Novel antimicrobial biomaterials

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Novel Antimicrobial Biomaterials

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To my children

Anas and Sara

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List of Abbreviations

MRSA	-	Methicillin-resistant *Staphylococcus aureus*
MSSA	-	Methicillin-sensitive *S. aureus* strains
PMMA	-	Polymethylmethacrylate
BAI	-	Biomaterial associated infections
ICUs	-	Intensive care units
US ICUs	-	United States intensive care units
MK	-	Microbial keratitis
PEO	-	Polyethylene oxide
PEG	-	Polyethylene glycol
PLL-g-PEG	-	Poly(l-lysine)-grafted-poly(ethylene glycol)
PLL-g-PEG/PEG-RGD	-	Poly(l-lysine)-grafted-poly(ethylene glycol) (PLL-g-PEG) functionalized with RGD
AMPs	-	Antimicrobial peptides
DNA	-	Deoxyribonucleic acid
tRNA	-	transfer-ribonucleic acid
AOT	-	*bis (2 ethylhexyl) sodium sulfosuccinate*
hBD	-	Human beta defensins
NP	-	Neutrophil peptide
VRE	-	Vancomycin-resistant *Enterococcus*
B-LFcin	-	Bovine lactoferricin
CVC	-	Central venous catheter
CLPU	-	Contact lens induced peripheral ulcer
CLARE	-	Contact lens-induced acute red eye
EDC	-	1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride
MHB	-	Muller Hinton Broth

MIC	-	Minimum inhibitory concentration
MBC	-	Minimum bactericidal concentration
CFU	-	Colony-forming units
SDS	-	Sodium dodecyl sulphate
CHAPS	-	3-[(3-cholamidopropyl) dimethylammonio]propanesulphonic acid
CD	-	Circular dichroism
TFE	-	Trifluoroethanol
EDTA	-	Ethylene diamine tetra-acetic acid

Abstract

Although the widespread use of biomaterials has resulted in significantly improved quality of life and patient survival, biomaterial-associated infections (BAI) remain a vital concern and treatment options are limited. In this study the ability of a novel antimicrobial peptide (AMP) melimine to prevent bacterial colonisation of contact lenses when covalently attached to the material surface was examined against a range of ocular clinical isolates. Various sequences derived from melimine were also analysed to determine if activity comparable to the parent peptide could be achieved with a shorter sequence. The solution mechanism of melimine was studied using circular dichroism and fluorescence spectra in membrane mimetic solvents and the interactions of melimine with bacterial membranes was examined using scanning electron, fluorescence and Atomic force microscopy. Perturbation of membrane integrity was tested by measurement of melimine mediated dye release from bacteria. Additionally, mechanism of action of surface bound melimine and a shorter derivative (melimine 4) was explored.

Melimine and melimine 4 covalently-bound to contact lenses effectively reduced adhesion of clinically relevant bacterial species. In solution, melimine assumes a predominantly random coil conformation but when it is solubilised in SDS micelles, which are bacterial membrane mimetic, the α-helical content increases to approximately 40%. A major effect of melimine was on the integrity of the cytoplasmic membrane for both *P. aeruginosa* and *S. aureus*. However, for *P. aeruginosa* the rapid loss of cytoplasmic membrane integrity correlated directly with loss of cell viability, while for S. aureus maximal dye release was obtained at

concentrations where there was no significant loss of viability. There have been few studies to date investigating differences in the action of cationic peptides towards Gram-positive and Gram-negative bacteria. For covalently-bound peptides displacement of cations on bacterial surfaces and damage to cytoplasmic membrane resulted in loss of cell viability.

These results indicate that the melimine and its shorter derivative melimine 4 are excellent candidates for further development as coatings to prevent bacterial colonisation of contact lenses and biomaterials. Further, mechanistic differences between Gram-negative and Gram-positive bacteria may further inform design of novel peptides with improved broad-spectrum activity.

Chapter 1: Literature Review

1.1 An introduction to Biomaterials

Continuing progress in medical science has increased the use of biomaterials. A biomaterial can be simply defined as "any substance (other than a drug) or combination of substances synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ or function of the body." (von Recum and LaBerge, 1995). From simple use of sutures that assist in wound healing to more complex orthopaedic devices and artificial heart valves, the use of biomaterials has revolutionised patient care (Coburn and Pundit, 2007). Evolved over a period of more than fifty years, the modern biomaterial industry has a turnover of more than $100 billion each year (Ratner and Bryant, 2004).

Earliest forms of biomaterials, dating back to more than 2000 years, included artificial teeth made up of gold or wood, glass eyes, and sutures made from animal sinew (Langer and Tirrell, 2004; Ratner *et al*, 1996; Coburn and Pundit, 2007). Tremendous progress has been made since that time to accommodate other materials including polymethylmethacrylate (PMMA) in dentistry, Dacron in vascular grafts and stainless steel and PMMA in total hip replacements (Peppas and Langer, 1994; Ratner *et al*, 1996). Today, biomaterial science is not only about the type of material and the site where it could be implanted, but also about

the input from wide range of disciplines including but not limited to chemistry, biology, bioengineering, material science, mechanics and surface science (Ratner and Bryant, 2004).

1.2 Biomaterial associated infections (BAI)

Undoubtedly, the extensive use of biomaterials has resulted in significant improvement in quality of life for patients. However, biomaterial associated infections (BAI) are still a vital concern despite progress in biomaterial manufacturing technology combined with the elimination of risk factors such as contamination during implantation, preventive antibiotic therapy and prompt treatment of peripheral infections (Campoccia *et* al, 2006; Qiu *et al*, 2007). Darouiche, (2007) suggested that of the two million nosocomial infections that occur in the United States (US) annually, almost half were device-related. A survey of medical intensive care units (ICUs) in the US conducted between 1992 and 1997 found 95% of urinary tract infections were catheter-related while 87% of primary bloodstream infections were associated with central lines (Richards *et al*, 1999).

Device-related infections were found to be significantly higher in ICUs from other parts of the world compared to ICUs from US (US ICUs). Rosenthal *et al*, (2008) in their surveillance study from 2002 through 2007 on International Nosocomial Infection Control Consortium (INICC) that comprised of 98 ICUs of 19 countries from Latin America, Asia, Africa and Europe, found approximately 2-3-fold higher rates of central-line associated bloodstream infections (9.2 cases per 1000 device-days) as compared to the US ICUs (2.4 – 5.3 cases per 1000 device-days). Additionally, ventilator-associated pneumonia (19.5 cases per 1000 ventilator-days for INICC vs. 1.1–3.6 cases per 1000 ventilator-days for US ICUs) and

catheter-associated urinary tract infections (6.5 cases versus 3.4–5.2 cases per 1000 catheter-days) were also significantly higher as compared to US ICUs. In a similar study, Rosenthal *et al*, (2006) compared the rates of device-associated infections from 2002 through 2005 from 55 ICUs of 8 developing countries (DC ICUs) with that of the US ICUs and reported higher rates of central-venous catheter related bloodstream infections (12.5 cases for DC ICUs per 1000 devices-days vs. 4.0 cases for US ICUs per 1000 devices-days), ventilator-associated pneumonia (24.1 cases per 1000 ventilator-days for DC ICUs vs. 5.4 cases per 1000 ventilator-days for US ICUs) and catheter-associated urinary tract infections (8.9 cases for DC ICUs per 1000 devices-days vs. 3.9 cases for US ICUs per 1000 devices-days) from ICUs from eight developing countries as compared to ICUs from the US.

Among contact lens wearers, microbial keratitis (MK) is a severe complication affecting approximately 5 cases per 10,000 lens wearers (Poggio, *et al*, 1989; Cheng *et al*, 1999; Lam *et al*, 2002). It has been reported that the occurrence of MK rarely happens in healthy eyes (Coster and Badenoch, 1987) and contact lens wear is the primary risk factor associated with MK (Dart *et al*, 1991; Erie *et al*, 1993). Although the incidence of lens-associated MK is rare, its impact is devastating and could result in irreparable visual loss from corneal scarring or perforation (Dart, 1988). Importantly, the risk and the incidence of MK have been reported to be independent of the type of contact lenses (Dart *et al*, 2008; Stapleton *et al*, 2008). Dart *et al*, (2008) found no difference in the relative risk of MK for daily-disposable and silicone hydrogel contact lenses, while Stapleton *et al*, (2008) in their 12-month, prospective, surveillance study reported no reduction in incidence of MK by newer types of soft contact lenses as compared to the old types.

The cost to the community of BAI is high, with total additional expenses exceeding $US11 billion per annum for biomaterial infections (Henderson and Levy, 1997). For example, the cost for replacement of just one type of device, a central venous line, as a result of device infection is $US 14,000 per patient or approximately $US 5.6 million per annum in the USA alone (Thomas *et al*, 2006). Consequently when all implantable device types are considered, the costs to the community are enormous. These figures consider only the direct medical costs to the community and not costs associated with reduced work productivity or quality of life. The incidence of microbial keratitis (infection of the cornea) due to contact lens wear is around 25,000–300,000 cases annually, and the cost of treatment as a result has been estimated to be between $15 and $30 million (Khatri *et al*, 2002). In Australia, costs associated with single cases of microbial keratitis (MK) were reported to be AU$5515 (2784 to 9437) for severe cases with vision loss, AU$1596 (774 to 4888) for severe cases without vision loss, and AU$795 (527 to 1234) for the mild MK (Keay *et al*, 2008). With around 140 million contact lens wearers world-wide, the cost to treating this disease is large.

1.3 Microorganisms implicated in BAI and their sources

A wide variety of microorganisms including bacteria, fungi, protozoa and viruses are involved in BAI (Rimondini *et al*, 2005). The most frequent group of microorganisms implicated in BAI is bacteria particularly Gram-positive *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and species belonging to the viridans *Streptococci* group; and Gram-negative *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumonia* (Donlan, 2001; Hall-Stoodley, *et al*, 2004; Rimondini *et al*, 2005).

The sources of these organisms vary. For example, sutures, vascular catheters and orthopaedic devices are most commonly infected at the skin insertion site predominantly by Gram-positive bacteria, primarily *S. epidermidis* and *S. aureus* (Maki *et al*, 1997; Costerton *et al*, 1999; Yorganci *et al*, 2002). Another skin contaminant, *Candida albicans* along with environmental contaminants such as *Aspergillus* species have been reported as a common cause of persistent fungal infections in artificial heart valves (Costerton *et al*, 1999).

Dental implants could be potentially colonised by the water system in the dental unit: Walker *et al* (2004), found high levels of microbial contamination (including *P. aeruginosa, Lagionella pneumophila* and *Candida* species) in substantial proportions of dental unit water systems across seven European countries. These organisms along with hepatitis virus could become a potential source of contamination and subsequent infection in dental implants (Rimondini *et al*, 2005).

The most frequently isolated bacteria from corneal ulcers among contact lens wearers include *P. aeruginosa* followed by S. *aureus* and *S. epidermidis* (Alfonso *et* al, 1986; Cohen *et al*, 1991; Costerton *et al*, 1999; Green *et al*, 2008). Contact lens-related fungal keratitis has been attributed to environmental contaminant *Fusarium* species (Alfonso *et al*, 2006), while *Acanthamoeba*, another environmental contaminant, has been implicated in significantly increased cases of keratitis in recent years with a high proportion among contact lens wearers (Ku *et al*, 2009).

1.4 Significance of *S. aureus* and *P. aeruginosa* in contact lens-related infections

Among bacteria, *P. aeruginosa* and *S. aureus* are of major significance in contact lens related infections due to their ability to produce a variety of virulence factors that help their initial adhesion to lens material and subsequent penetration into corneal epithelium (Wu *et al*, 1999; Willcox and Hume, 1999; Vallas *et al*, 1999). Pili and flagella produced by *P. aeruginosa* help initial adhesion of the bacteria to contact lenses and host epithelium, while enzymes including alkaline protease and elastase assist in migration of bacteria by degrading extracellular matrix molecules (Nicas and Iglewski, 1986; Gupta *et al*, 1994; Feldman *et al*, 1998; Nordlund and Pepose, 2005). Additionally, a number of proteins produced by the bacteria such as leukocidin and phospholipase C destroy key precursors of inflammatory cytokines thereby negating the host immune response (Nicas *et al*, 1986; Nordlund and Pepose, 2005). These factors combined with the ability of bacteria to develop resistance to antibiotics makes treatment even more difficult (Nordlund and Pepose, 2005). Based on their interactions with the corneal epithelial cells, *P. aeruginosa* strains have been further subdivided into invasive and cytotoxic strains (Fleiszig *et al*, 1996; 1997). Invasive strains have the ability to invade and reside in the host epithelial cells (Fleiszig *et al*, 1994; 1995), while cytotoxic strains remain extracellular and induce death of various mammalian cells (Evans *et al*, 1998).

Although *S. aureus* can be isolated from conjunctiva of healthy individuals, it can initiate an infection in case of any breaks in epithelial layers (Nordlund and Pepose, 2005). Some of the most important virulence factors of *S. aureus* as reviewed by Nordlund and Pepose, (2005)

include: hyaluronidase that breaks down hyaluronic acid (a key component of connective tissue in the cornea) and hence promotes spread of bacteria; coagulase that produce plasma clots and thereby reduce the access of white blood cells to the site of infection; catalase that hydrolyse hydrogen peroxide a key component used by phagocytic cells for oxidative killing of bacterial cells; leukocidin that kills leucocytes; and hemolysins that enhance the availability of iron for bacterial growth by degrading red blood cells (Nordlund and Pepose, 2005). Additionally, fibronectin binding proteins that assist in invasion of human corneal epithelial cells is also another important virulence factor (Jett and Gilmore, 2002). Finally, *S. aureus* also produce variety of exotoxins, such as α toxin that is cytotoxic and produce necrosis and apoptosis of corneal epithelial cells (Callegan *et al*, 1994; Moreau *et al*, 1997).

1.5 Importance of biofilms in BAI

One of the major causes of device related infections is the ability of bacteria to form surface-associated biofilms (Mack *et al,* 2006). Although biofilm formation was suggested in the fossil record dating back approximately 3.25 billion years, so far, a complete understanding of this form of bacterial growth is yet to be fully accomplished (Hall-Stoodley *et al*, 2004). Donlan and Costerton (2002) define biofilm as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription."

The first step in biofilm formation is attachment of bacterial cells to the surface of a biomaterial and is largely dependent on the bacterial load, the rate at which cells move over a surface and the physical as well as chemical characteristics of the surface (Donlan, 2001; Subramani *et al*, 2009). The second step involves production of extracellular polysaccharides by the bacteria that ultimately result in the formation of biofilm. The growth of biofilm is dependent on the availability of nutrients, concentration of antibacterial drug if any, velocity of shear forces and the surrounding temperature (Donlan, 2001; Donlan and Costerton, 2002).

Bacteria residing within biofilms behave differently to planktonic cells towards environmental stressors such as depletion of nutrients, temperature shifts, pH, and osmolarity (Lewis, 2001; Novick, 2003). Importantly, they become more resistant to antimicrobial compounds including antibiotics, disinfectants and germicides (Hill *et al*, 2005; Donlan and Costerton, 2002). The mechanism by which bacterial cells resist antimicrobial action could be one or a combination of: (a) difficulty of penetration through the matrix resulting in a late penetration of antimicrobial compound, (b) a slower growth rate of bacteria associated with biofilm than the planktonic cells that result in slow uptake of antimicrobial agents, (c) changes in gene expression that leads to sub-populations of phenotypes resistant to antimicrobial agents and (d) other alterations of physiological nature caused by cells growing in biofilm such as high local concentrations of catalase produced by *P. aeruginosa* to neutralize toxic levels of hydrogen peroxide (Stewart *et al*, 2000; Donlan and Costerton, 2002; Hall-Stoodley *et al*, 2004).

1.6 Current measures to prevent BAI

The current measures to prevent BAI include:

- Antisepsis
- Physical modifications of biomaterial surfaces
- Use of antibiotics

1.6.1 Antisepsis

The most important consideration in prevention of BAI is that at the time of its insertion in the body it has to be free of all types of microorganisms (Costerton, 2005; Schierholz and Beuth, 2001). However, despite attempts to maintain high levels of sterility in operating theatres it is not possible to create a predictably sterile wound, even under unidirectional air flow conditions (Nelson, 1979). Potentially pathogenic bacteria such as *Staphylococcus aureus* can be recovered from 90% of clean wounds at the time of closure (Schierholz and Beuth, 2001). Consequently, even in the most carefully controlled environments, low numbers of bacteria are introduced to the implant site. These numbers have been estimated to be in the order of 10^2 - 10^5 cfu/cm^2, but are strongly influenced by environmental factors including the number of personnel present in the operating theatre and the complexity of the surgical procedure (Fitzgerald, 1979; Gristina, 1987; Subbiahdoss, *et al*, 2009). This problem is compounded by alterations in host defences associated with the peri-implant region (Gristina, 1987; Zimmerli *et al*, 1984; Kaplan *et al*, 1992) which result in increased susceptibility to infection as bacteria may not be cleared from the peri-implant tissue (Broekhuizen *et al*, 2007).

1.6.2 Physical modifications of biomaterial surfaces

Another way of reducing bacterial contamination is to physically modify the biomaterial surfaces. This can be done in a variety of ways and includes functionalized surfaces, polishing of the surfaces to reduce surface roughness, modifying surfaces with coatings such as polyethylene oxide (PEO) or polyethylene glycol (PEG) coatings and altering the surface-free energy (Flemming *et al*, 2000; Rimondini *et al*, 2005; Qiu *et al*. 2007; Harbers *et al*, 2007; Norowski and Bumgardner 2009). Surface free energy can be estimated using a contact angle goniometer and is observed as their wetting behaviour towards various solvents. Surfaces that are strongly hydrophilic represent one example of alteration in surface energy. These surfaces by means of their hydrophilicity rapidly develop a monolayer of water molecules. Attachment of bacteria is disrupted because of their inability to form hydrogen bonds with the water molecules (Norowski and Bumgardner 2009).

PEO or PEG coatings are strongly hydrophilic and form a thin hydrous layer that repel bacteria and other proteins (Norowski and Bumgardner 2009; Qiu *et al*, 2007). Titanium surfaces coated with PEG have been reported to substantially reduce the adhesion of many bacteria including *S. aureus* and *P. aeruginosa* (Park *et al*, 1998; Razatos *et al*, 2000). Harris *et al*, (2004) demonstrated that poly (l-lysine)-grafted-poly (ethylene glycol) (PLL-g-PEG) functionalized with RGD (Arg–Asp–Gly) type peptide (PLL-g-PEG/PEG-RGD) inhibited bacterial adhesion whilst maintaining fibroblast and osteoblast attachment which is necessary for integration of implants with the host tissues. On the other hand, PLL-g-PEG without functionalized RGD type inhibited both bacterial as well as fibroblast and osteoblast gowth (Harris *et al*, 2004).

However, the study did not take into account the long-term stability of PEG-coated implants as surfaces coated with that strategy have been reported to resist protein deposition and cell adhesion for only 8 – 12 days (VandeVondele *et al*, 2003). Another major issue is that the wound healing process after implantation results in the formation of granulation tissue followed by fibrous encapsulation of the implant that consist of monocyte-derived macrophages which could fuse into foreign-body giant cells that have phagocytic as well as degradative properties leading to implant damage and subsequent failure (Ziats *et al*, 1988; Zhao *et al*, 1991).

Biomaterials surfaces could also be altered to change their surface charge. Methacrylate polymers and copolymers with varying charges have been found to have antimicrobial effects (Harkes *et al*, 1991; Gottenbos *et al*, 2001; 2002). Harkes *et* al, (1991) found the greatest adhesion of bacteria to surfaces that were positively charged and lowest adhesion on surfaces that were negatively charged. However, Gottenbos *et al*, (2001; 2003) found positively charged surfaces with antimicrobial properties towards bacteria. They reported that although bacteria with anionic surfaces readily attached to positively charged surfaces by electrostatic attraction, further growth was severely impeded because of the very strong adhesive nature of positively charged surfaces (Gottenbos *et al*, 2001; 2003).

1.6.3 Use of Antibiotics

Antibiotics have been used extensively to prevent BAI in various forms including: antibiotic releasing coatings, antibiotic dipping, antibiotic covalently-bound to biomaterial surfaces and the use of antibiotics in more conventional oral or parenteral forms (Jose *et al*, 2005; Costerton, 2005; Darouiche, 2007). Various reports have linked reduction in BAI to

antibiotics released from coatings that contained single or combination of antibiotics such as vancomycin, rifampin, minocycline, nalidixic acid and teicoplanin (Hamilton *et* al, 1997; Hampl *et* al, 1995, 2003; Han *et al*, 2005; van de watering and van Woensel, 2003; Darouiche *et al*, 2007). Limited information is available on the successful use of antibiotic dipping method as means of reducing BAI. In one report, Actis Dato *et al*, (1992) found significantly reduced rates of prosthetic valve endocarditis among those valves that were dipped in antibiotic solution as compared to undipped valves.

Systemic administration of antibiotics prophylactically has been found to be beneficial before implantation of various biomaterials (Bengtson *et al*, 1989; Blackburn and Alarcon, 1991; Lucke *et al*, 2005) including internal shunts (Ratilal *et al*, 2008). Boxma *et al*, (1996) conducted a randomised controlled trial of antibiotic prophylaxis in surgical treatment of fractures and reported significant reduction in fracture-associated infections with administration of a single-dose broad-spectrum prophylactic antibiotic. Recently, Darouiche *et al*, (2009) demonstrated superior efficacy of therapeutic doses of telavancin, an antibiotic that belongs to lipoglycopeptide class, to vancomycin in prevention of subcutaneous implant infection by *S. aureus*.

However the use of antibiotics in different forms as mentioned above has its own drawbacks. A major problem with antibiotic release coatings is the fact that after a rapid onset, the release of antibiotics slows down over time to levels below therapeutic concentrations, potentially allowing the development of resistant strains of bacteria (Norris *et* al, 2005; Costerton, 2005). The duration of antimicrobial effects for antibiotic dipping is even shorter than the antibiotic release coatings as very little antibiotic is actually available on the surface

by this approach, resulting in antimicrobial activity for no more than a few hours (Darouiche, 2007). Similarly, some studies suggest a failure of systemic prophylactic antibiotics to cause a significant reduction in the incidence of BAI (Yerdel *et al*, 2001; Jensen *et al*, 1985).

