 8.1. 2D gel analysis of *E. coli* before (A) and after (B) 10 passages in the presence of triclosan. Green indicates protein spot FabI, black indicates ClpB and red indicates EcoKI.

Analysis of protein expression profiles in *E. coli* after exposure to triclosan revealed an upregulation in target enzyme FabI, as well as increased expression of NADP dependent malic enzyme EcoK1 and heat shock associated chaperone protein ClpB (Figure 8.1). Overexpression of target enzyme FabI may help explain the increase in

MIC observed in *E. coli,* after triclosan exposure. Triclosan forms a FabI-NAD+-triclosan stable tertiary complex in bacteria, due to the interaction of the phenol ring of the triclosan with the nictotinamide rings of NAD+, within the active site of FabI (McMurry*, et al.*, 1998). It has been suggested that over expression of enzymes, for which NADP is a cofactor, for example EcoK1, may lead to increased binding to free triclosan, lowering the effective concentration and decreasing the resulting MIC (Yu*, et al.*). ClpB is a chaperone protein involved in a generalized stress response in *E. coli* (Ma, 1995). ClpB works with DnaK to resolubilise toxic aggregate proteins produced in response to a stress, such as exposure of bacteria to an antimicrobial agent. This accumulation of upregulated proteins gives insight into the response of *E. coli* to sub-lethal triclosan exposure.

Chapter 9

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