1.7 Other promising antimicrobial substances

As there are a number of drawbacks to the use of antibiotics a great deal of research has been directed towards the development of alternatives for the reduction of BAI. Some of the most promising of these antimicrobial compounds that have shown to be successful in reducing adhesion and/or colonization of bacteria to biomaterials include: silver; chlorhexidine; triclosan; and benzalkonium chloride.

1.7.1 Silver

Silver has been extensively explored for antibacterial activity when coated on biomaterials (reviewed by Monteiro *et al*, 2009). It has been reported to be effective when coated to percutaneous silver wire implants (Spadaro *et al*, 1986), urinary catheters (Johnson *et al*, 1990), dialysis catheters (Tobin and Bambauer, 2003), prosthetic heart valves as silver threads (Shevchenko *et al*, 1999) and silver-coated endotracheal tubes (Olson *et al*, 2002). The disadvantages of using silver coated implants include allergy (Holmstrup, 1991; McKenna *et al*, 1995) and its potential to cause chronic inflammation (Tozzi *et al*, 2001). The latter resulted in the withdrawal of the Silzone heart valves from the market in 2000 (Tozzi *et al*, 2001). An interesting development in the contact lens field has been the commercial release of a contact lens storage case (the vessel in which lenses are disinfected when not

worn over night) that has silver incorporated into it. Amos and George, (2006) have reported that use of one of the commercially available silver lens cases resulted in only 26% of cases being contaminated whereas 67% of non-silver cases were contaminated.

1.7.2 Chlorhexidine

Chlorhexidine impregnated dressings have been reported to prevent vascular and epidural catheter colonization and infection (Ho and Litton, 2006) and prevent colonization of central venous catheters in infants and children (Levy *et al*, 2005). In addition, central venous catheters impregnated or coated with chlorhexidine in combination with silver-sulfadiazine, have been found to be effective in reducing colonization of bacteria (Jaeger *et al*, 2005; Ostendorf *et al*, 2005). However, effectiveness of chlorhexidine is questionable as Sherertz *et al*, (1996) in a randomized double-blind trial reported that chlorhexidine-coated triple-lumen catheter was inefficacious in preventing infection. In another study, Harnet *et al*, (2009) found that overnight soaking of suture material in chlorhexidine did not prevent colonization by *E.* coli. Another disadvantage of using chlorhexidine is its potential to induce anaphylaxis (Terazawa *et al*, 1998; Stephens *et al*, 2001).

1.7.3 Triclosan

Triclosan belongs to the bis-phenol group and is particularly active against Gram-positive bacteria (Savage, 1971). Most commonly, it has been used in surgical hand wash solutions (Boyce and Pittet, 2002). With reference to prevention of BAI, triclosan has been shown to be effective as a coating for sutures (Ford *et al*, 2005; Edmiston *et al*, 2006), impregnated stent segments (cadieux *et al*, 2006) and urinary catheters (Jones *et al*, 2006). A major concern

with the use of triclosan is bacterial resistance as there have been reports of triclosan resistance emerging in medically important bacteria such *S. aureus* (Fan *et al*, 2002), methicillin-resistant *S. aureus* (Seaman *et al*, 2007; Bayston *et al*, 2007) and *E. coli* (McMurry *et al*, 1998).

1.7.4 Benzalkonium chloride

Benzalkonium chloride, a cationic surface-active agent that belongs to the quaternary ammonium group has been used as an antimicrobial compound since 1935 (Block, 1991) and is commonly used preservative in ophthalmic preparations (Pisella *et* al, 2000; Noecker, 2001). It has been explored for its potential to prevent BAI and the results of one study using a benzalkonium chloride impregnated polymer suggested its value in the prevention of biliary stent blockage (Rees *et al*, 1998). It has also been used as a coating on internal and external surfaces of central venous catheters causing significant reduction of the incidence of bacterial colonization (Moss *et al*, 2000). Additionally, central venous catheters impregnated with benzalkonium chloride have also been shown to be effective in reducing bacterial adhesion (Tebbs and Elliott, 1993).

There is a growing concern about the increasing incidence of benzalkonium chloride - resistant microorganisms. For instance, *P. aeruginosa* has shown adapted resistance to benzalkonium chloride and interestingly, resistant cells exhibited co-resistance to other quaternary ammonium compounds such as cetylpyridinium chloride and cetrimide (Loughlin *et al*, 2002). Further, benzalkonium chloride has also been associated with cytotoxicity (Baudouin, 2008).

Why there is a need to develop alternative approaches to prevent BAI?

The current preventive measures, alone or in various combinations, are not enough to prevent BAI as even the most carefully implanted devices are contaminated by small numbers of bacteria (Schierholz, and Beuth, 2001). There is a growing need to find suitable alternatives for the prevention of BAI. One emerging area of research to address this need is the use of antimicrobial peptides: a new class of therapeutic antibiotics.

1.8 Antimicrobial peptides

Antimicrobial peptides (AMPs) are widely distributed in nature. They have been isolated from primitive forms of life such as microorganisms and invertebrates and from higher forms such as plants and mammals including humans (Jenssen *et al*, 2006). They are a key component of the innate immune response. Most of them are relatively short (12-50 amino acid residues) with a net positive charge and are amphiphilic (Hancock and Chapple, 1999; Hancock and Diamond 2000; Jenssen *et al*, 2006; Brown and Hancock 2006). The spectrum of activity of these peptides ranges from bacteria (including Gram-positive and Gram-negative) to fungi (Zasloff, 2002), protozoa (Aley *et al*, 1994; Rivas *et al*, 2009) and some viruses including HIV (Masuda *et al*, 1992; Murakami *et al*, 1991).

The role of these peptides is not limited to their wide range of antimicrobial activity but they also act as effector molecules (Ganz, 2003; Brown and Hancock 2006), that result in enhanced expression of chemokines and integrins, increased phagocytosis and recruitment of immune cells at inflammation sites (Agerberth *et al*, 2000; Zasloff *et* al, 2002; Yang *et al*,

2004). They have also been shown to accelerate angiogenesis (Steinstraesser, *et al*, 2006). These immunomodulatory effects may occur at levels below that required for antimicrobial activity (Niyonsaba et al, 2007).

1.8.1 Structure and classification of AMPs

To date more than 1200 AMPs have been isolated from various sources (Wang *et al* 2009). There are four major classes of AMPs: α helical; cysteine-containing or β sheet; loop; and extended structures peptides (Hancock, 1997; 2001). Each will be discussed briefly in the following sections.

1.8.1.1 α helical AMPs

Several hundred AMPs estimated to belong to this group have been described in plants, invertebrates and vertebrates including humans (Wang *et al*, 2009). They have been shown to kill bacteria, both Gram-positive and Gram-negative, fungi and protozoa. However, some of the AMPs from this group are lytic to eukaryotic cells (Bulet *et al*, 2004). α helical AMPs adopt a random conformation in aqueous solutions, however upon interaction with lipid surfaces such as membranes or membrane-mimic environments they assume an amphipathic α helical secondary structure (Bechinger *et al*, 1993). The activity of α helical AMPs is dependent on: net charge; ability to assume amphipathic conformations; hydrophobicity; size; and their amino acid sequence (Bulet *et al*, 2004). The ability of cerain AMPs to assume amphipathic *α* helix structures (fig. 1.1B) is important for their interaction with bacterial membranes (Marion *et al*, 1988; Yeaman and Yont, 2003).

Cecropins isolated from insects (Steiner *et al*, 1981) and magainins isolated from frogs (Simmaco *et al*, 1998; Zasloff, 2002) are two of the most frequently studied AMPs that belong to α helical class. Generally, cecropins have two important characteristics: a tryptophan residue at position one or two; and a C-terminal amidated residue. The former confers activity towards both Gram-positive and Gram-negative bacteria while the latter strengthens the stability and increases the cationicity of the peptide (Bulet *et al*, 2003; 2004).

Melittin, isolated from the venom of the European honey bee *Apis mellifera* and composed of 26 amino acid residues (NH2-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH2) is another important peptide that belongs to α helical group (Habermann, 1972). Although predominantly hydrophobic, it has a net charge of +6 at physiological pH due to the presence of 2 residues each of lysine and arginine at C-terminus and 1 residue of lysine at N-terminus. When aligned in an α helical configuration, melittin has an asymmetric distribution of polar and non polar amino acids that makes it amphipathic (figure 1.2; Dathe and Wieprecht, 1999). At low ionic strength and low physiological pH, melittin adopts a random coil conformation, while high peptide concentration and/or high ionic strength at neutral pH promotes its self association into a tetrameric structure due to the formation of a hydrophobic core (Talbot *et al*, 1979). Self association of melittin has also reported to be favoured by reduction in electrostatic repulsion between the positive charges that occurs at high pH and/or high ionic strength (Wilcox and Eisenberg, 1992).

Melittin has been shown to disrupt the barrier function of membranes. It has also been reported to induce channel formation in plannar bilayers (Dempsey, 1990). Similar to

detergents, permeabilization of membranes by melittin at high concentrations has been shown to cause the breakdown of membranes into micelles (Raghuraman and Chattopadhyay, 2007).

However, melittin is highly haemolytic even at sub-micromolar concentrations with the ability to bind to and rupture erythrocytes very rapidly (Dempsey, 1990). The dissociation constant for melittin varies between 10^5 and 10^7 M with approximately 1.8×10^7 binding sites per erythrocyte (Lee *et al*, 2001; Degrado *et al*, 1982).

Other important members of AMPs that produce some form of α helices include protamines and poly-L-arginine. Protamines belong to a group of small proteins (molecular weight: 4000 – 8500) that are strongly basic due to the presence of approximately 70% of arginine residues mainly in clusters (Ando *et al*, 1973). One of the main functions of the protamines includes condensation of sperm chromatin and protection of deoxyribonucleic acid (DNA) from enzymatic degradation (Hud *et al*, 1994). Removal of protamines from the native protamine-DNA complex results in denaturation of the DNA due to absence of electrostatic forces that exist between positively-charged arginine in protamine and negatively-charged phosphoric acids in DNA (Raukas and Mikelsaar, 1999). Although protamine exhibits a random coil conformation in aqueous solutions, there is a clear evidence that up on binding to transfer-ribonucleic acid (tRNA), it assumes secondary structures that consists of α helices bound to a shallow groove in the double helical portion of tRNA (Warrant and Kim, 1978). The helical regions in a protamine molecule correspond to arginine-rich clusters (Raukas and Mikelsaar, 1999).

Protamine has broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria (Johansen *et al*, 1995; Aspedon and Groisman, 1996). It lacks the ability to assume amphipathic structures required for channel formation in bacterial cells. The protamine-mediated killing is attributed to disruption of cytoplasmic membrane function without cell lysis (Aspedon and Groisman, 1996).

Poly-L-arginine remains unstructured in aqueous solutions due to strong forces of electrostatic repulsion. Ordered structure of the polymer is induced by the addition of alcohol, salt or surfactant to produce electrostatic shielding and/or damage to the hydration shell. Seno *et al*, (1984) using an anionic surfactant, bis (2 ethylhexyl) sodium sulfosuccinate (AOT) revealed that poly-L-arginine conformed to α helical structure upon addition of AOT. However, further addition of AOT resulted in the formation of β structure.

1.8.1.2 Cysteine-containing or β sheet AMPs

The AMPs belonging to this class contain cysteine residues that form one or more disulfide bonds (Hwang and Vogel, 1998; Bulet *et al*, 2004). The number of cysteine residues and their pairing determine the final conformation of the peptide as either a β-sheet formed by three disulfide bonds (mostly seen in vertebrate defensins) or β- hairpin-like structures that result from the formation of two disulfide bonds (mostly arthropod defensins), or mixed structure containing both α helix and β sheet that result from the formation of two disulfide-linked α helices and two antiparallel β sheets (mostly invertebrate and plant defensins; Hancock and Lehrer, 1998; Bulet *et al*, 2004).

Among AMPs that form β sheets, defensins are the best characterised (fig. 1.1A). They are composed of 29-34 amino acids with high frequency of arginine (Boman, 1995). Although defensins are found to be active against both Gram-positive and Gram-negative bacteria, the antibacterial activity seems to be greater against Gram-positive bacteria (Lehrer *et al*, 1993; Boman 1995). They are only active at physiological concentrations of NaCl (150 mM; Harder *et al*, 2001) and their antimicrobial activity is inferior to cecropins (Boman, 1995).

1.8.1.3 AMPs with loop structure

The AMPs from this class form one intramolecular disulfide bond mostly at the C-terminus that results in a loop structure within the molecule which contains as few as seven amino acid-residues and a longer tail (Boman, 1995). Bactenecin (fig. 1.1C) was the first AMP reported from this class (Romeo *et al*, 1988). Isolated from bovine neutrophils, bactenecin (also called bovine dodecapeptide) is a small peptide composed of 12 amino acids including 4 arginine and 2 cysteine residues (Romeo *et al*, 1988; Wu and Hancock, 1999). The loop is made up of nine amino acids held together by a single disulfide bond with one residue on one side while two on the other (Romeo *et al*, 1988; Boman *et al*, 1995). The peptide is active against both Gram-positive and Gram-negative bacteria (Boman *et al*, 1995). Other AMPs that form loop structures include brevinins, esculentins and ranalexins (Morikawa *et al*, 1992; Clark *et al*, 1994)

1.8.1.4 Extended structures peptides

Extended structures peptides have a peculiar amino acid composition with certain regular amino acids in unusually high proportion. This characteristic pattern of amino acids results in a peptide structure that is different to α helix or β sheet (Brewer *et al*, 1998; Tsai and Bobek,

1998). For example, the anticandidal peptide histatin is very rich in histidine residues (Xu *et al*, 1991) while two peptides isolated from bovine neutrophils, Bac5, and Bac 7, and one peptide from porcine neutrophils, PR-39, are high in proline (45-49%) and arginine (24-29%) residues (Gennaro *et al*, 1989; Frank *et al*, 1990; Agerberth *et al*, 1991; Boman 1995). All three proline and arginine rich peptides (Bac5, Bac7 and PR-39) have demonstrated antibacterial activity against Gram-negative bacteria (Boman, 1995).

Another AMP indolicidin (fig. 1.1D) has been reported to have abundant tryptophan residues, a trait that is not common among other peptides or proteins (Selsted *et al*, 1992). The importance of tryptophan lies in its ability to partition peptides into membranes due to its tendency to place itself near the membrane/water interface (Kachel *et al*, 1995, Persson *et al*, 1998). Although indolicidin may assume a random conformation in solution, upon interaction with phospholipids that random conformation changes to a structured one, thereby significantly improving the peptide's ability to interact with the bacterial membranes (Ladokhin *et al*, 1997; 1999).

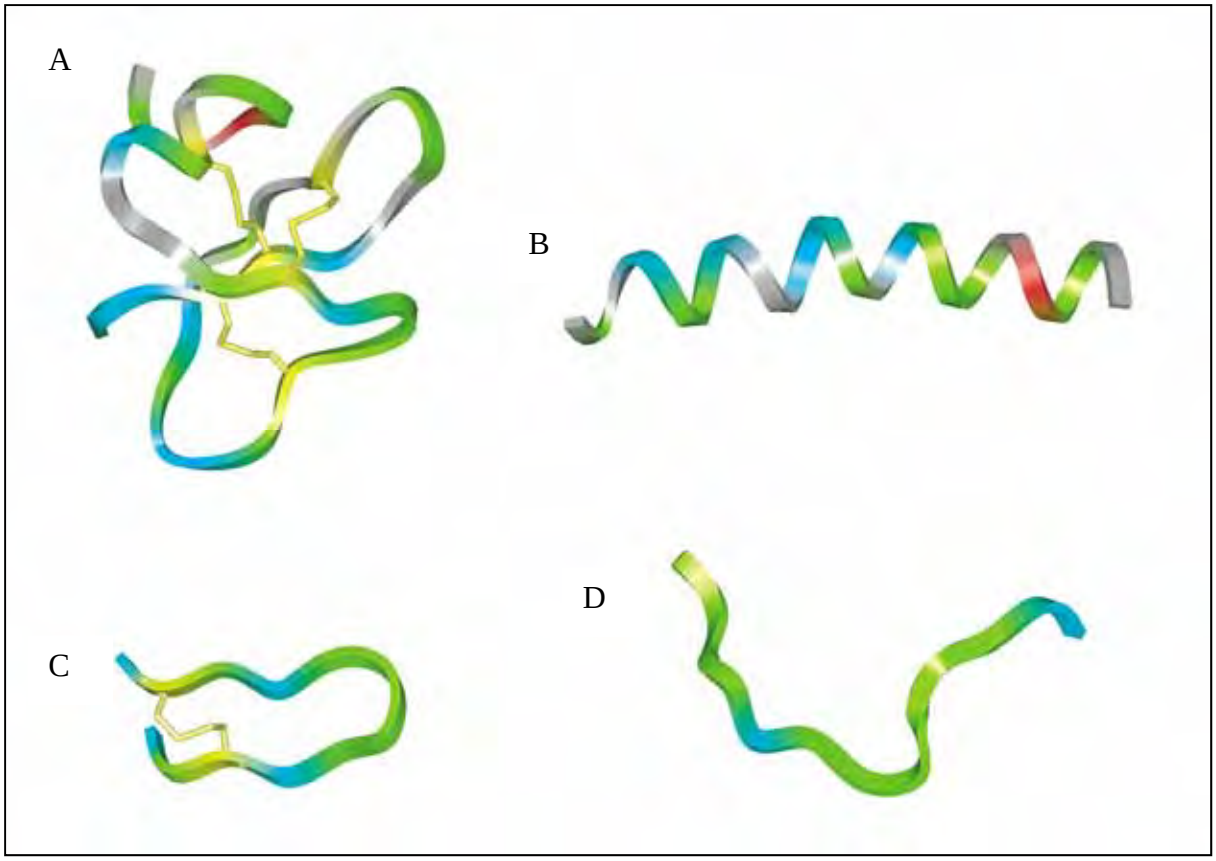


Figure 1.1: Molecular models of different structural classes of AMPs (taken from Hancock, 2001). All models are based on either two-dimensional nuclear magnetic resonace spectroscopy of the peptides in aqueous solutions for human β-defensin-2 (HBD-2) or a membrane mimetic condition (for other peptides). (A) HBD-2, which forms a triple-stranded β-sheet structure (containing a small α-helical segment at the N-terminus) stabilised by three cysteine disulphide bridges. (B) The amphipathic α-helical structure of magainin 2. (C) The β-turn loop structure of bovine bactenecin. (D) The extended boat-shaped structure of bovine indolicidin. The backbone structures are shown with the charged regions in blue and the hydrophobic residues in green.

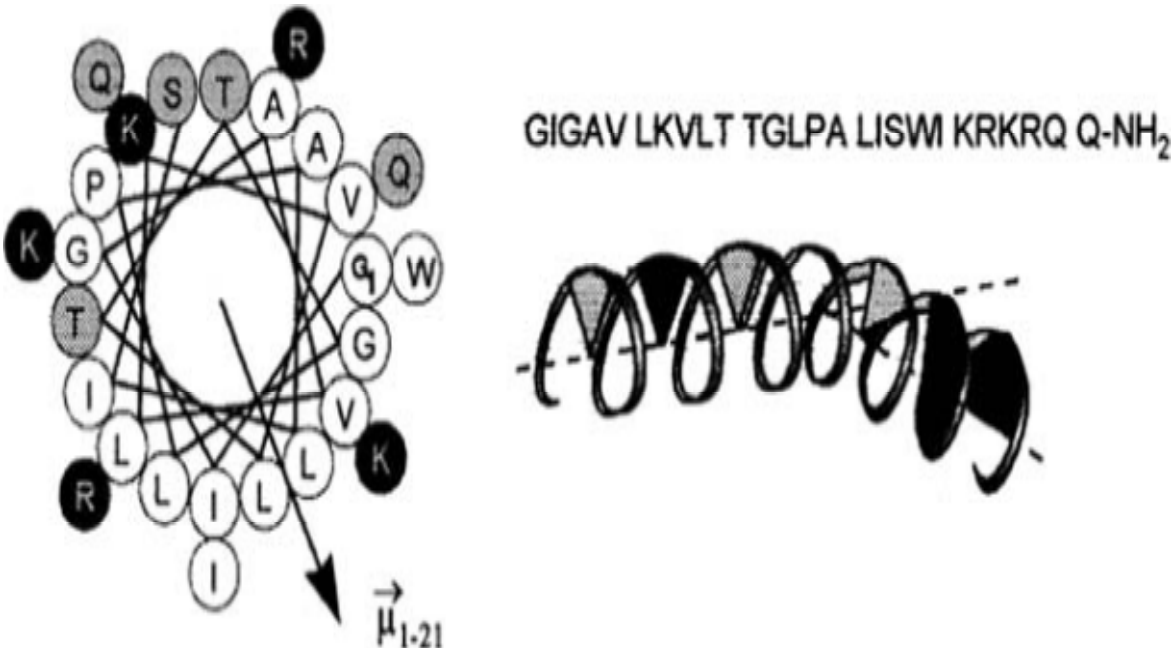


Figure 1.2: Helical wheel projection and schematic representation of the amphipathic helix of melittin showing various structural features. The one letter code for amino acids is used. Hydrophobic residues are shown in white, polar residues in gray and cationic residues in black circles. The hydrophobic moment (l) for the a-helical region is shown. Taken from (Dathe and Wieprecht, (1999) and modified by Raghuraman and Chattopadhyay (2007)

1.8.2 Mechanism of action of AMPs

A considerable amount of work has been done over the years to discern the exact mechanism by which AMPs exert their antimicrobial effects and it is generally agreed that an important element of their activity is interaction with the cell membrane (Falla *et al*, 1996; Wu *et al*, 1999; Shai, 2002). Electrostatic forces govern the interaction of peptides which have a net positive charge and bacterial membranes which contain anionic components such as phosphate groups in the lipopolysaccharides of Gram-negative bacteria, or lipoteichoic acid in the case of Gram-positive bacteria. In addition, in Gram-negative bacteria antimicrobial peptides may cause local disruption of the outer membrane through 'self promoted uptake' which describes the displacement of magnesium ions that partially neutralize the negative charge of the outer membrane and pave the way for the AMPs to interact with anionic

lipopolysaccharides causing its distortion. The whole process causes a destabilization of the outer membrane that result in uptake of peptide by the otherwise impermeable outer membrane and its subsequent accessibility to the cytoplasmic membrane (Wu *et al*, 1999; Zhang *et al*, 2000).

The next step for interaction of AMPs with both Gram-positive and Gram-negative bacteria involves the permeabilisation and/or translocation of peptide across the membrane (Wu *et al*, 1999; Zhang *et al*, 2000). The last step is the disruption of the cytoplasmic membrane permeability barrier either by the complete lysis of the cell caused by membrane rupture or by changes in the lipid bi-layer of the membrane resulting in the leakage of vital cell components and subsequent cell death (Wu *et al*, 1999; Zhang *et al*, 2000; 2001).

Various models have been proposed to explain the steps that lead to membrane permeabilisation and subsequent cell death. These models include the barrel-stave, carpet, and toroidal pore models (reviewed by Brogden, 2005; Sato and Feix, 2006).

1.8.2.1 The barrel-stave model

First described by Ehrenstein and Lecar (1977), the barrel-stave model describes the formation of pores in membrane by bundles of peptides (fig. 1.3A) that have their hydrophobic surfaces aligned with the lipid core of the membrane while their hydrophilic surfaces are aligned inwards. This results in the formation of a water-filled channel, much like the staves of a barrel (Shai and Oren, 2001; Sato and Feix, 2006). The transmembrane pore disrupts cytoplasmic membrane potential leading to leakage of intracellular material and

subsequently cell death (Sato and Feix, 2006). Alamethicin is a classic example of an AMP that forms barrel-stave channel (Bechinger, 1999).

In order to act via the barrel-stave model, the peptides must: be composed of predominantly hydrophobic residues in order to interact with the lipid core of the membrane; be able to self-associate to form bundles of transmembrane pores; be composed of a minimum of 22 residues if an α-helical peptide or 8 residues if the peptide assumes a β-sheet conformation; and be able to span the lipid bilayer (Shai, 2002).

1.8.2.2 The carpet model

In the carpet model (fig 1.3B), the peptides cover the membrane in a carpet-like fashion with their hydrophobic faces aligned parallel to the lipid bilayer while the hydrophilic face is directed towards the solvent. After reaching a threshold concentration, the peptides cause a disruption of lipid bilayer into micelles in a detergent-like manner with accompanying cell death (Pouny *et al*, 1992; Shai, 2002; Jenssen *et al*, 2006). However, unlike detergents, AMPs that act via the carpet mechanism accumulate on membranes in a high proportion and changes in membrane fluidity and/or alteration in barrier function results in membrane disruption (Yeaman, and Yount, 2003). Many AMPs have been reported to act by carpet mechanism for example, dermaseptin S (Pouny *et al*, 1992), cecropin (Shai, 1995) and melittin (Naito *et al*, 2000). Melittin has also been reported to form transmembrane pores; yet a considerably higher concentration of the peptide is required (Allende *et al*, 2005). At low concentrations, melittin forms monomeric α helices parallel to the lipid bi-layer (John and Jahnig, 1991). However, published reports contradict on the exact location of melittin in the membrane interface. Lin and Baumgaertner, (2000) in their molecular dynamics simulation

study suggested its position was on the outside of the lipid head group region while others have reported its location deep within the membrane interface (Berneche *et al*, 1998; Bachar and Becker, 2000). Hristova *et al*, (2001) using a novel X-ray absolute scale refinement method, suggested that the α helical axis of the monomeric peptide aligned itself parallel to the bilayer plane near the glycerol groups and the lipid perturbations induced by the peptide at low concentrations were modest in contrast to larger perturbations of lipid caused by dimeric forms of the peptide. Papo and Shai, (2003) using surface plasmon resonace concluded two different modes of actions for melittin on the basis of its binding with the zwitterionic and anionic membranes. They found 25-fold higher affinity of melittin to zwitterionic bilayers than to monolayers suggesting a deep insertion of the peptide in the membrane. As the interaction of melittin to zwitterionic membranes is primarily hydrophobic, the authors suggested a case for pore formation as the most likely event. On the other hand, 8.5-fold higher affinity of melittin for anionic bilayers than to monolayers suggested lack of insertion of the peptide in the bilayer and pointed towards a carpet mechanism.

Interestingly, unlike the barrel-stave model, the carpet model does not require the peptides: to be composed of predominantly hydrophobic residues; to insert into membranes to form pores; and to recognise between membrane-bound peptide monomers (Shai and Oren, 2001; Shai, 2002). Many peptides can fulfil the criteria of the carpet model as compared to the barrel-stave model due to the less stringent requirements of the former. Additionally, the carpet mechanism also explains the antimicrobial nature of hundreds of peptides irrespective of their size and sequence that fail to fulfil the criteria of barrel-stave model (Shai and Oren, 2001; Shai, 2002).

1.8.2.3 The toroidal pore model

In the toroidal pore model (fig. 1.3C), aggregates of the peptides orient themselves to be perpendicular to the membrane and induce the merging of the two leaflets of the lipid bilayer to form a curve which is continuous from top to bottom with the pore resembling that of a torus (Brogden, 2005; Jenssen *et al*, 2006). Examples of AMPs that have been reported to act via the toroidal pore mechanism include magainin (Matsuzaki *et al*, 1996), protegrin (Yamaguchi *et al*, 2002), and LL-37 (Henzler Wildman *et al*, 2003).

The toroidal pore model is different to the barrel stave model as in the former the peptides remain attached with lipid head groups at all times in such a way that they are located always towards the inner side of the transmembrane pore rather than forming the "walls" of the pore. Consequently, it has also been termed a 'supramolecular complex' because it is characterized by a transmembrane pore lined with polar surfaces of peptides and lipid head groups (Yang *et al*, 2001; Yeaman and Yont, 2003).

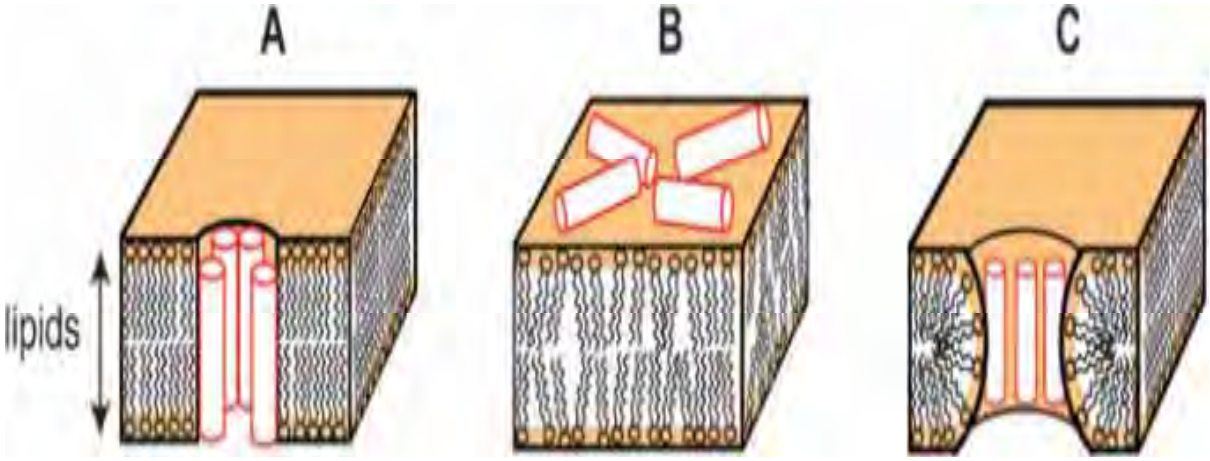


Figure 1.3: Three models of membrane disruption by antimicrobial peptides model (taken from Tang and Hong, 2009). (A) Barrel-stave model. (B) Carpet model. (C) Toroidal pore

1.8.2.4 Other mechanisms of action: Intracellular targets

Although events that follow membrane permeabilisation such as ion channels and formation of transmembrane pores as well as extensive membrane damage are enough to kill bacteria, there are growing reports that these events are not always the sole cause of cellular death (Friedrich *et al*, 2000; Brogden, 2005; Xiong *et al*, 2005; Hale and Hancock, 2007; Shin *et al*, 2009). Some of the other cellular targets that have been linked to bacterial killing include: activation of bacterial autolysins by pep5 and nisin (Bierbaum and Sahl, 1985); inhibition of cell wall synthesis by human β defensins (Sahl *et al*, 2005); inhibition of nucleic-acid synthesis by pleurocidin (Patrzykat *et al*, 2002); inhibition of protein synthesis by indolicidin (Friedrich *et al*, 2001); inhibition of enzymatic activity by histatins (Andreu and Rivas, 1998) and ixodidin (Fogaca *et al*, 2006).

1.8.3 Optimization and development of AMPs

Factors affecting antimicrobial activity

The antimicrobial activity of AMPs is dependent on several factors that include sequence length, net charge, hydrophobicity and ability to assume amphipathic structures (Dathe and Wieprecht, 1999; Tossi *et al*, 2000). An understanding of these factors and their dependence on each other combined with the ways to regulate them could be helpful to improve the antimicrobial potency of future AMPS (Tian *et al*, 2009). Truncation, substitution and deletion of amino acids are some of the approaches used to improve antimicrobial activity (Fink *et* al, 1989; Gopal *et al*, 2009; Okuda *et al*, 2009). Additionally, peptide hybridization has also been used successfully to improve antimicrobial activity (Andreu *et al*, 1992; Tian *et al*, 2009)

However, is it difficult to predict empirically the spectrum of activity and mechanism of action of a peptide, as peptides with similar secondary structures or small changes in the primary sequence can have quite different antimicrobial activities (Abrunhosa *et al*, 2005). This unpredictability may reflect a delicate balance between hydrophobicity and charge. In addition, the differing membrane potentials of bacterial cells (Ruhr and Sahl, 1985) are likely to be important and so it is probable that the mode of action may vary according to the target cell.

Reduction in size

One potential hurdle for naturally occurring AMPs to be developed further into marketable drugs is their large size, which not only makes them costly to manufacture, but also opens the door for various issues such as low bioavailability, poor stability, slow elimination and immunogenicity (Hancock, 2001; Otvos, 2008; Sharma *et al*, 2009). One way to resolve this problem is to design peptidomimetics (smaller synthetic AMPs composed of unnatural residues; Sharma *et al*, 2009). Using this approach, Shin *et al*, (2009) designed 9-mer peptide analogues of the insect defensin protaetiamycine, a 43-amino-acid-residue peptide and reported a four to eight-fold improvement in antibacterial activity without any increase in cytotoxicity. Similarly, Sharma *et al*, (2009) used synthetic peptide analogues that were based on amino acids tryptophan-histidine and histidine-arginine and found antibacterial activity against many strains of Gram- positive and Gram-negative bacteria as well as antifungal activity against one fungal strain with MIC levels of 5 – 20µg/ml.

Improvement of activity under physiological conditions

Another important consideration is the ability of AMPs to remain effective under physiological conditions since many peptides such as human β defensin 2 (Tomita *et al*, 2000), histatin 5 (Helmerhorst *et al*, 1999), lactoferricin B (Bellamy *et al*, 1992), and human cathelicidin LL-37 (Turner *et al*, 1998) are salt sensitive; their antimicrobial activity is reduced or lost at high salt concentrations. As membrane disruption of AMPs is concentration dependant and adsorption of positively charged AMPs on negatively charged membranes is optimal at low ionic strength, so under physiological salt conditions a loss in potency of AMPs is inevitable (Ringstad *et al*, 2007; Stromstedt *et al*, 2009b). For example, the antimicrobial activity of human β-defensin-1 is severely compromised in an environment of high salt concentrations such as those present in lungs of cystic fibrosis patients (Goldman *et al*, 1997). One possible way to overcome this problem is to increase overall hydrophobicity of the AMP. However, AMPs with increased hydrophobicity have shown increased cytotoxicity towards mammalian cells (Tossi *et al*, 2000). Stromstedt *et al*, (2009b) demonstrated that the problem could be resolved by linking a tryptophan pentamer tag at the *C*-terminal end. Using the same approach, Pasupuleti *et al*, (2009) designed AMPs with increased potency at high electrolyte concentrations against *P. aeruginosa* in a contact lens as well as skin wound model. Additionally, the bulkiness and the aromatic properties of tryptophan residues prevent binding of tagged peptides to membranes containing cholesterol and hence result in limited cytotoxicity (Pasupuleti *et al*, 2009).

Park *et al*, (2004a) showed that the antimicrobial activity of the α-helical peptides under physiological salt concentrations could be improved by adding helix capping motifs at each end of α-helices. An important feature of α-helical AMPs is the presence of repeated i → i –

4 hydrogen bonds that form between each amide hydrogen group and a carbonyl oxygen group from the adjacent helical turn (Pauling and Corey, 1951). However, at the helix termini, that pattern is interrupted due to unavailability of any further turn in the helix to provide pairing for additional hydrogen bonds. Helix-capping motifs provide those alternative hydrogen bond patterns that can be paired at both *N* and *C*- termini to stabilise the α-helices (Richardson & Richardson, 1988, Presta & Rose, 1988). Park *et al*, (2004a) further tested the ability of helix-capping motifs to improve antimicrobial activity of magainin 2, a known salt sensitive peptide (Lee *et al*, 1997), and found it to be active at salt concentrations of up to 200 mM NaCl compared to magainin without the added helix-capping motif that lost its antimicrobial activity at that concentration.

Research has been conducted to determine whether the naturally occurring defensins on the ocular surface would likely be antimicrobial given the relatively high salt concentration of tears. *In vitro*, NaCl markedly attenuated, and tears almost completely inhibited the activity of human beta defensins (hBD) 1 or 2 and thymosin-beta 4, but not that of hBD-3 (Huang *et al*, 2007a). Other studies by the same group demonstrated that it was probably not only the salt concentration of tears that inhibited the action of the AMPs, but interaction of hBD-2 with mucin 5AC was also involved (Huang *et al*, 2007b). The alpha defensins of rabbit tears, neutrophil peptide (NP) 1 and 3a are also inhibited by tears, although NP-1 did retain some activity in 70% v/v tears (McDermott *et al*, 2006). However, the cathelicidin LL-37 retains its anti-*P. aeruginosa* activity in human tears (Huang *et al*, 2006).

Enhancement of cell-selectivity

Reduction in cytotoxicity to host cells is another important goal for the successful development of AMPs (Chen *et al*, 2005). Numerous studies have correlated high amphipathicity, increased conformational stability and high hydrophobicity to increased cytotoxicity (Dathe *et al*, 1997; Kondejewski *et al*, 1999; Zelezetsky and Tossi, 2006). In fact, stabilization of peptides beyond a certain point could result in decreased antimicrobial activity with an associated increase in haemolytic activity (Zelezetsky and Tossi, 2006). Therefore, a balance between high antimicrobial activity and reduced cytotoxicity is required. Polyansky *et al*, (2009) demonstrated that *N*-terminus amphipathic helices of peptides with hydrophobic properties exhibited haemolytic and other cytotoxic activity. By substituting three hydrophobic amino acids from the *N*-terminus amphipathic helices with polar residues, they successfully designed AMPs free of haemolytic activities from those AMPs that were originally haemolytic (Polyansky *et al*, 2009).

Resistance to proteolytic enzymes

In vivo susceptibility of AMPs to proteolytic degradation poses another challenge to their development in systemic applications (Jenssen *et al*, 2006). For example although LL-37 has excellent antibacterial activity even at physiological salt conditions, its development as a potential drug is severely hampered due to its susceptibility to proteolytic degradation (Travis et al, 2000; Stromstedt et al, 2009a). One possible way to increase resistance of AMPs to degradation by proteolytic enzymes is to modify the *C*-terminus by amidation. Svenson *et al*, (2008) reported that amide modification at *C*-terminus and introduction of a bulky synthetic side chain at the central amino acid position resulted in increased stability of the peptides. Other possible ways of improving stability of peptides against proteolytic degradation include

modification of the *N*-terminus through acetylation and substitution of natural amino acids with unnatural residues at known cleavage sites for proteases (Oyston *et al*, 2009; Stromstedt et al, 2009a).

1.8.4 Mechanism of resistance to AMPs

Development of resistance to AMPs by an otherwise susceptible microbial strain is unlikely due to essential changes in membrane structure required by the target organisms to evade antimicrobial action of AMPs (Zasloff, 2002). On the other hand, resistance to AMPs has been reported as a fundamental constituent of some bacteria (Guo *et al*, 1997; McPhee *et al*, 2003; Fedtke *et al*, 2004). Some common resistance mechanisms of bacteria include: modification of surfaces (Guo *et al*, 1997; McPhee *et al*, 2003); active efflux of the AMPs from the cells (Parra-Lopez *et al*, 1994); degradation of AMPs by proteolytic enzymes (Sieprawska-Lupa, *et al*, 2004); peptide-mediated modifications of host processes such as degradation of β-defensins by host proteases (Taggart *et al*, 2003).

1.8.5 Potential role of AMPs in prevention of BAI

AMPs prospective role in prevention of BAI could be further subdivided into: effectiveness of unbound AMPs; and effectiveness of AMPs bound to biomaterial surfaces.

1.8.5.1 Effectiveness of unbound AMPs

<u>In solution</u>

Most studies conducted thus far have assessed the antimicrobial activities of these peptides in solution form where they have been very effective against a wide range of pathogens (including antibiotic-resistant strains) implicated in BAI such as *S. aureus*, methicillin-resistant *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. Faecalis*, vancomycin-resistant *Enterococcus* (VRE) and *E. coli* (Steinberg *et al*, 1997; Kwakman *et al*, 2006; Sparo *et al*, 2009; Augustin *et al*, 2009).

In systemic infections *in vivo*

AMPs have also been reported to be effective *in vivo* for the treatment of systemic infections involving methicillin-resistant *S. aureus*, *P. aeruginosa* and vancomycin-resistant *E. faecium* (Steinberg *et al*, 1997; Deslouches *et al*, 2005a; 2007). In one such study, mice infected by intravenous injection of *S. aureus* and vancomycin-resistant *E. faecium* were given a single dose of intravenous Protegrin-1 at 0 to 60 minutes after infection. Compared to controls (mortality of 73 to 93%), intravenous injection of Protegrin-1 resulted in significant reduction in mortality (7 to 33%) by *S. aureus*. In the case of vancomycin-resistant *E. faecium*, the mortality reduced from 87% in controls to 33% in mice that were treated with a single dose of Protegrin-1 (Steinberg *et al*, 1997).

In experimental BAI models *in vivo*

Additionally, BAI models have also been used to test efficacy of AMPs (Faber *et al*, 2005; Brouwer *et al*, 2006; Kwakman *et al*; 2006). In an experimental mouse BAI model, Kwakman *et al*, (2006) injected bactericidal peptide 2 (BP2) through a catheter's lumen three hours prior to challenge with *S. epidermidis* to determine the prophylactic ability of the

peptide and reported a reduction of 85% and 90% colony forming units (cfu) from implants and peri-implant tissue respectively. In the same study treatment efficacy of BP2 was also assessed by injection of the peptide along subcutaneous implants one hour post-challenge with *S. epidermidis*. Compared to controls that were injected with saline, BP2 reduced the culture-positive implants by 80% and recovery of *S. epidermidis* from peri-implant tissue by 100-fold reduction in cfu (Kwakman *et al*, 2006).

In synergy with antibiotics

Synergism of AMPs with conventional antibiotics both *in vitro* and *in vivo* has also been reported in the literature (Darveau, *et al*, 1991; Park *et al*, 2004b; Oo *et al*, 2010). For example, antimicrobial peptide A3 in synergy with chloramphenicol showed excellent antibacterial activity against *S. aureus* and *P. aeruginosa* (Park *et al*, 2004b). Furthermore, an *in vivo* study of the peptide magainin 2 in synergy with the conventional antibiotic cefepime, a cephalosporin, demonstrated a significant increase in survival rate of mice, while magainin 2 alone was found to be completely ineffective (Darveau, *et al*, 1991). More recently, Oo *et al*, (2010) reported synergistic effects of using the AMP bovine lactoferricin (B-LFcin) with antibiotics as a topical treatment for corneal infections caused by multi-drug resistant strains of *P. aeruginosa*. In that study, mouse corneas were challenged topically with multi-drug resistant strains *P. aeruginosa*. After nine hours, infected corneas were treated with hourly topical applications of b-LFcin in combination with either ciprofloxacin or ceftazidime. After eight hours of treatment, B-LFcin was found to act synergistically with both antibiotics in reducing bacterial numbers. In addition, myeloperoxidase activity in infected corneas was reduced indicating the potential of B-LFcin as both an antibacterial and anti-inflammatory for the treatment of corneal infections (Oo *et al*, 2010).

1.8.5.2 Effectiveness of AMPs bound to biomaterial surfaces

Effectiveness of AMPs adsorbed to surfaces

Physical adsorption of peptides to biomaterials has been used successfully *in vivo* as well as *in vitro* for many peptides (Giacometti *et al* 2000; Ghiselli *et al*, 2002; Cirioni *et al*, 2006a; Willcox *et al*, 2008). In one *in vitro* study, soaking of contact lenses in the antimicrobial peptide melimine resulted in approximately 80% reduction in viable counts of *S. aureus* and *P. aeruginosa* (Willcox *et al*, 2008). In another study in a rat model, Giacometti *et al*, (2000) found that Dacron grafts soaked in either of the two AMPs buforin II or ranalexin were more efficacious in preventing vascular graft infections due to *S. epidermidis* than the grafts soaked in vancomycin or teicoplanin alone.

While some *in vivo* studies used biomaterials soaked in AMP only, most studies used a combination of AMP-soaked biomaterial along with prophylactic antibiotics. For example, vascular grafts soaked in Temporin A along with prophylactic vancomycin (Ghiselli *et al*, 2002) or linezolid (Giacometti *et al*, 2004), resulted in prevention of infection by methicillin-resistant *S. aureus* and *S. epidermidis* respectively in rat pouch models. In both studies, the efficacy of vascular grafts soaked in Temporin A alone was comparable to prophylactic vancomycin and linezolid. Cirioni *et al*, (2006b), using a rat model of central venous catheter (CVC)-associated infection, showed that that the bacterial load in CVC filled with Citropin 1.1 in combination with antibiotic treatment with rifampicin or monocycline reduced to 10^1 cfu/ml as compared to 10^7 cfu/ml in untreated controls. Whereas, citropin 1.1 alone caused a

reduction of bacterial load to only 10^3 that was comparable to either of the two antibiotics alone.

Effectiveness of AMPs covalently-bound to surfaces

Several reports suggest that AMPs retain their activity while covalently bound to surfaces and represent an excellent candidate for further development as antimicrobial coatings on biomaterial surfaces (Harris *et al*, 2004; Gabriel *et al*, 2006; Willcox *et al*, 2008; Hilpert *et al*, 2009; Chen *et al*, 2009; Humblot *et al*, 2009; Bagheri *et al*, 2009; Cole *et al*, 2010).

Most of the studies conducted thus far on covalently bound peptides are *in vitro* studies where the emphasis has been on improving the methods of attachment of these peptides on various surfaces while retaining activity against microorganisms. For example, LaPorte *et al*, (1977), found antimicrobial activity of agarose beads covalently bound with polymyxin B against *E. coli* and *P. aeruginosa*. Haynie *et al*, (1995) produced polymer-bound AMP coatings by linking the carboxy-terminal amino acid with an ethylenediamine-modified polyamide resin and demonstrated antimicrobial activity against *S. aureus* and *E. coli*. Hilpert *et al*, (2009) used a cellulose-amino-hydroxypropyl ether linker chemistry to synthesize peptides on a cellulose support that retained antimicrobial activity in covalently bound form. Similarly, Chen *et al*, (2009) used glass surfaces to covalently attach melimine via two azide linkers and demonstrated the ability of peptide to reduce adhesion of *P. aeruginosa* and *S. aureus*. This chemistry is readily applicable to a range of biomaterial plastics. Others have explored the use of a modification of this chemistry to covalently attach magainin to metal surfaces following functionalisation with 11-mercaptoundecanoic acid (Humblot *et al*, 2009). In addition, covalent incorporation of an antimicrobial peptide into the bulk polymer has been report to retain activity *in vivo* (Cole *et al*, 2010). Gabriel *et al*, (2006) developed a method of

covalent attachment of LL-37 on titanium surface via flexible hydrophilic poly (ethylene glycol) spacer that resulted in formation of a surface peptide layer with antimicrobial potential.

Willcox *et al*, (2008) in an *in vitro* study reported a 70% reduction in adhesion of bacteria to contact lens surfaces with covalently bound melimine. Cole *et al*, (2010) further tested the efficacy of the peptide melimine *in vivo* to prevent contact lens induced peripheral ulcer (CLPU) in a *S. aureus* rabbit model and contact lens-induced acute red eye (CLARE) in a *P. aeruginosa* guinea pig model. Melimine incorporated into the bulk polymer of the lens resulted in a significant reduction in ocular symptom scores and in the degree of corneal infiltration in the CLPU model whereas the CLARE model demonstrated a significant improvement in each of the ocular response parameters measured (Cole *et al*, 2010).

1.8.6 Mechanism of action of AMPs covalently bound to the surfaces

Although covalently bound AMPs have been found to be effective on various surfaces, very few studies have attempted to elucidate the exact mechanism by which they exert their anti-adhesive/antimicrobial effects (Humblot *et al*, 2009; Chen *et al*, 2009; Hilpert *et al*, 2009). The limited information that is currently available suggests that outer membrane perturbation of Gram-negative bacteria by the attached peptides triggers subsequent alterations in cytoplasmic membrane permeability and subsequent killing of bacterial cells (LaPorte *et al*, 1977; Haynie *et al*, 1995).

However, covalently bound peptide's ability to directly interact with the cytoplasmic membrane is debatable. Hilpert *et al,* (2009) suggested that bacterial killing by an attached peptide composed of only 9 amino acid-residues could not be possible by direct interaction of the peptide with the cytoplasmic membrane due to length constraints. Hence the killing event is independent of peptide penetration in the cytoplasmic membrane of the bacteria. However Gabriel *et al,* (2006) showed that titanium surfaces coated with LL-37 *via* a long and flexible linker i.e. functionalized poly(ethylene glycol; PEG) molecules, were bactericidal activity towards *E. coli*, while in the absence of PEGs no bactericidal activity was detected. Therefore, bactericidal activity of the peptide is likely to depend on its ability to interact with the cytoplasmic membrane (Gabriel *et al*, 2006). Clearly, more work is required to determine the mechanism of action of bound AMPs.

1.8.7 Current clinical status of AMPs

Although more than thousand AMPs have been identified to date (Wang *et al*, 2009), only four are being used clinically in two topical and two systemically administered formulations for the treatment and prophylaxis of various diseases and infections (Evans *et al*, 1999; Marr *et al*, 2006). Gramicidin S and Polymyxins including polymyxin B and polymyxin E are used for treatment of infections involving *P. aeruginosa* and *Acinetobacter baumannii*. Polymyxins B in combination with gramicidin S and bacitracin as topical formulations are used clinically for wound, eye and ear infections (Evans *et al*, 1999; Marr *et al*, 2006)

Colomycin, a derivative of the polymyxins, and daptomycin, an anionic lipopeptide, are two AMPs that are used clinically for systemic infections (Conway *et al*, 1997; Schriever *et al*, 2005**;** Marr *et al*, 2006). Colomycin is indicated for lung infections in patients with cystic

fibrosis (Conway *et al*, 1997) while daptomycin is approved by the FDA for complicated skin and skin-structure infections (Schriever *et al*, 2005).

Several other AMPs are currently at various stages of development. Among them, CLS001, a 12-mer analog of indolicidin and formerly developed as Omiganan, has been shown to prevent colonisation of catheters as well as for the treatment of acne and rosacea (an inflammatory condition), is currently in phase III b clinical trials (Hamil *et al*, 2008). Another peptide, hLF-1-11, originally derived from human lactoferrin as an AMP with weak antimicrobial activity, is in phase II clinical trials for its immunomodulatory potential against bone marrow stem cell transplant–associated infections (Hancock and Sahl, 2006; Hamil *et al*, 2008). MX-594 AN, in topical formulation for the treatment of acne vulgaris, has completed phase II b trial (Giuliani *et al*, 2007). P113, an AMP based on histatins and composed of 12 amino acids is currently under phase I/II clinical trial for its potential role to treat candidiasis in patients infected with HIV (Zaiou, 2007; Giuliani *et al*, 2007).

In addition to these peptides in various clinical phases, several others are at preclinical testing phases. Some of them include: SB006, a potent, protease-stable AMP under development for Gram-negative multidrug resistant clinical isolates (Pini *et al*, 2005); heliomycin, an antifungal AMP derived from insects (Zaiou, 2007); and plectasin, a peptide belong to the defensins class with a strong antibacterial activity against multidrug resistant bacteria and against *S. pneumoniae* (Zaiou, 2007; Giuliani *et al*, 2007). A list of AMPs at various stages of clinical development is presented in table 1.1.

	Product	Company	Trials outcome and applications	Planned trials
Available on the market	Polymixin B-Colistin-Colomycin (prodrug)	RX Generic drugs	Indicated for G- skin infections	none
	Daptomycin (Cubicin ®)	Cubist Pharmaceuticals	Indicated for G+ skin infections	not announced
Late clinical development (Phase III and above)	Pexiganan	Genaera Plymouthmeeting	Failed FDA approval no advantage over convent. antibiotics	none
	Iseganan	Intrabiotics Pharmaceuticals	Failed two Phase III stomatitis and pneumonia	Phase IIa trial as Rx for CF patients
	Omiganan	Migenix	Failed Phase III trial as Rx for catheter related bloodstream infections	Repeat Phase III trial
Early clinical development (Phase I, II, pre-clinical)	LTX Serie	Ltfx Biopharma	Several G+, G- and fungal infections. Preclinical	not announced
	SB006	SpiderBiotech	G- infections Under preclinical trials	not announced
	Product-I serie	Iramex	Several hospital bacterial infections	not announced
	MX594AN	Migenix	Phase IIb showed efficacy as topical Rx for acne	Phase III trial
	Plectasin	Novozyme	G- infections Under preclinical trials	not announced
	P113/P113D	Damégen/Pacgen	Phase IIb showed efficacy for oral candidasis	Inhalation Rx for CF patients under consideration
	hLF1-11	AM-Pharma	Positive Phase I.Under Phase II for severe fungal and bacterial infection	not announced
	XMP.629	Xoma	Failed Phase II clinical trial as topical Rx for acne	not announced
	Neuprex	Xoma	Failed Phase III as Rx in pediatric meningococcemia	Planned Phase I/II in pediatric indications
	Mersacidin	Novacta Biosystems	Systemic anti-MRSA and other G+ pathogens Under preclinical trials	not announced
	PTX serie	PeriTx	G- infections and LPS neutralization (antiseptic). Under preclinical trials	not announced
	HB Serie	Helix-Biomedix	Preclinical trials showed efficacy for cystic fibrosis	not announced

Table 1.1: AMPs at various stages of commercial development (taken from Giuliani *et al*, 2007).

1.9 The development of antimicrobial contact lenses

Zhu *et al*, (2008) have demonstrated that fimbrolides can reduce the adhesion of *P. aeruginosa* by 67% and of *S. aureus* by 87% once covalently bound to contact lenses. Fimbrolides (also known as furanones), initially isolated from the marine alga *Delissia pulchra*, have been shown to interfere specifically with quorum sensing systems of *P. aeruginosa*, and these quorum sensing systems are involved in biofilm formation (Hentzer *et al*, 2003; Kirisits *et al*, 2006). Willcox *et al*, (2008) reported that the cationic peptide melimine adsorbed or bound to a contact lens surface could reduce the adhesion of *P. aeruginosa* by 92% and of *S. aureus* by 76%. A study by Mathews *et al*, (2006) reported that selenium-coating of contact lenses reduced the adhesion of *P. aeruginosa*. Finally, silver-coated contact lenses have been tested in the laboratory and shown to be effective at reducing the colonisation by *P. aeruginosa* but not as effective against *S. aureus* (Nissen and Furkert, 2000).

1.10 Rationale for using melimine in the current study

Melimine (T L I S W I K N K R K Q R P R V S R R R R R R R G G R R R R) was chosen for the current study because previous studies have demonstrated its excellent potential to be further developed as coating on surfaces of biomaterials (Willcox *et al*, 2008; Chen *et al*, 2009). This synthetic peptide designed by incorporating active regions of two AMPs, protamine (from salmon sperm) and melittin (from bee venom), has been found to be effective against both Gram-positive and Gram-negative bacteria *in vitro* (Willcox *et al*,

2008; Chen *et al*, 2009) as well as *in vivo* (Cole *et al*, 2010). Melimine was found to be non-cytotoxic as it showed only 50% haemolysis of sheep blood cells at concentrations several times greater than the MICs (Willcox *et al*, 2008). It demonstrated limited potential to induce resistance and was found to be heat-sterilisable (Willcox *et al*, 2008). Its activity is modulated by the method by which it is attached to biomaterial surface (Chen *et al*, 2009) and therefore it represents an excellent candidate for further development as coating on biomaterial surfaces.

The aim of the study was to test the efficacy of melimine in covalently bound form on surfaces to reduce adhesion of bacteria commonly implicated in contact lens-related infections, to determine the shortest sequence of melimine with efficacy comparable to parent peptide and to explore the mechanism of action of melimine and its shortest derivative.

The specific aims of the study were:

- To test the efficacy of contact lenses soaked in melimine (physical adsorption) for their ability to reduce adhesion of bacteria implicated in lens-related infections.
- To produce coatings of melimine, in covalently bound form, on contact lenses and to test the efficacy of the coatings against both Gram-positive and Gram-negative bacteria.
- To synthesise several shorter derivatives of melimine and to determine the shortest sequence of the peptide with activity comparable to the parent peptide melimine in covalently bound form.

- To determine mechanism of action of parent peptide melimine in solution form and to further explore mechanism of action of parent peptide melimine and its shortest derivative while covalently bound to the surfaces.

Chapter 2: Antibacterial activity of melimine when bound to biomaterials

2.1 Introduction

A higher life expectancy combined with significant improvement in quality of life has led to an increase in use of biomaterials (Engelsman *et al*, 2009). However, infections remain a major cause of implant failure that results in removal of the device and its subsequent replacement (Gristina, 1987; Verkerke *et al*, 1997; Qiu *et al*, 2007; Engelsman *et al*, 2009). The whole procedure adds to the distress of the patient. Additionally, associated cost becomes a burden on the healthcare system (Qiu *et al*, 2007). For example, in USA alone, the cost associated with the replacement of just one type of device, a central venous line, as a result of infection is $US 14,000 per patient or approximately $US 5.6 million per annum (Thomas *et al*, 2006), while the estimated cost of treatment of contact lens-related keratitis is $US 15 – 30 million annually (Khatri *et al*, 2002). In Australia, the average costs associated with a single case of contact lens-related keratitis were reported to be AU$5515 for severe cases with vision loss, AU$1596 for severe cases without vision loss and AU$795 for the mild cases of microbial keratitis (Keay *et al*, 2008).

Current preventive measures to reduce biomaterial associated infections (BAI) include: antiseptic cleansing to eradicate the bacterial contamination by skin microbiota; improvement in surgical procedures; and the use of antibiotics in various forms (Darouiche *et al*, 2007; Qiu

et al, 2007; Costerton, 2005). Yet biomaterial associated infections are difficult to overcome (Qiu *et al*, 2007).

One possible way to prevent BAI involves coating of biomaterials with antimicrobial substances including antibiotics and there have been various reports that link reduction in BAI to coatings that contained single or combinations of antibiotics such as vancomycin, rifampin, minocycline, nalidixic acid and teicoplanin (Hamilton *et* al, 1997; Hampl *et* al, 1995, 2003; Han *et al*, 2005; van de watering and van Woensel, 2003; Darouiche *et al*, 2007). Among alternative coatings, silver (Johnson *et al*, 1990; Tobin and Bambauer, 2003), chlorhexidine (Ho and Litton, 2006), triclosan (Edmiston *et al*, 2006; Jones *et al*, 2006) and quaternary ammonium compounds (Moss *et al*, 2000) have shown promising results. However, a major drawback with the use of antibiotics as coating is that after a rapid onset, the release of antibiotics slows overtime to levels far below the therapeutic concentrations, potentially allowing the development of microbial resistance (Norris *et* al, 2005; Costerton, 2005). The efficacy of silver and chlorhexidine as coatings on biomaterial surfaces is also questionable. For example, Amos and George, (2006) found that there was still 26% of silver-coated contact lens cases contaminated with bacteria (as compared to 67% non-silver cases). Harnet *et al*, (2009) reported that overnight soaking of suture material in chlorhexidine did not prevent colonization by *E.* coli. A major concern with the use of triclosan as well as quaternary ammonium compounds is the emergence of microbial resistance (Fan *et al*, 2002; Loughlin *et al*, 2002).

Clearly, there is a growing need to find suitable alternatives and one emerging area of research to address this need is the use of antimicrobial peptides: a new class of therapeutic antibiotics.

Antimicrobial peptides (AMPs) are relatively short (6 - 50 amino acid residues) with a net positive charge and are amphiphillic (Wang and Wang 2004). They have been found to be active against bacteria, fungi, protozoa and viruses (Murakami *et al*, 1991; Masuda *et al*, 1992; Aley *et al*, 1994; Zasloff, 2002). The most important features of antimicrobial peptides are their relatively low cytotoxicity towards mammalian cells (Hwang and Vogel, 1998) and their ability to rapidly kill microorganisms (Hancock, 1997). The latter property may help to reduce the emergence of resistance to these peptides by microbes (Hancock, 1997).

Most studies conducted thus far have assessed the activity of these peptides *in vitro* where they have been very effective against a wide range of pathogens including multi-drug resistant bacteria. Various peptides have been found to be effective in the treatment of systemic infections involving methicillin-resistant *S. aureus* (Steinberg *et al*, 1997) and *P. aeruginosa* (Gough *et al*, 1996; Steinberg *et al*, 1997). The rate of bacterial killing by AMPs is very rapid. For instance, Steinberg *et al* (1997) reported a more than three-log reduction in colony forming units per millilitre (cfu/ml) of both methicillin-resistant *S. aureus* and *P. aeruginosa* in less than 15 minutes after addition of an AMP Protegrin -1 to the assay medium. In addition, other reports suggest synergistic activity of cationic peptides with conventional antibiotics against *P. aeruginosa* infections (Darveau *et al*, 1991).

Physical adsorption of these peptide types to biomaterials has been employed by researchers in recent years to assess their efficacy. In one study, adsorbed Temporin A (FLPLIGRVLSGIL-NH2) combined with the antibiotic linezolid greatly reduced bacterial numbers and prevented vascular graft infection in a subcutaneous rat pouch model of infection (Giacometti *et al*. 2004). Temporin A was also found to be an effective prophylactic agent when combined with vancomycin against methicillin-susceptible and methicillin-resistant *S. epidermidis* vascular graft infection (Ghiselli *et al*. 2002). In another study when Temporin A was combined with another peptide: RNA III inhibiting peptide (YSPWTNF-NH2), the combination successfully eliminated infection of staphylococci by 100% in a rat pouch model of graft infection (Cirioni *et al*. 2003). Similarly, vascular grafts soaked in Buforin II (TRSSRAGLQFPVGRVH RLLRK) - Ranalexin (NH2-FLGGLIKIVPAMICAVTKKC-COOH) combination were found to be effective as prophylactic agents against *S .epidermidis* vascular graft infection (Giacometti *et al*. 2000).

The current study aimed at using the antimicrobial peptide melimine in both adsorbed and covalently-bound forms on contact lens surfaces to assess its ability to inhibit adhesion of Gram-positive and Gram-negative bacteria. Melimine is a synthetic peptide composed of portions of protamine (PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR) and melittin (NH2-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH2) and has shown excellent activity while attached to surfaces (Willcox *et al.* 2008; Chen *et al.* 2009). Bacteria were found to possess a very limited potential to become resistant to melimine. Additionally, melimine was also shown to be heat stable (Willcox *et al.* 2008). This study aimed to further assess melimine's potential to reduce adhesion of bacteria on surfaces of contact lenses.

The specific aims of the study included:

- To determine activity of antimicrobial peptide melimine adsorbed or covalently bound to contact lens surfaces against various strains of *S. aureus* and *P. aeruginosa*.
- To assess the ability of melimine in adsorbed form to reduce adhesion of methicillin-resistant *S. aureus* and antibiotic-resistant strains of *P. aeruginosa*
- To quantify the amount of melimine attached to the surface of contact lenses in both adsorbed and covalently-bound forms.

2.2 Materials and Methods

2.2.1 Synthesis of melimine

Melimine (T L I S W I K N K R K Q R P R V S R R R R R R G G R R R R-NH2) was synthesized by conventional solid-phase peptide synthesis protocols and was purchased from the American peptide company, Inc (Sunnyvale, CA, USA) at a purity of >90%.

2.2.2 Bacterial strains and growth conditions

Ten strains of *S. aureus* including five methicillin-resistant *S. aureus* strains (MRSA) and eight strains of *P. aeruginosa* including three strains resistant to multiple antibiotics were used in the adhesion assays (Table 2.1 and 2.2).

Table 2.1: Strains of *S. aureus* used in adhesion assay

Bacteria	Strain	Description
S. aureus	ATCC 12600	Standard strain also known as NTCC 8532
	Sa 009	Isolated from case of CLARE* α and β toxin producer (Wu *et al*, 1999)
	Sa 31	Isolated from a CLPU# patient (Jalbert *et al*, 2000)
	Sa 38	a clinical isolate from human corneal ulcer (Hume *et al*, 2005)
	Sa 45	Laboratory strain (alph toxin negative) (Wu *et al*, 2005)
	Sa 101	Resistant to: Methicillin, Tobramycin, Erythromycin and fluoroquinolone (Schubert *et al*, 2008)
	Sa 103	Resistant to: Methicillin, Tobramycin, Erythromycin and fluoroquinolone (Schubert *et al*, 2008)
	Sa 111	Resistant to: Methicillin, Tobramycin, Erythromycin and fluoroquinolone (Schubert *et al*, 2008)
	Sa 112	Resistant to: Methicillin, Tobramycin, Erythromycin and fluoroquinolone (Schubert *et al*, 2008)
	Sa 113	Resistant to: Methicillin, Tobramycin, Erythromycin and fluoroquinolone (Schubert *et al*, 2008)

*CLARE: contact lens induced acute red eye is an acute inflammatory reaction associated with bacterial colonisation of contact lenses during overnight wear (Holden et al, 1996). Some of the symptoms of this condition include pain and redness of the eyes, ocular discomfort and photophobia (Sweeney et al, 2003)

Contact lens induced peripheral ulceration (Jalbert et al, 2000)

Table 2.2: Strains of *P. aeruginosa* used in adhesion assay

Bacteria	Strain	Description
P. aeruginosa	Paer001	Isolated from a case of CLARE (Holden *et al*, 1996)
	Paer 6294	Isolated from human corneal ulcer (Fleiszig *et al*, 1997)
	ATCC 19660	Originally isolated from wound infection and has been widely used in virulence studies (Hazlett *et al*, 1985)
	Paer 023	Isolated from corneal ulcer at Manchester Royal infirmary (Taylor *et al*, 1998)
	Paer 6206	Cytotoxic strain (Fleiszig *et al*, 1996)
	Paer 033	Resistant to Ciprofloxacin, Gentamicin and Cefazolin (Zhu *et al*, 2006)
	Paer 034	Resistant to Ciprofloxacin and Gentamicin (Zhu *et al*, 2006)
	Paer 035	Resistant to Ciprofloxacin (Zhu *et al*, 2006)

*CLARE: contact lens induced acute red eye is an acute inflammatory reaction associated with bacterial colonisation of contact lenses during overnight wear (Holden *et al*, 1996). Some of the symptoms of this condition include pain and redness of the eyes, ocular discomfort and photophobia (Sweeney *et al*, 2003)

Bacterial strains were grown overnight in Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK), harvested by centrifugation and washed three times with phosphate-buffered saline (PBS) containing one part TSB in one thousand parts of PBS for *P. aeruginosa* strains and one part TSB in fifty parts of PBS for *S. aureus* strains. Cells were resuspended in the same buffer to an OD_{660} of 0.1 that corresponds to 1×10^8 cells/ml of suspension (confirmed by

retrospective plate counts). For *S. aureus*, cell suspension was serially diluted to obtain a final concentration of 1×10^3 cells/ml of suspension. To avoid a lower initial adhesion of *S. aureus* to contact lens surfaces as demonstrated by Bruinsma *et al*, (2001), a previously published method (Zhu *et al*, 2008) was used with comparatively lower initial inoculums but a longer adhesion time (24 hours).

2.2.3 Attachment of melimine on contact lens surfaces

Melimine was attached to the surfaces of contact lenses by two approaches:

a) Adsorption (soaking)
b) Covalent attachment

a) Adsorption

Acuvue 2 contact lenses (Etafilcon A; Johnson & Johnson Vision Care, Jacksonville, FL, USA) were washed 3 times with PBS. The washing procedure included aseptically placing a lens in PBS followed by shaking at 175 rpm for 30 seconds. Lenses were allowed to soak for 24 hours at 37^o C on a shaker in 1 mg/ml or 0.5 mg/ml final concentrations of melimine for *P. aeruginosa* and *S. aureus* respectively. After that period, lenses were washed three times in PBS to remove any loosely bound melimine before use in adhesion assay.

b) Covalent attachment

Melimine was covalently attached to contact lenses (Etafilcon A) by a method adapted from Willcox *et al* (2008), with some modifications. Briefly, lenses were washed three times in PBS followed by soaking in 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) at a final concentration of 5mg/ml in PBS. After one hour of incubation at room temperature, lenses were washed thrice in PBS and resuspended in 0.5 mg/ml and 1 mg/ml

final concentrations of melimine in PBS. Melimine was allowed to attach to lenses for a period of 24 hours at 37^{o}C with shaking. After three additional washes in PBS to remove any loosely associated melimine lenses were used for adhesion assay.

2.2.4 Adhesion assay

Adhesion assays were performed using a protocol adapted from Williams *et al* (2003). The final concentration of melimine used in covalently bound or adsorbed form was 0.5 mg/ml for *S. aureus* and 1 mg/ml for *P. aeruginosa*. Contact lenses with or without associated (adsorbed or bound) melimine were washed three times in PBS and inoculated with 1 ml of bacterial suspension (final concentration of 1×10^{8} cells of *P. aeruginosa* and 1×10^{3} cells of *S. aureus*). Lenses inoculated with *P. aeruginosa* were incubated for a period of 10 minutes at 37^{o}C with shaking while for *S. aureus* the incubation period was 24 hours. A longer incubation period (adapted from Zhu *et al*, 2008) was chosen for *S. aureus* because pilot studies suggested a very low initial adhesion of *S. aureus* (<10 cells per lens after one hour of incubation). After incubation, lenses were washed three times with PBS to remove any loosely bound bacteria followed by homogenisation in 2 ml PBS by means of vortexing at high speed for 1 minute. After serial dilutions in Dey-Engley neutralizing broth (DE broth; Difco, Becton Dickinson), aliquots of 20 µl were plated on nutrient agar and incubated at 37^{o}C overnight. Bacterial viability was determined by colony counts and expressed as colony forming units per mm^2. The number of colonies per mm^2 lens surface (cfu/mm^2) was calculated by using the following formula:

$$\frac{\text{(Average number of bacteria per ml} \times \text{dilution factor)}}{\text{Area of contact lens}}$$

Where the whole lens surface area of an Acuvue contact lens is 387.2 mm^2

Lenses were tested in triplicate in each adhesion assay for control (Etafilcon A without peptide) and test (Etafilcon A with adsorbed or covalently bound melimine). Additionally, where melimine covalently bound to lenses were tested three process control lenses were also used (EDC-coupled lenses without melimine). The adhesion assay was repeated twice and results were expressed as percentage reduction in adhesion of bacteria on melimine-treated lenses compared to control non-melimine treated lenses.

2.2.5 Quantification of peptide on lenses

A protocol adapted from Cole and Ralston, (1994) was used for quantification of peptides on lenses. A standard curve was made from 16 1-cm pieces of gel (polyacrylamide gel; BIO-RAD, USA) soaked in difference concentrations (0, 2, 4, 6, 8, 10, 15, and 20 µg/µl; in duplicate) of melimine. Contact lenses with adsorbed or covalently bound melimine and gel standards were rinsed in fixative (10% v/v acetic acid, 10% v/v isopropanol) and stained with 0.025% Coomassie blue stain (GE Healthcare, Sydney, Australia) in 10% acetic acid and 10% isopropanol overnight. Next day, destaining was performed using 10% acetic acid, 10% isopropanol solution at room temperatue. The destaining procedure was repeated for several days until solution or control lenses were clear. Extraction of the Coomassie stain was accomplished by placing lenses and gels into eppendorf tubes to which 25% pyridine was added and left overnight. Next day, absorbance was recorded from supernatants at 600nm with 25% pyridine used as blank. The test lens readings were compared with the standards.

2.2.6 Statistical analysis

A two-tailed t test was employed to compare the differences of means obtained from lenses treated with melimine as compared to untreated lenses with $P \leq 0.05$ considered as statistically significant.

2.3 Results

2.3.1 Effect of adsorbed melimine on adhesion of *S. aureus*

Melimine adsorbed to the contact lens surfaces appeared to significantly inhibit the adhesion of all *S. aureus* strains. The bacterial viability counts for melimine-adsorbed lenses after 24 hour of adhesion showed a two-log inhibition in adhesion for all strains tested in the study (fig.2.1a). Compared to controls, this effect was statistically significant for all strains ($P \leq 0.01$). The percentage inhibition of adhesion was found to be more than 98% for all the strains used in the study (fig. 2.1b). The standard deviations ranged from a minimum of ± 0.14 for Sa 001 to maximum of ± 0.39 for Sa 31 (fig. 2.1b).

2.3.2 Effect of covalently-bound melimine on adhesion of *S. aureus*

Covalent attachment of melimine on the surface of lens resulted in complete inhibition of adhesion of all *S. aureus* strains (fig. 2.2a). Compared to controls, no viable cells could be recovered from lenses with covalently bound melimine (fig. 2.2a). Viable counts obtained from process controls (EDC without melimine) were not different ($P > 0.05$) when compared to controls (lenses in PBS). Inhibition of adhesion of all the strains tested in the study was found to be 100% (fig. 2.2b).

2.3.3 Effect of adsorbed melimine on adhesion of MRSA

The viability counts after 24 hour adhesion of MRSA strains on melimine adsorbed lenses are presented in fig. 2.3a. As seen with the methicillin-sensitive *S. aureus* strains (MSSA) (fig. 2.1 and 2.2), significant inhibition in adhesion was observed for all the MRSA used in the study ($P \leq 0.01$). Similarly, the percentage inhibition of adhesion of all MRSA strains was more than 99% with a maximum standard deviation of ± 0.15 for Sa 101 (fig. 2.3b).

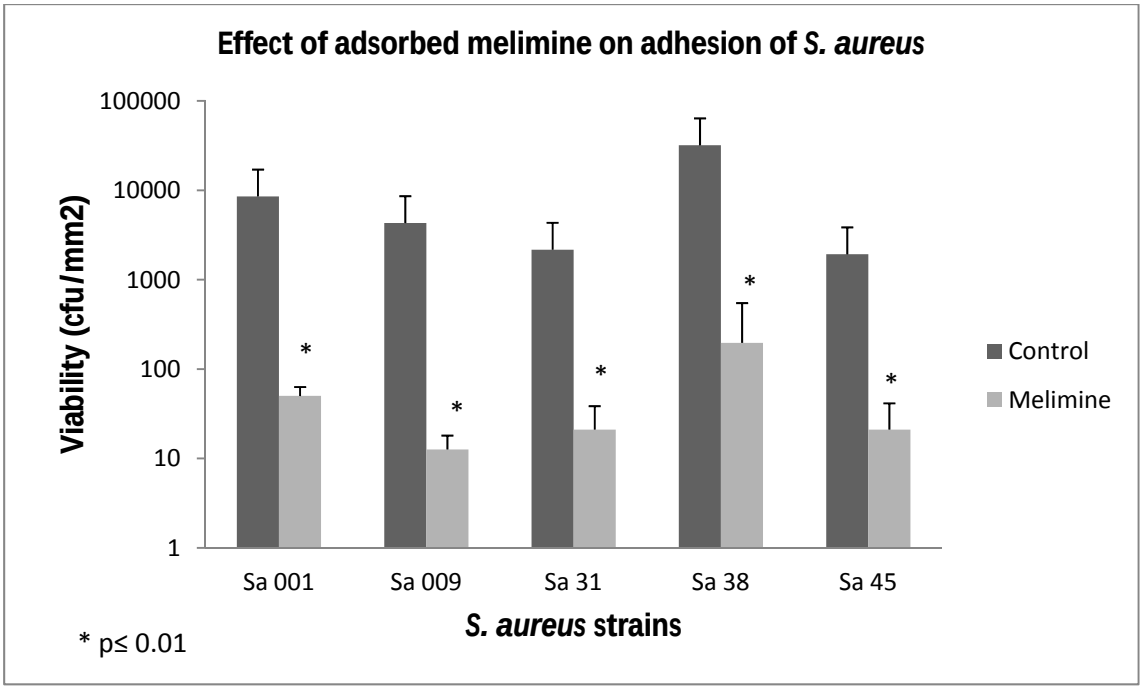


Figure 2.1a: Effect of adsorbed melimine on adhesion of *S. aureus*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

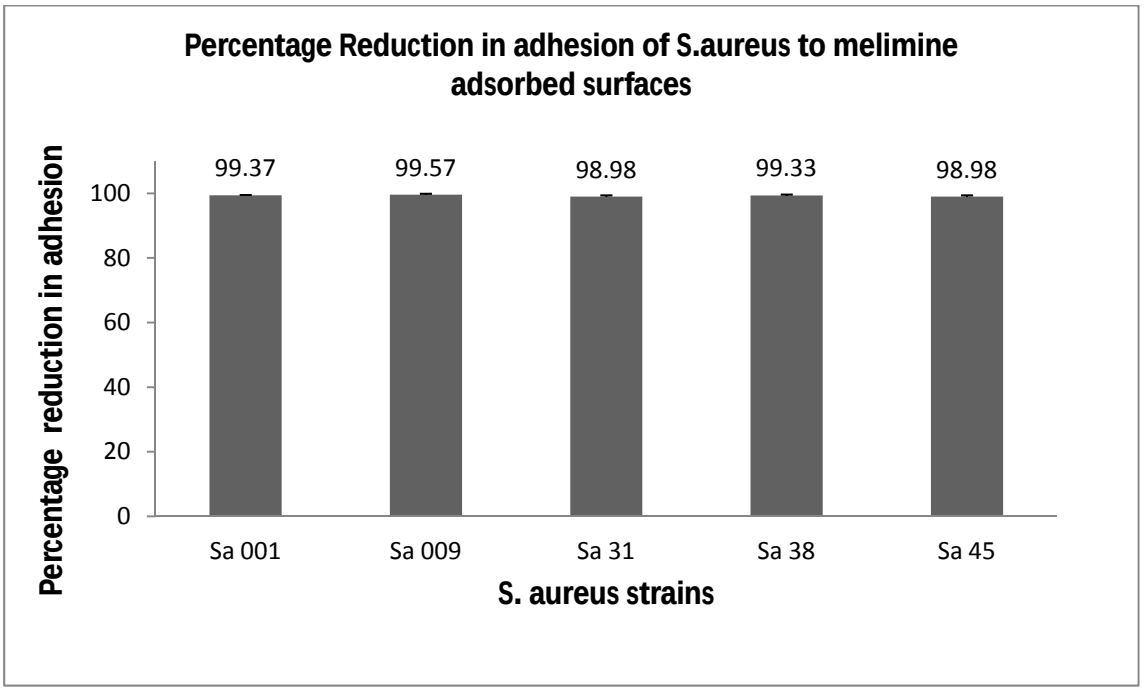


Figure 2.1b: Percentage reduction in adhesion of *S. aureus* to melimine adsorbed surfaces Standard deviation is shown by the vertical bars (n = 9).

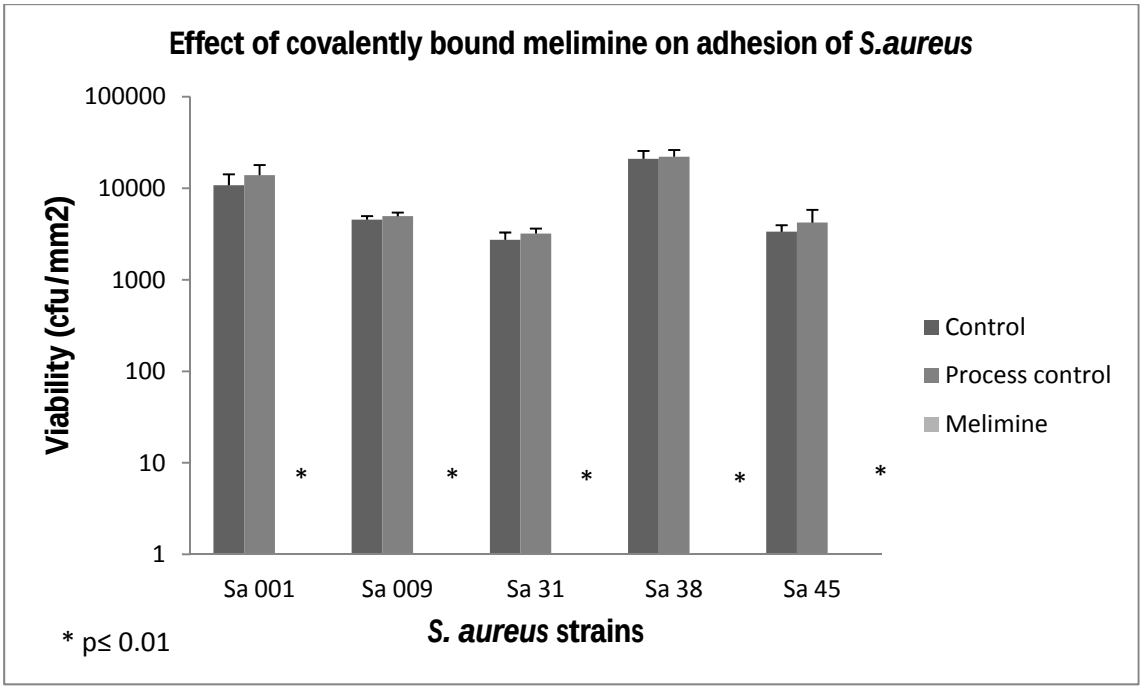


Figure 2.2a: Effect of covalently-bound melimine on adhesion of *S. aureus*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

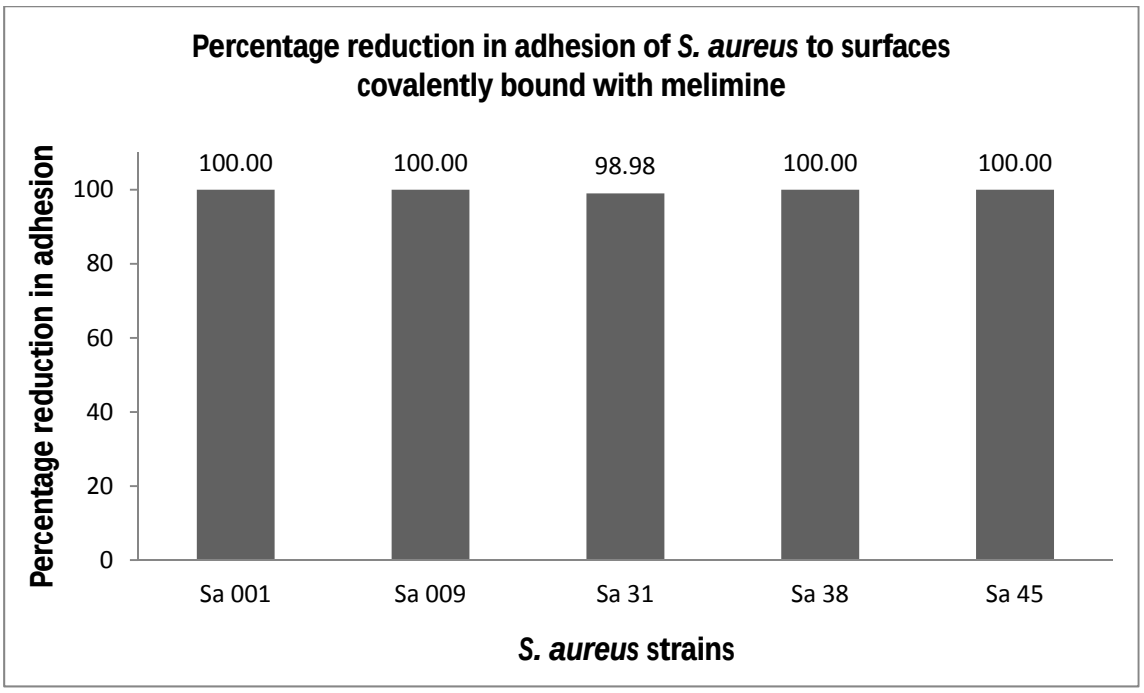


Figure 2.2b: Percentage reduction in adhesion of *S. aureus* to surfaces covalently-bound with melimine (n = 9).

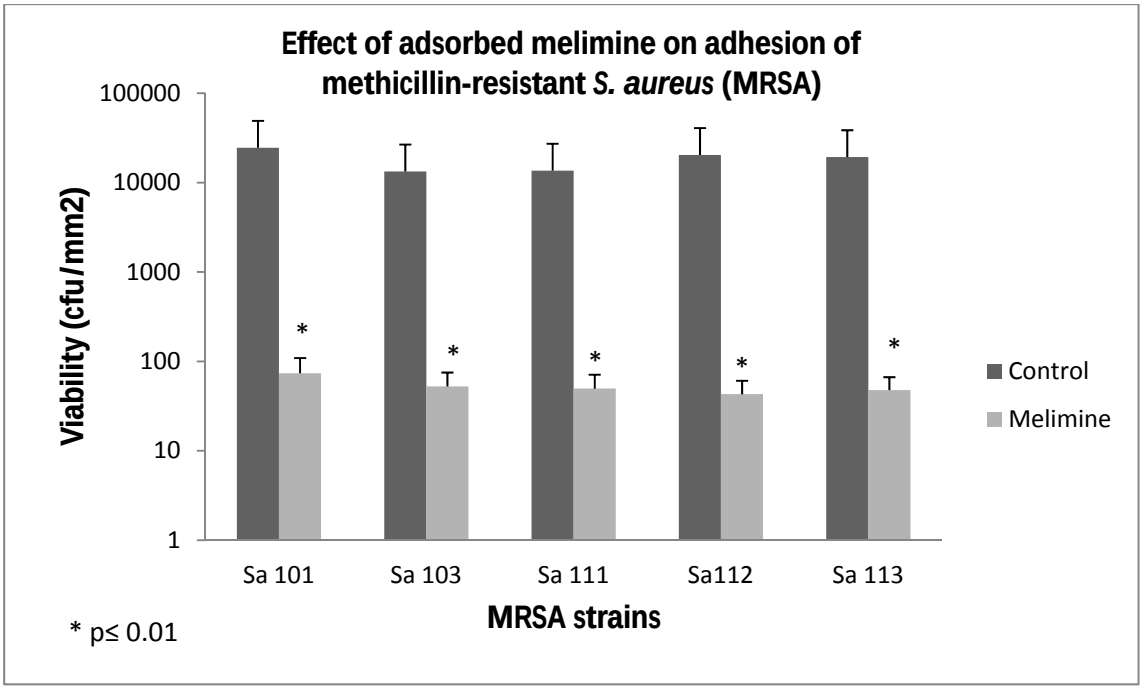


Figure 2.3a: Effect of adsorbed melimine on adhesion of methicillin-resistant *S. aureus* (MRSA). Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

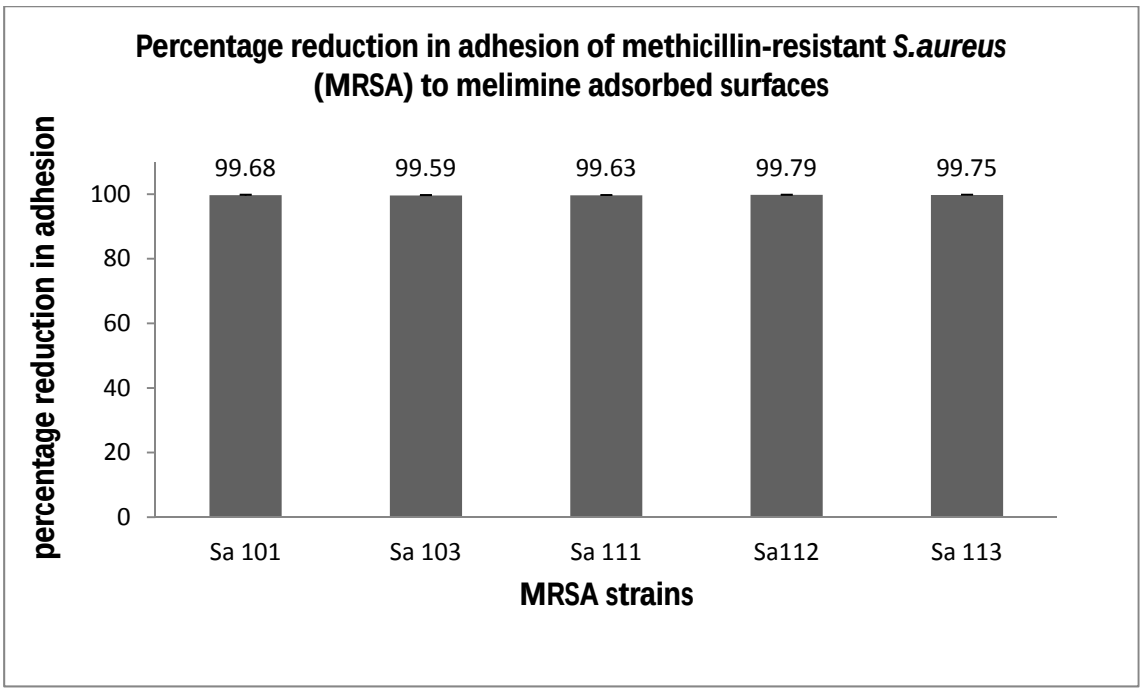


Figure 2.3b: Percentage reduction in adhesion of methicillin-resistant *S. aureus* (MRSA) to melimine adsorbed surfaces. Standard deviation is shown by the vertical bars (n = 9).

2.3.4 Effect of adsorbed melimine on adhesion of *P. aeruginosa*

Fig. 2.4a shows the viability count data obtained after 10-minutes adhesion of *P. aeruginosa* on lens surfaces adsorbed with melimine. Compared to controls, melimine-adsorbed lenses reduced the attachment of *P. aeruginosa*. The effect was consistent and statistically significant for all the strains tested in the study ($P \leq 0.01$). However, the percentage inhibition of adhesion varied among different strains. Three of the five strains (Paer 001, 023 and ATCC 19660) showed inhibition in adhesion in excess of 90%, while for Paer 6206 and 6294 the percentage inhibition of adhesion was found to be 71 ± 9 and 50 ± 10 respectively (fig. 2.4b).

2.3.5 Effect of covalently-bound melimine on adhesion of *P. aeruginosa*

Viable counts obtained after 10-minutes adhesion of *P.* aeruginosa to lenses covalently bound with melimine are shown in fig. 2.5a. No viable bacteria were observed with two of the five strains tested i.e. 6206 and ATCC 19660. A two-log reduction in viability was observed for strains Paer 001($P \leq 0.01$) and Paer 023 ($P \leq 0.01$).

The percentage inhibition in adhesion to contact lenses covalently bound with melimine of *P. aeruginosa* strains is presented in figure 2.5b. The highest percentage inhibition in adhesion was observed in by Paer 001 (98 ± 0.1), followed by ATCC19660 (97 ± 1). Paer 023 and Paer 6206 showed percentage inhibition of 92 ± 5. The lowest percentage inhibition in adhesion was observed with 6294 (88 ± 4).

2.3.6 Effect of adsorbed melimine on adhesion of resistant strains of *P. aeruginosa*

The viable counts and percentage inhibition in adhesion of three antibiotic resistant strains of *P. aeruginosa* is shown in figure 2.6a and 2.6b respectively. The viable counts for all the strains used in the study reduced significantly after 10 minutes adhesion to contact lenses adsorbed with melimine ($P \leq 0.01$; fig 2.6a). The percentage inhibition in adhesion varied from 71 ± 8 for Paer 033, to 68 ± 5 for Paer 034 and 63 ± 6 for Paer 035 (fig. 2.6b).

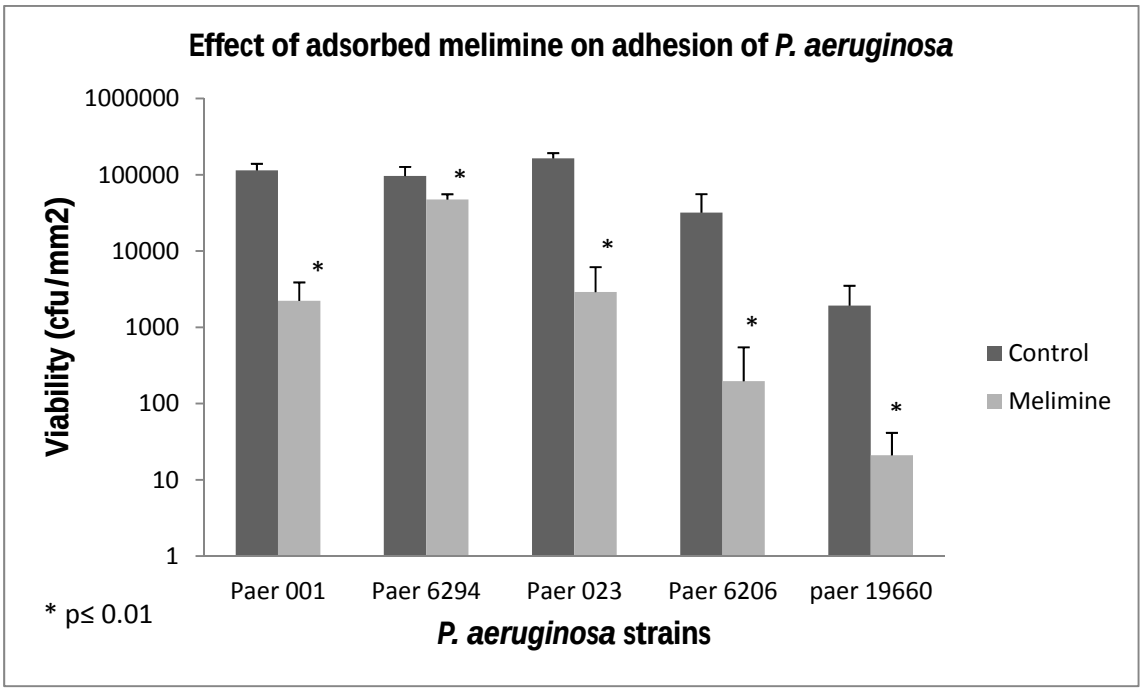


Figure 2.4a: Effect of adsorbed melimine on adhesion of *P. aeruginosa*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

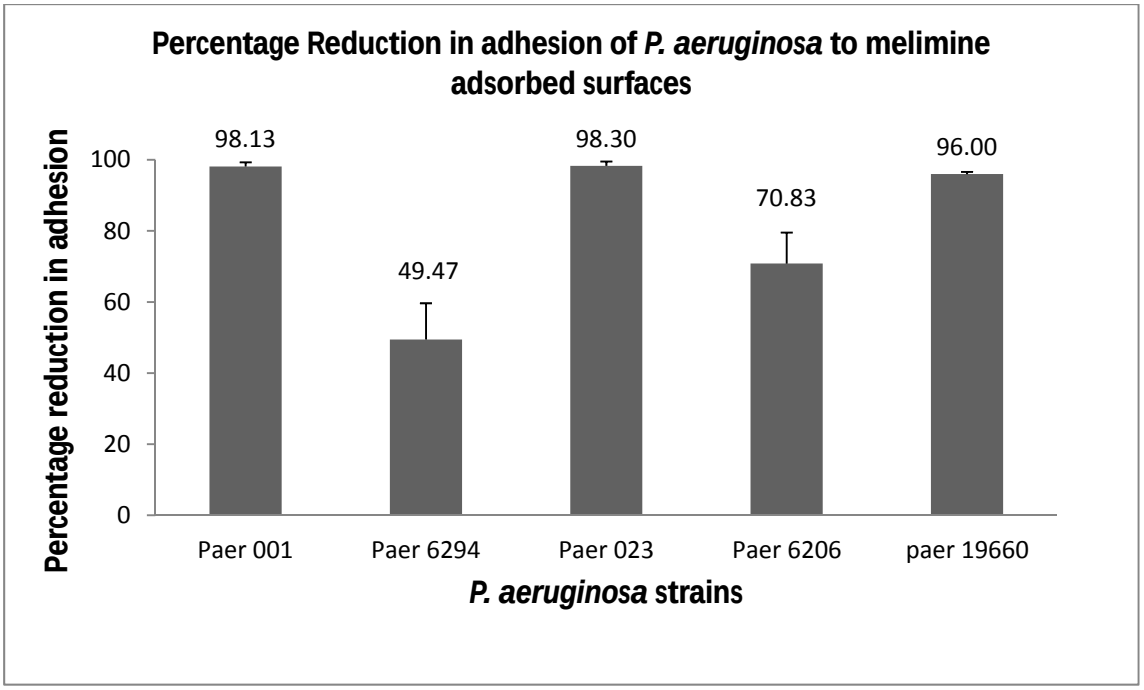


Figure 2.4b: Percentage reduction in adhesion of *P. aeruginosa* to melimine adsorbed surfaces. Standard deviation is shown by the vertical bars (n = 9).

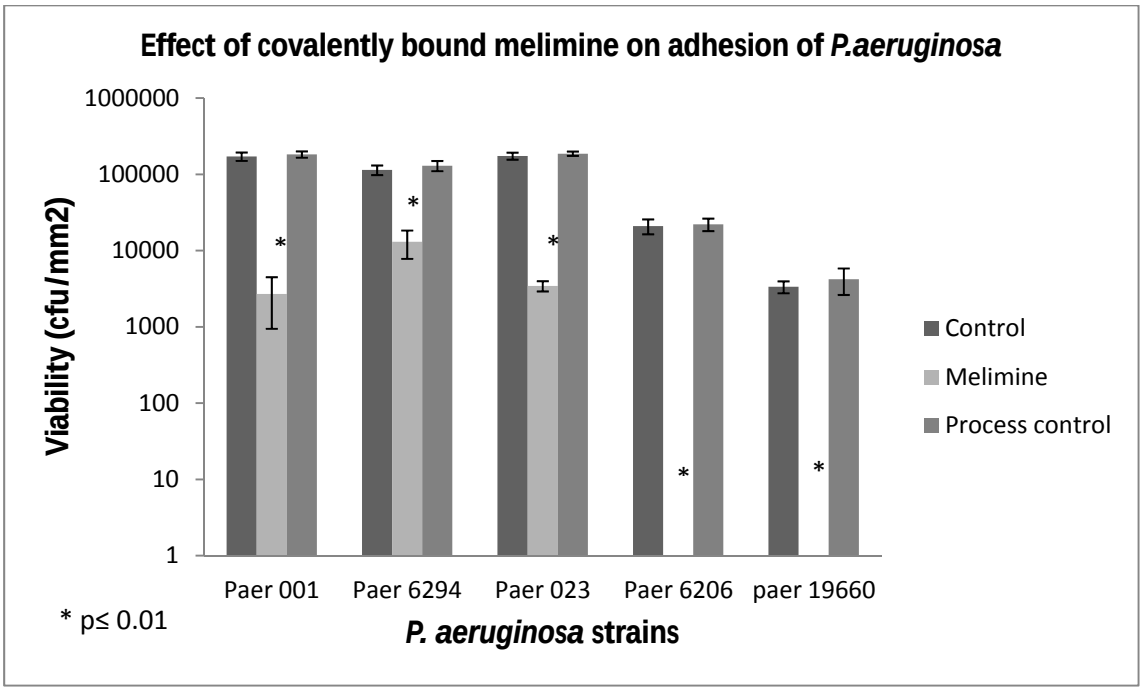


Figure 2.5a: Effect of covalently-bound melimine on adhesion of *P. aeruginosa*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

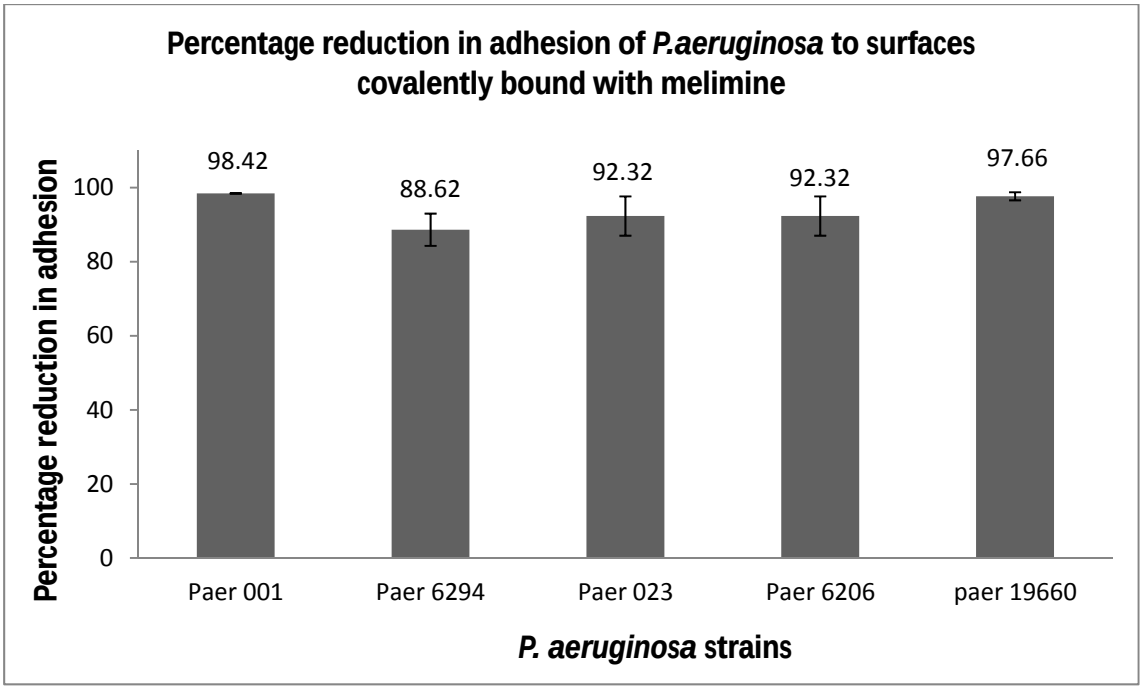


Figure 2.5b: Percentage reduction in adhesion of *P. aeruginosa* to surfaces covalently-bound with melimine. Standard deviation is shown by the vertical bars (n = 9).

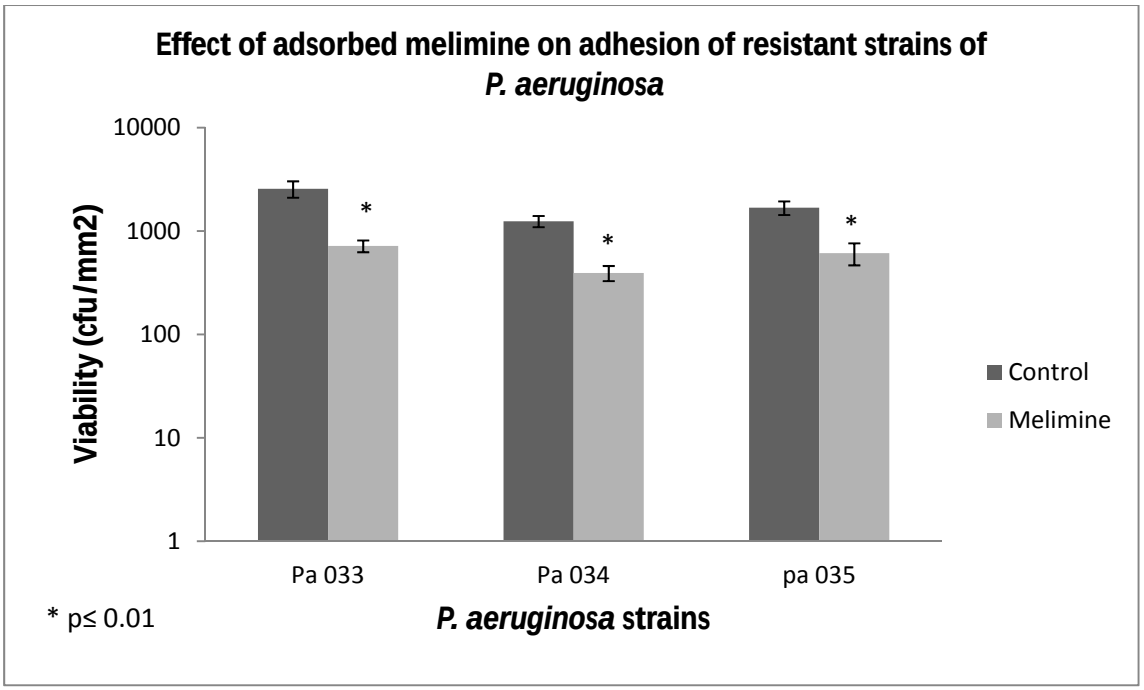


Figure 2.6a: Effect of adsorbed melimine on adhesion of resistant strains of *P. aeruginosa*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

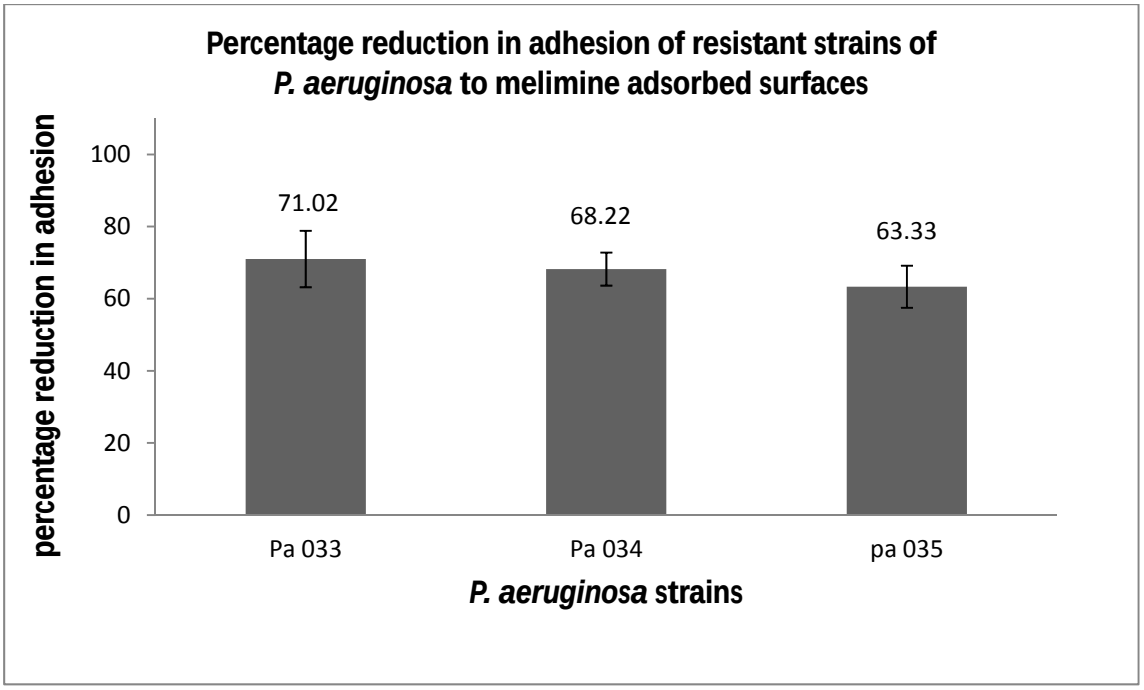


Figure 2.6b: Percentage reduction in adhesion of resistant strains of *P. aeruginosa* to melimine adsorbed surfaces. Standard deviation is shown by the vertical bars (n = 9).

2.3.7 Quantification of peptide on lenses

The amount of melimine attached to contact lens surfaces in both adsorbed and covalently bound form was measured by a method adapted from Cole and Ralston, (1994). The estimated amount of melimine is presented in Table 2.3.

Table 2.3: Estimation of final concentration of melimine on contact lens surfaces

Attachment type	**Initial concentration of melimine (µg/ml) per lens**	**Final estimated amount of melimine (µmol/lens)**
Adsorbed	500	8.6×10^{-3}
Adsorbed	1000	8.8×10^{-3}

Covalently-bound	500	4.0×10^{-3}
Covalently-bound	1000	4.9×10^{-3}

2.4 Discussion

The current study demonstrated the ability of melimine to significantly reduce adhesion of various ocular strains of *S. aureus* and *P. aeruginosa* to contact lens surfaces. This effect was consistent with both adsorbed and covalently-bound melimine. Importantly, lenses with melimine in attached covalent form appeared to be superior in comparison with the adsorbed melimine in case of *P. aeruginosa* strains while for *S. aureus* this difference was not significant (although both forms of attached melimine reduced adhesion by almost 100% for *S. aureus*). The results are in direct agreement with earlier published reports by Willcox *et al* (2008). However, in this study a considerably higher number of strains of both organisms were used (10 strains versus 4 strains). Furthermore, a total of eight strains resistant to antibiotics including five methicillin-resistant strains (MRSA) were also tested with melimine-adsorbed lenses. Indeed, melimine was found to reduce in adhesion of these resistant strains.

The most important aspect of the study was the ability of melimine to reduce adhesion of bacteria while attached to surface in a covalently-bound form. Covalent binding not only gives stability to peptide for long period of times, but also it is less toxic compared to the other systems where peptide is incorporated in to release-based systems (Venter, 1982). Moreover, there are several limitations to accomplish this type of attachment, such as limited affinity of the surface for the peptide; possible alterations of the mechanical properties of the

biomaterial; the limited availability of the antimicrobial to be covalently attached to surfaces (Bagheri *et al*, 2009). Bacterial adhesion to surfaces of biomaterials is the first step to initiate biofilm formation (Donlan, 2001). Melimine's ability to reduce initial adhesion could pave way for its future development as a potential coating on biomaterial surfaces.

It is also interesting to compare the quantification of melimine achieved by covalent binding as opposed to adsorption. Adsorption is a physical phenomenon in which the peptide is attached in random loosely bound fashion, whereas covalent attachment involves EDC coupling of the peptide to the polymer surface (Willcox *et al*, 2008). In the current study, the quantified amount of adsorbed melimine was found to be approximately twice as much as covalently bound melimine. Yet, the efficacy of latter was superior to the former. The reason could be the increased surface availability of the covalently bound form of melimine compared to adsorbed form where there was a possibility of uneven distribution of peptide on the lens surface. Additionally, there was a high probability of adsorbed peptide being washed away during the washing steps markedly reducing the amount of active peptide on lens surfaces (Willcox *et al*, 2008). Finally, orientation differences of melimine in covalently bound and physically adsorbed forms could also be of importance. While the adsorbed form is potentially more likely to adopt a random orientation this may not always be the case as the orientation of adsorbed peptides is determined by interactions of the side groups with surfaces. Some studies have suggested that cationic side chains are likely to interact with quartz surfaces reducing their availability at the surface (Baio *et al*, 2010). However others have demonstrated that orientation differences between adsorbed and covalent attachement through the C-terminus of the antimicrobial peptide Cecropin has large effects on its antimicrobial efficacy (Strauss *et al*, 2010) with the covalently linked forms being more ordered (Ye *et al*, 2010). The contribution of orientation to difference in efficacy of

melimine could be investigated by the use of techniques such as sum-frequency generation vibrational spectroscopy or time-of-flight secondary-ion mass spectrometery.

The activity of melimine against both *P. aeruginosa* and *S. aureus* was determined by using cells from the stationary phase of growth for two reasons: firstly, Williams *et al* (2003), in their study on viability of bacteria on contact lens surfaces, suggested no difference in proportion of viable cells harvested from stationary phase and exponential phase for most of the strains tested. In fact, they reported a higher number of viable stationary phase bacteria adhered to surface of contact lens as compared to viable exponential phase cells. Secondly, the effect of quorum sensing was taken into consideration. Many bacteria regulate gene transcription through quorum sensing (Williams *et al*, 2003). In both *S. aureus* and *P. aeruginosa* quorum sensing appears to occur in stationary phase (Wright *et al*, 2005; Medina *et al*, 2003).

The adhesion times used for *P. aeruginosa* differed from those of *S. aureus* (10 minutes for *P. aeruginosa* and 24 hours for *S. aureus*). The simple reason was the lower initial adhesion of *S. aureus* to contact lens surface as compared to *P. aeruginosa*. Bruinsma *et al*, (2001) reported a ten-fold higher initial deposition rate of *P. aeruginosa* than *S. aureus* on hydrophilic lenses that persisted for two hours. Indeed, the present study demonstrated a lower adhesion of *S. aureus* as compared to *P. aeruginosa* even after 24 hour of adhesion. For all the strains tested the maximum adhesion observed with *S. aureus* was approximately nine times less than that of *P. aeruginosa* strains. Another reason for lower number of counts obtained with *S. aureus* could be the higher rate of detachment of these cells during the washing process (Bruinsma *et al*, 2001). The current study focused on initial adhesion of bacteria to lens surfaces coated with melimine where the maximum adhesion time was 24

hours (for *S. aureus*), however Chen *et al* (2009), demonstrated that melimine coated surfaces reduced adhesion of both *P. aeruginosa* and *S. aureus* for up to 48 hours suggesting an excellent potential for melimine to be further developed as antimicrobial coating.

Results of the present study using three multi-drug resistant strains of *P. aeruginosa* demonstrated their increased resistance to the effects of melimine in adsorbed/soaked form on contact lens surfaces as compared to non multi-drug resistant strains. One possible explanation for these results is that all three strains possess the Type III secretion system-associated cytotoxin *exoU* and cytotoxic phenotype (Zhu *et al*, 2006). Type III secretion systems, produced by many Gram-negative bacteria including *P. aeruginosa*, are complex secretion pathways to transfer exoproducts such as toxins from the bacterial cytoplasm to host cells (Yahr *et al*, 1997; Kubori *et al*, 1998). Lakkis and Fleiszig, (2001) studied the resistance of *P. aeruginosa* to contact lens disinfectants and found that bacterial resistance was correlated well with the cytotoxic strains and with a gene *exsA*, that encodes a protein responsible for cytotoxicity via a type III secretion system. Interestingly, *P. aeruginosa* strain 6294, an invasive strain, also demonstrated increased adhesion on contact lens surfaces adsorbed with melimine. However, a significantly lower reduction was observed for the same strain when melimine was covalently attached to contact lens surfaces indicating, in latter, a possibility of increased surface area and minimal loss of the peptide during the washing steps (Willcox *et al*, 2008). As multi-drug resistant strains of *P. aeruginosa* were only tested with melimine-adsorbed lenses and not with covalently-bound lenses, it is safer to assume that the efficacy of the former could be significantly improved if used in covalently-attached form.

In conclusion, the present study demonstrated the ability of melimine in both adsorbed and covalently-attached form, to reduce adhesion of both *S. aureus* and *P. aeruginosa* and has shown the potential to act as protective coating on surfaces of biomaterials to reduce biomaterial-associated infections.

Chapter 3: Determination of the optimum size and sequence of melimine

3.1 Introduction

Antimicrobial peptides (AMPs) are a promising class of antimicrobial agents to prevent biomaterial associated infections (Kwakman *et al*, 2006). Widely distributed in nature, they are shown to be active against bacteria, fungi, protozoa and viruses (Hancock and Lehrer, 1998; Lehrer and Ganz, 1999; Zasloff *et al*, 2002). It is generally agreed that they target the cytoplasmic membrane in bacteria that leads to alterations in permeability and subsequent failure of membrane to act as an active barrier (Duclohier *et al*, 1989; Boman, 1995; Hancock and Lehrer, 1998).

Although AMPs differ significantly in terms of origin, sequences and structures, most of them are small (12 – 50 amino acids), positively charged (+2 to +9 amino acids), amphipathic (≥ 30% hydrophobic amino acids) and with antimicrobial activity (Giuliani *et al*, 2007; Chen *et al*, 2007; Hilpert *et* al, 2008). However, two of the most common characteristics shared by most AMPs are net positive charge and the ability to adopt amphipathic conformations. The former feature assists the initial interaction of AMPs with the negatively-charged outer layer of bacteria while the latter is important in interaction with the cytoplasmic membrane (Zasloff *et al*, 2002; Giangaspero *et al*, 2001).

Despite the huge potential of the AMPs, currently, only four are being used clinically for treatment as well as prophylaxis (Marr *et al*, 2006). They lacked certain pharmacological characteristics considered essential for drug development that include very low survival in

serum, insufficient bioavailability at the site of infection, poor metabolic stability and slow elimination. These factors in combination of high cost of production contributed to the unwillingness of pharmaceutical industries to invest heavily in this growing field (Hancock, 1999; Otvos, 2008). Compared with conventional antibiotics, the cost of using antimicrobial peptide could run as high as five to twenty times. Currently known AMPs, for example, would cost US $ 50 – 400 /day/patient to provide a dose of 1 mg/kg of body weight (Marr *et al*, 2006; Giuliani, 2007).

Andreu *et* al (1992) noted that length and/or complexity of structures of AMPs are the two most important factors hindering their development into chemically synthesizable drugs. For example, insect peptide cecropin A, although active against both gram-positive and gram-negative bacteria, is unsuitable for further development as a clinical drug because of high cost involved in its production. The simple reason is the fact that it is composed of 37-residues, far too long to be considered for further development as drug (Andreu *et* al, 1983; 1992).

One practical approach to reduce cost associated with AMPs use is to reduce the size without compromising effectiveness against infective agents (Giuliani, 2007). Deslouches *et al* (2005b), using multimers composed of 12-residue lytic base unit (LBU) peptide, demonstrated that LBU2, a 24-residue AMP produced antibacterial activity against *P. aeruginosa* that was comparable to that of 36-residue LBU3. Andreu *et al* (1992), managed to reduce the size of 26-residue long cecropin A and melittin hybrids to a 15-residue analogs with comparable antibacterial activity to that of parent peptide.

The results obtained from chapter 2 clearly demonstrated the potential of melimine to prevent adhesion/reduce viability of both gram-positive and gram-negative bacteria on biomaterial surfaces. The next step in the study is to optimize the length and sequence of melimine. The parent melimine is composed of 29 residues and needs further reduction in size to be considered for further development as an effective coating on surfaces of biomaterials.

The aims of the following experiments include:

- To adsorb various sequences of melimine on surfaces of contact lenses and determine the best sequence of melimine in terms of least number of amino acids with activity comparable to that of parent melimine in reducing adhesion of both *S. aureus* 38 and *P. aeruginosa* 6294.
- To covalently attach the best sequence of melimine on surfaces of contact lenses and to assess whether it remains active in reducing adhesion of both *S. aureus* 38 and *P. aeruginosa* 6294.
- To determine minimum inhibitory concentrations of the best sequence of melimine and to compare it with the parent peptide.

3.2 Materials and Methods

3.2.1 Rationale for alterations in sequences of melimine

The data from chapter 2 suggested an excellent antibacterial activity of melimine bound to biomaterials. The next step involved in the development of melimine was to explore whether comparable antibacterial activity could be achieved by shortening the sequence length of peptide. Melimine is composed of a portion of protamine and a peptide based on melittin (Willcox *et al.* 2008). The sequence of melimine is shown below with melittin and protamine portions shown in italics and bold respectively:

*TLISWIKNKRKQ***RPRVSRRRRRRGGRRRR**-NH_2

Four sequences of melimine with varying net charge, amphipathicity, hydrophobicity and length (based on a peptide sequence analysis tool set up by Tossi *et al*, 2002) used in the study were generated by removal of amino acids from *N* and *C* termini as well as the amino acids internal to the peptide. Table 3.1 shows all the sequences as well as the changes made to the parent peptide. For the sake of convenience peptides were named Melimine 1, 2, 3 and 4. The characteristics of various sequences of melimine and its derivatives are presented in table 3.2.

3.2.2 Synthesis of Peptides

Melimine (TLISWIKNKRKQRPRVSRRRRRRGGRRRR-NH2) and its variants: melimine 1 (TLISWIQRPRVS), melimine 2 (TLISWIKNKRKQRPRVS), melimine 3 (TLISWIQRPRVSRRRRRRGGRRRR) and melimine 4 (KNKRKRRRRRRGGRRRR) were

all synthesized by conventional solid-phase peptide synthesis protocols and were purchased from the American peptide company, Inc (Sunnyvale, CA, USA) at a purity of >90%.

Table 3.1: Various sequences of melimine synthesized to determine optimum size of the peptide

Peptide/Alterations made to the peptide	**Final sequence**
Melimine	TLISWIKNKRKQRPRVSRRRRRRGGRRRR
Removal of KNKRK from melittin portion and RRRRRRGGRRRR from protamine portion	TLISWIQRPRVS (Melimine 1)
Removal of RRRRRRGGRRRR from protamine portion	TLISWIKNKRKQRPRVS (Melimine 2)
Removal of KNKRK from melittin portion	TLISWIQRPRVSRRRRRRGGRRRR (Melimine 3)
Removal of TLISWI and Q from melittin portion and RPRVS from protamine portion	KNKRKRRRRRRGGRRRR (Melimine 4)

.

Table 3.2: Characteristics of various sequences of melimine synthesized to determine optimum size of the peptide

Peptide	Net charge	Amphipathicity	% Hydrophobicity	length
Melimine	+16	0.25	17	29
Melimine 1	+2	0.38	41	12
Melimine 2	+6	0.31	29	17
Melimine 3	+12	0.15	20	24
Melimine 4	+14	0.02	0	17

3.2.3 Bacterial strains and growth conditions

P. aeruginosa 6294 and *S. aureus* 38 used in the study are isolates from cases of human corneal ulcers (Fleiszig et al, 1997; Hume et al, 2005). Bacterial growth conditions used in chapter 2 were maintained in these experiments (section 2.2.2, page 88).

3.2.4 Adsorption and covalent attachment of peptides to contact lens surfaces

Melimine and its variants: Melimine 1; Melimine 2; Melimine 3; and Melimine 4 were adsorbed to contact lens surfaces (Etafilcon A) in final concentrations of 500 µg/ml and 1000 µg/ml by the method described in chapter 2 (section 2.2.3 page 91). Covalent attachment of peptides in final concentrations of 500 µg/ml and 1000 µg/ml to contact lens surfaces (Etafilcon A) via EDC was carried out by a method adapted from Willcox *et al* (2008), with

some modifications. For detailed methods please refer to section 2.2.3 of chapter 2. Controls lenses were reacted with EDC only.

3.2.5 Adhesion assays

Adhesion assays were performed using a protocol adapted from Williams *et al* (2003) and described in detail in chapter 2 (section 2.2.4). EDC-reacted lenses without peptide served as controls. For comparison purposes, in addition to various melimine sequences, the parent melimine sequence was also used in all adhesion assays. Lenses were tested in triplicate in each adhesion assay for control (Etafilcon A without peptide) and test (Etafilcon A with adsorbed or covalently bound melimine). Additionally, where melimine covalently bound to lenses were tested three process control lenses were also used (EDC-coupled lenses without melimine). Adhesion assays were repeated twice.

3.2.6 Statistical analysis

For determination of the shortest melimine sequence with activity comparable to parent peptide melimine, a two-way mixed ANOVA was used with $P \leq 0.05$ considered as statistically significant. Once the shortest peptide sequence was selected, a two-tailed t test was employed for further statistical analysis to compare the differences of means obtained from lenses treated with melimine and its variant to untreated lenses with $P \leq 0.05$ considered as statistically significant.

3.2.7 Selection of melimine variants

All melimine variants adsorbed to contact lens surfaces were tested for their ability to reduce adhesion of both *S. aureus* 38 and *P. aeruginosa* 6294. The percentage reduction in adhesion by each melimine variant was compared with parent melimine. The shortest peptide (least

number of amino acids) with the activity comparable to melimine ($P \leq 0.01$) was used for covalent attachment and subsequent adhesion experiments.

3.2.8 Determination of the minimum inhibitory concentrations

The minimal inhibitory concentrations (MIC) of melimine and the selected melimine variant for *P. aeruginosa* 6294 and *S. aureus* 38 were determined by using the modified broth microdilution assay described by Freidrich *et al*. (2000). Briefly, serial dilutions of the peptide were made in 0.2% bovine serum albumin, 0.1% acetic acid in a 96-well polypropylene microtitre plate (Nunc, F96, Denmark). Each well was inoculated with 200µl of the test organism in Muller Hinton Broth (MHB) to a final concentration of 5×10^5 colony forming units (cfu)/ml. MHB is used as it contains high concentrations of dicarboxylic or phosphocarboxylic acids and polyanionic peptides (Turner *et al*. 1998) and gives a useful estimation of the efficacy of the peptide in the presence of these species. The MIC was taken as the lowest peptide concentration at which growth was inhibited after 24 h of incubation at 37^oC.

3.2.9 Quantification of peptide on lenses

Melimine and its selected variant were quantified in both adsorbed and covalently-bound form by a protocol adapted from Cole and Ralston (1994) as described in chapter 2 (see section 2.2.5, page 70).

3.3 Results

3.3.1 Effect of adsorbed peptides on adhesion of *S. aureus*

Four altered sequences of melimine were tested for the ability to reduce adhesion of *S. aureus* 38 on surfaces of contact lenses. Compared to controls, three of the four sequences, melimine

2, melimine 3, and melimine 4, showed significant reduction in staphylococcal viability ($P \leq 0.01$), after 24 hours of adhesion (fig.3.1). The percentage reduction in adhesion was found to be 99.93 ± 0.07 for melimine; 5.56 ± 4.94 for melimine 1; 96.42 ± 1.39 for melimine 2; 99.97 ± 0.03 for melimine 3; 99.99 ± 0.01 for melimine 4 (fig. 3.2).

The results obtained were compared with the parent peptide melimine. Compared with melimine, melimine 2 appeared to be significantly less effective in reducing adhesion of Sa 38 on lens surfaces ($P \leq 0.01$). The activity of melimine was not found to be statistically different to melimine 3 and melimine 4 ($P > 0.01$). Similarly, no significant difference ($P > 0.01$) was observed in activities of melimine 3 and melimine 4 (fig. 3.1).

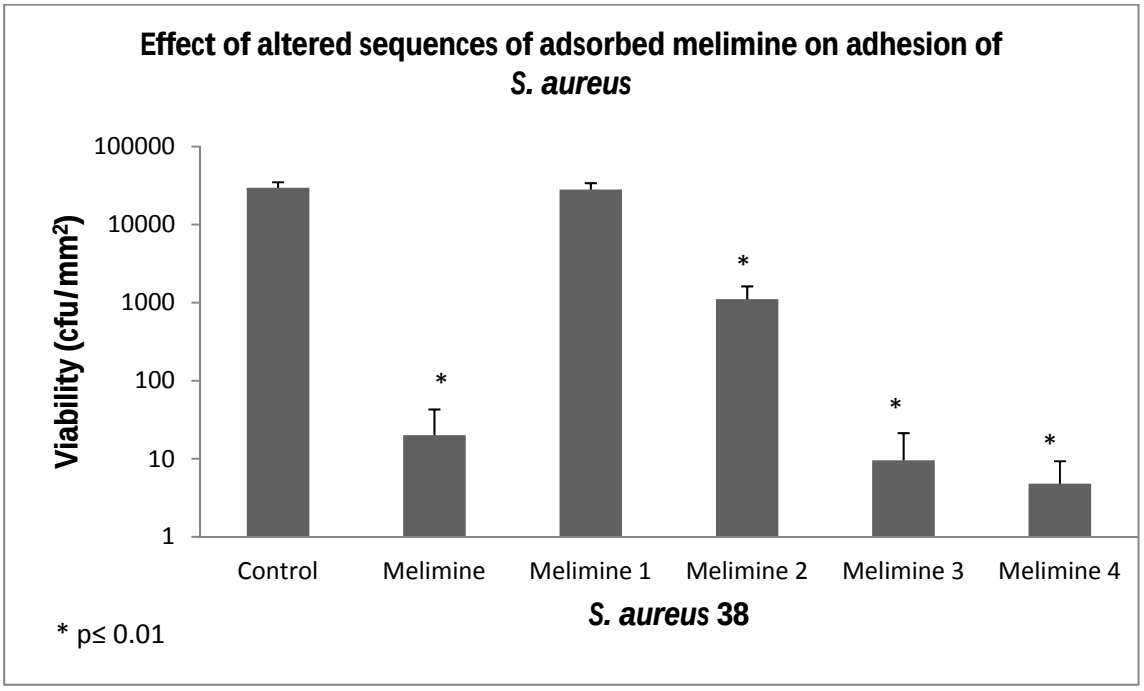


Figure 3.1: Effect of altered sequences of adsorbed melimine on adhesion of *S. aureus*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

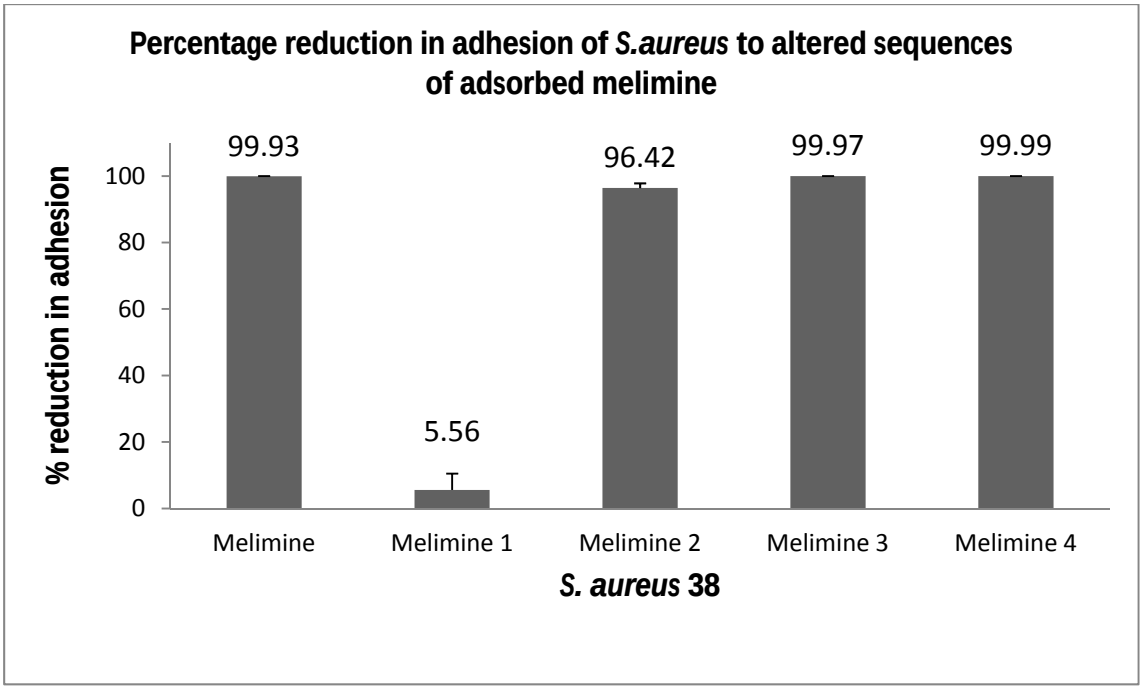


Figure 3.2: Percentage reduction in adhesion of *S. aureus* to altered sequences of adsorbed melimine. Standard deviation is shown by the vertical bars (n = 9).

The above result suggested no significant difference in activities among melimine, melimine 3 and melimine 4 in reducing adhesion of Sa 38 on lens surfaces. Of the three peptides, melimine 4 is composed of least number of amino acids i.e. 17, while melimine 3 consists of 24 amino acids and the parent melimine has 29 amino acids. Based on these observations, melimine 4 was chosen for covalent attachment and subsequent adhesion experiments.

3.3.2 Effect of covalently-bound melimine 4 on adhesion of *S. aureus*

Melimine 4 was covalently attached to lens surfaces (*etafilcon A*) by a method adapted from Willcox *et al* (2008). No significant difference in viable counts ($P = 0.349$) was observed between controls (lenses in PBS) and process controls (lenses treated with EDC but without melimine or melimine 4). Compared to controls both EDC-Melimine and EDC-Melimine 4 showed significant reductions in adhesion of Sa 38 viable counts ($P \leq 0.01$; fig. 3.3). The percentage inhibition in adhesion was found to be 99.3 ± 0.05 for EDC-Melimine and 98.20 ±

1.57 for EDC-Melimine 4 (fig. 3.4). When the activities of EDC-Melimine and EDC-Melimine 4 were compared, no significant difference was observed in their ability to reduce adhesion of Sa 38 ($P > 0.01$; fig. 3.3 and fig. 3.4).

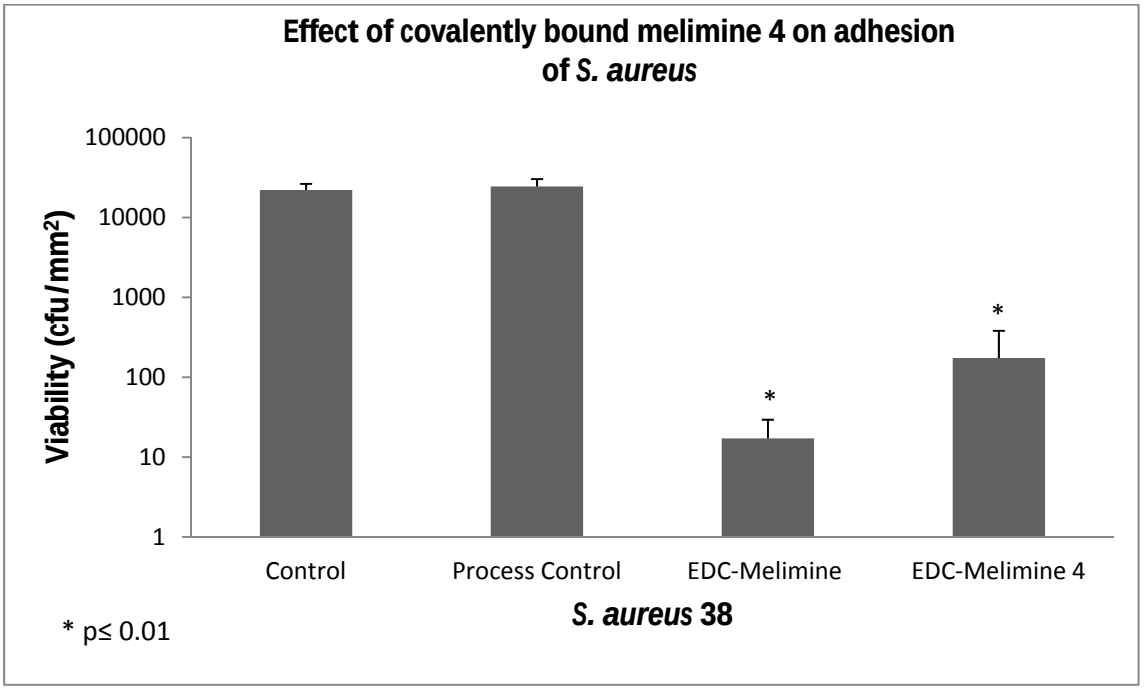


Figure 3.3: Effect of covalently bound melimine 4 on adhesion of *S. aureus*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

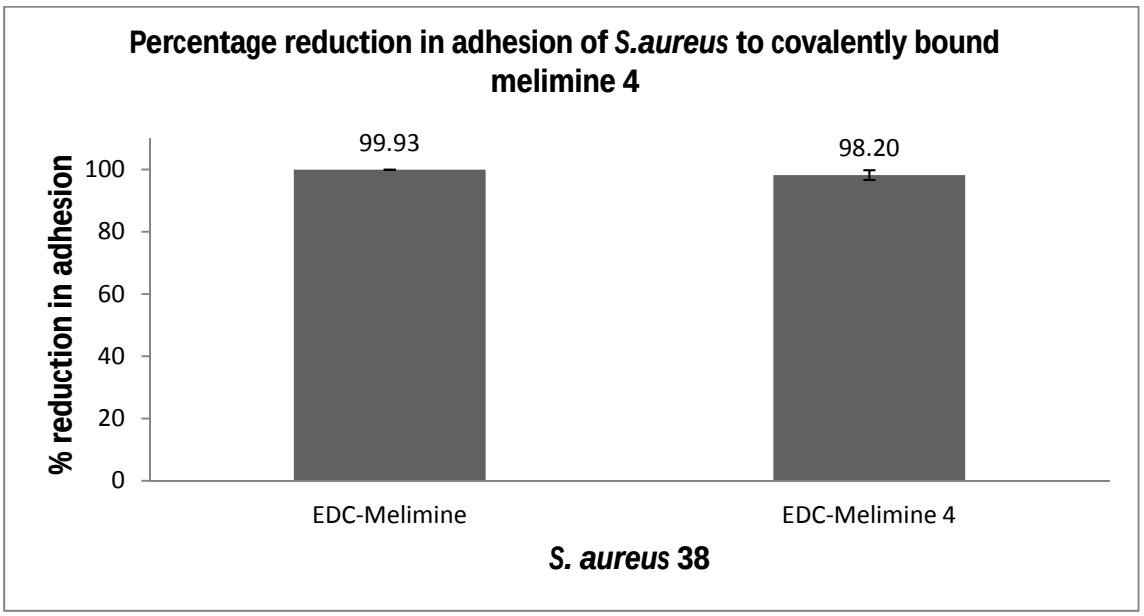


Figure 3.4: Percentage reduction in adhesion of *S. aureus* to covalently bound melimine 4. Standard deviation is shown by the vertical bars (n = 9).

3.3.3 Effect of adsorbed peptides on adhesion of *P. aeruginosa*

Of the four altered sequences of melimine tested in the study two appeared to cause no reduction in viability of *P. aeruginosa* 6294 after 10 minutes of adhesion. The viability counts obtained for both melimine 1 and melimine 2 were not found to be statistically different ($P = 0.117$ for melimine 1; and $P = 0.211$ for melimine 2) to untreated controls (fig. 3.5). However, both melimine 3 and melimine 4 showed significant reduction in viability counts ($P \leq 0.01$). Compared to parent melimine, a higher reduction in viability was observed for both melimine 3 as well as melimine 4 ($P \leq 0.01$; fig. 3.5).

The percentage inhibition in adhesion of Paer 6294 was 57.86 ± 2.67 for lenses adsorbed with melimine, 72.34 ± 2.33 for lenses adsorbed with melimine 3 and 82.30 ± 0.96 for lenses adsorbed with melimine 4 (fig. 3.6). The percentage inhibition observed with melimine 4 was statistically significantly different to melimine as well as melimine 3 ($P \leq 0.01$).

The above results clearly suggested that of the four melimine sequences tested, melimine 4 appeared to be the most effective sequence in reducing adhesion of Paer 6294 (fig. 3.5 and 3.6) and for this reason was used for covalent attachment on lenses.

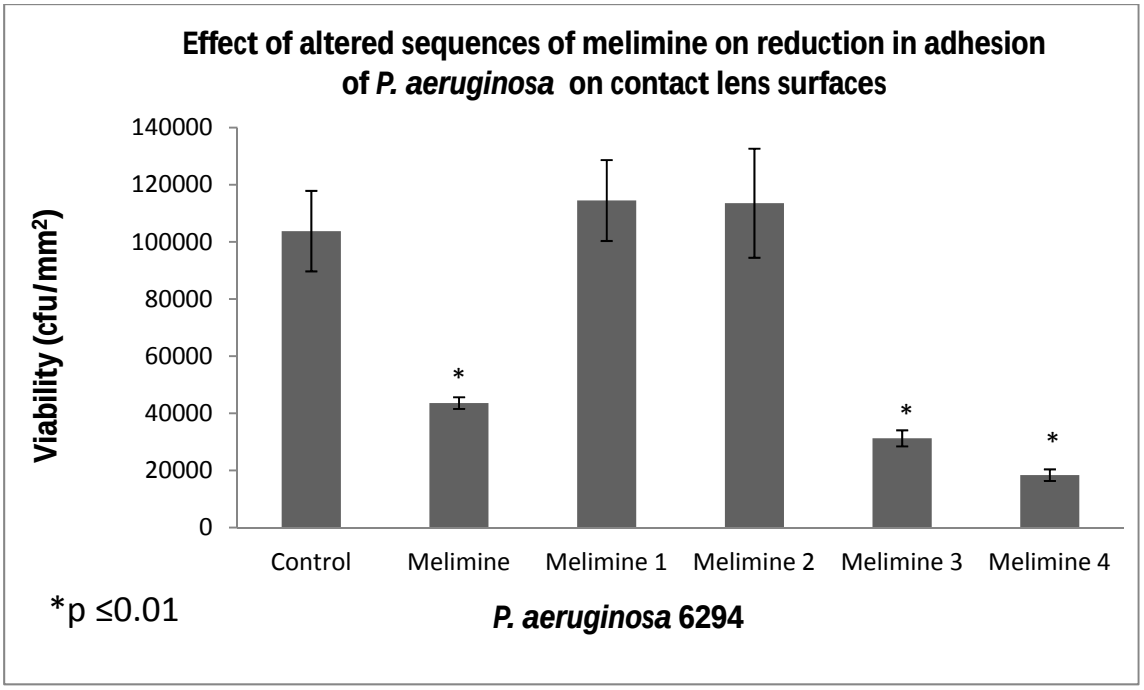


Figure 3.5: Effect of altered sequences of adsorbed melimine on adhesion of *P. aeruginosa*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

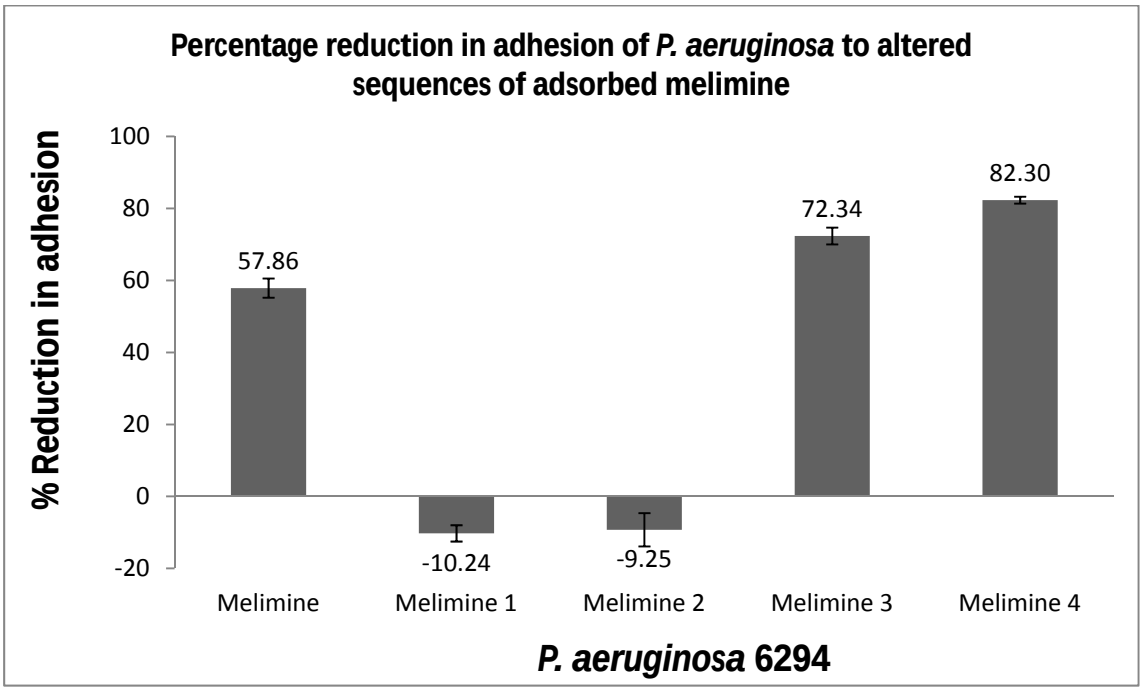


Figure 3.6: Percentage reduction in adhesion of *P. aeruginosa* to altered sequences of adsorbed melimine. Standard deviation is shown by the vertical bars (n = 9).

3.3.4 Effect of covalently-bound melimine 4 on adhesion of *P. aeruginosa*

Melimine 4 covalently attached to contact lens surfaces via EDC coupling was further tested for its potential to reduce adhesion of *P. aeruginosa* 6294 on contact lens surfaces. After 10-minutes adhesion, no significant difference in viability counts was found between untreated negative controls and EDC treated process controls ($P > 0.50$; fig. 3.7). Compared to controls, both parent melimine and melimine 4 caused significant reductions ($P \leq 0.01$) in viable counts (fig. 3.7). The reduction in viable counts obtained from adhesion of Paer 6294 to lenses covalently attached with melimine was not found to be statistically different ($P = 0.102$) to that of melimine 4 (fig. 3.7).

The percentage inhibition in adhesion by EDC-melimine was found to be 85.66 ± 5.88 when compared to controls while that of EDC-melimine 4 was found to be 83.19 ± 6.88 (fig. 3.8). No significant difference ($P = 0.66$) was obtained when EDC-melimine was compared with EDC-melimine 4 (fig. 3.8).

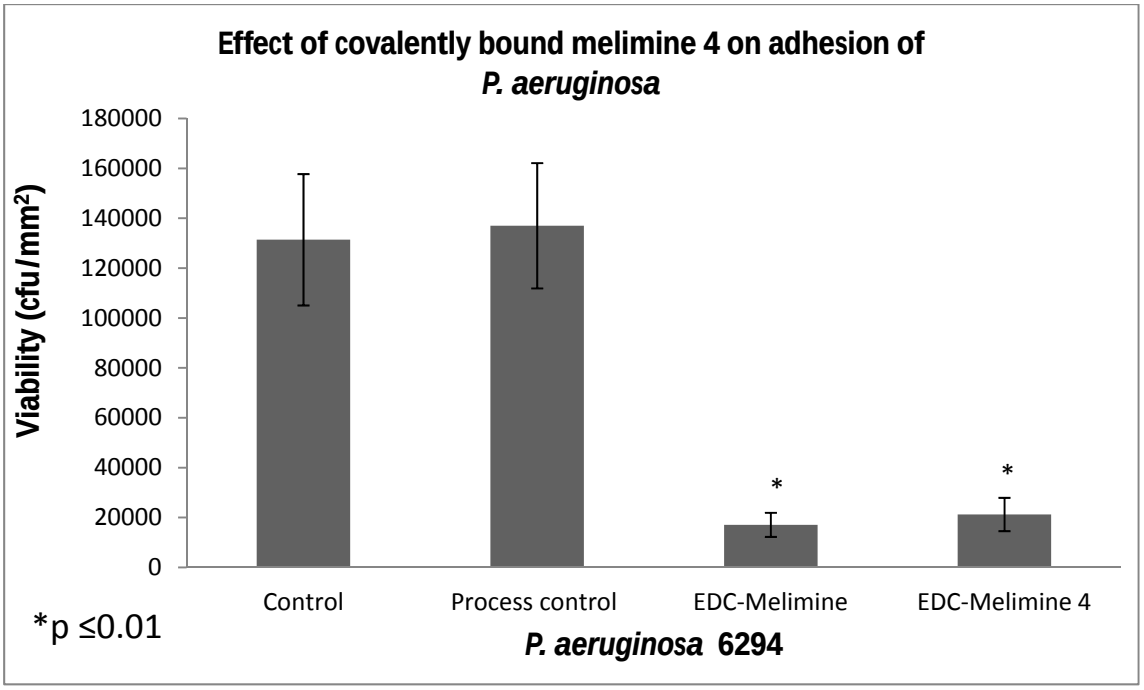


Figure 3.7: Effect of covalently bound melimine 4 on adhesion of *P. aeruginosa*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

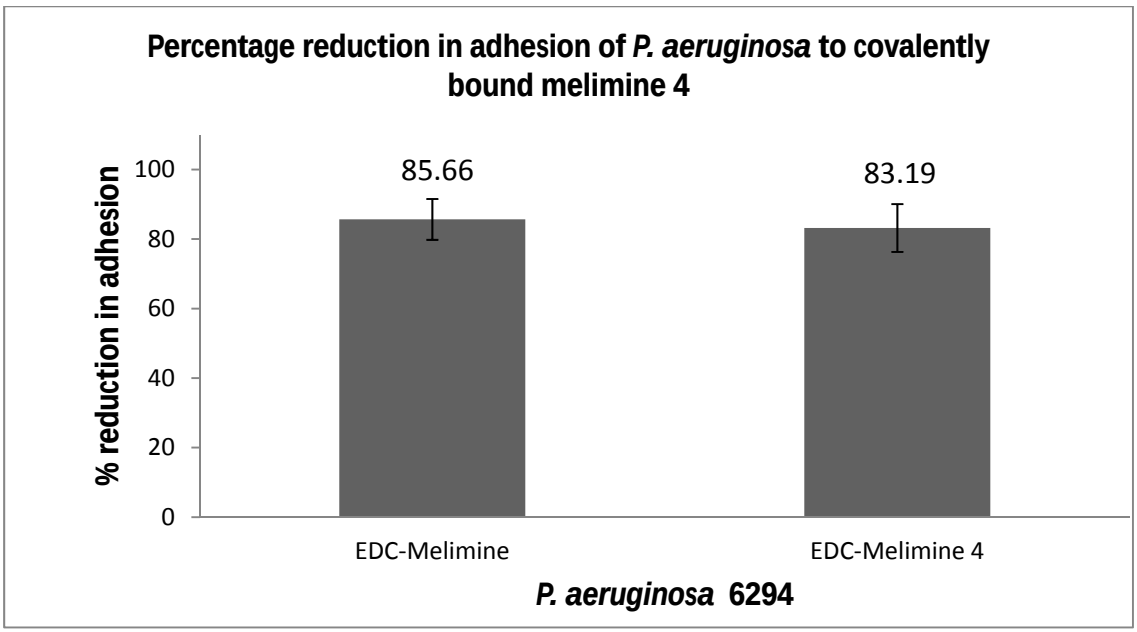


Figure 3.8: Percentage reduction in adhesion of *P. aeruginosa* to covalently bound melimine 4. Standard deviation is shown by the vertical bars (n = 9).

3.3.5 Minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of melimine and melimine 4 were found to be 3.9 µM and 15.6 µM respectively for *S. aureus* 38. For *P. aeruginosa*, MIC for both melimine and melimine 4 was found to be 125 µM.

3.3.6 Quantification of peptide on lenses

The amount of melimine attached to contact lens surfaces in both adsorbed and covalently bound form was measured by a method adapted from Cole and Ralston (1994). The estimated amount of melimine is presented in Table 3.3.

Table 3.3: Estimation of final concentration of melimine on contact lens surfaces

Attachment type	**Initial concentration of peptide (µg/ml) per lens**	**Final estimated amount of melimine (µmol/ lens)**	**Final estimated amount of melimine 4 (µmol/ lens)**
Adsorbed	500	8.8×10^{-3}	9.6×10^{-3}
Adsorbed	1000	8.9×10^{-3}	1.1×10^{-2}
Covalently-bound	500	4.2×10^{-3}	8.2×10^{-3}
Covalently-bound	1000	5.1×10^{-3}	9.4×10^{-3}

3.4 Discussion

Various studies have shown that the sequence length of antimicrobial peptides could be reduced by determination of the active regions of the peptide and deletion of those residues that appear to lack antimicrobial activity (Giangaspero *et al*, 2001; Li *et al*, 2006; Andra, *et al*, 2007). For example, Li *et al*, (2006) in their study on a 37-residue human LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) found that *N*-terminal fragment LL-37 (1 – 12) was inactive against *E. coli*, while the *C*-terminal fragment LL-37 (13 – 37) was antibacterial. Furthermore, they identified a 13-residue LL-37 (17– 29) with antibacterial activity against *E. coli* based on total correlated spectroscopy by trimming non-essential regions (TOCSY-trim), a technique that recognize disordered or weekly micelle binding regions in a peptide (Griesinger *et al*, 1988) that have little effect on overall antibacterial activity and hence their deletion leads to an overall reduction in the size of the peptide. The results from the current study suggested that the *N*- terminal region composed primarily of poly-arginine (RRRRRRGGRRRR) is vital to melimine activity as this region was retained in both melimine 3 (TLISWIQRPRVSRRRRRRGGRRRR) and melimine 4 (KNKRKRRRRRRGGRRRR).

Unlike melimine 3, the melimine 4 sequence also contains three lysine residues which could account for its superior activity in adsorbed form against *P. aeruginosa* (fig. 3.5). However, the fact that melimine 2 (TLISWIKNKRKQRPRVS) contained three lysine and three arginine residues but lacked the *N*- terminal poly-arginine region was active in adsorbed form against *S. aureus* and not against *P. aeruginosa*, suggests that the presence of lysine residues is important to inhibit adhesion of *S. aureus* only, while the presence of *N*- terminal poly-

arginine region is essential for activity against both organisms. Shafer *et al*, (1996) in their study on the role of individual amino acids on broad-spectrum antimicrobial activity of an AMP CG 117-36 (RPGTLCTVAGWGRVSMRRGT) reached somewhat similar conclusions. They reported that the replacement of any of the four arginine residues with lysine, alanine, or citrulline resulted in reduction in bactericidal activity against both *P. aeruginosa* and *S. aureus*. They hypothesized that arginine has a superior potential to form hydrogen bonding (five for arginine as compared to three for lysine) with the negatively–charged bacterial surfaces that helps its initial interaction with the bacterial envelope and subsequent killing (Shafer *et al*, 1996).

Despite variability in various physicochemical characteristics, a net positive charge and amphipathicity stands out as two of the most common characteristics shared by most AMPs (Zasloff *et al*, 2002; Giangaspero *et al*, 2001). Table 3.2 depicts the net charge of melimine and all of its derivatives. The parent peptide melimine has a net charge of +16. Interestingly, both melimine 3 and melimine 4, two of the best peptide sequences identified in the present study have higher net positive charges than the other two sequences, melimine 1 and melimine 2. The net positive charges for melimine 3 and melimine 4 were +12 and +14 respectively. Melimine 2 with a net positive charge of +6 was found to be effective in adsorbed form in reducing adhesion of *S. aureus* but not *P. aeruginosa*. Melimine 1, the peptide sequence with the least positive charge (+2) was found to be ineffective in adsorbed form against both *S. aureus* and *P. aeruginosa*. These results correlated well with a number of studies where an increase in net positive charge conferred an improvement in antimicrobial activity of many AMPs (Giangaspero *et al*, 2001; Jiang *et al*, 2009). Although some reports suggested no relationship between increased net charge and enhanced antimicrobial activity (Scott *et al*, 1999; Mangoni *et al*, 2000), many agreed that an increase in cationicity can lead

to increased bactericidal activity, although there is a threshold of cationicity beyond which there is no further improvement in activity (Dathe and Wieprecht 1999; Dathe *et al*, 2001).

Apart from cationicity, it is widely accepted that the ability to assume amphipathic structures is the second most important characteristics of any given AMP (Dathe et al, 1997; van't Hof et al, 2001). To compare amphipathicities of melimine and its variants, a peptide sequence analysis tool set up by Tossi *et al*, (2002) was used and the values for melimine and its derivatives are presented in table 3.2. The parent peptide melimine has a 25 % maximum possible amphipathicity, while two of the least active sequences of melimine variants: melimine 1 and melimine 2 have 38% and 31% maximum possible amphithicities respectively. Interestingly, melimine 3 and melimine 4, two of the best peptide variants of melimine have amphipathicities of 15% and 2% of maximum possible amphithicities respectively. Importantly, low amphipathicity values of melimine 4 combined with excellent antimicrobial activity both in adsorbed and covalently bound form, lead to an assumption of it being the least cytotoxic of all melimine variants as a high degree of amphipathicity is generally correlated with an increased toxicity towards zwitterionic eukaryotic cells (Dathe and Wieprecht 1999).

Another important parameter that determines the ability of AMPs to interact with bacterial membranes is the hydrophobicity which is defined as the percentage of hydrophobic amino acids in a peptide (Yeaman and Yount, 2003). Most antimicrobial peptides have between 30 to 50% of these residues (Yeaman and Yount, 2003; Hilpert *et* al, 2008). Table 3.2 represents the percentage hydrophobicities for melimine and its derivatives. No correlation was observed between hydrophobicity and antimicrobial activity for melimine or any of its

derivatives. Surprisingly, melimine 4, with no hydrophobic residues was found to be effective in reducing adhesion of both *S. aureus* and *P. aeruginosa*. This data suggest that both the parent melimine and its most successful variant, melimine 4, are relatively hydrophilic and non-amphipathic (< 30% hydrophobic residues) in contrast to typical AMPs with 30 – 50% hydrophobic amino acids (Yeaman and Yount, 2003; Hilpert et al, 2008).

The relatively hydrophilic and non- amphipathic nature of melimine 4 suggest a different mechanism of action from that of other common AMPs that assume amphipathic structures necessary for their interaction with the negatively charged bacterial membranes (Dathe et al, 1997; van't Hof et al, 2001). Although mode of action of melimine 4 was not explored in the current study, it could be assumed that a very high net positive charge resulted in strong interaction with the bacterial membranes rendering them inactive. The apparent mode of action was not too dissimilar to another hydrophilic, non-amphipathic antimicrobial peptide Androctonin that seemed to kill bacteria by binding only to the negatively charged surfaces of the bacteria without any penetration in the hydrophobic core that requires amphipathic structures (Hetru *et al*, 2000).

In conclusion, the current study established that melimine could be further reduced from 29 residues to 17 residues whilst retaining activity against *S. aureus* as well as *P. aeruginosa*. However, further studies need to undertaken on its potential cytotoxicity to eukaryotic cells. Another important aspect worth exploring is to find out how long it can retain its activity while attached to surfaces. Adhesion experiments conducted in the current study demonstrated that it was still active even after 24 hours of adhesion for *S. aureus*. Additionally, *in vivo* experiments where melimine is attached to surfaces and tested for

efficacy will be required before it could be established as a genuine candidate for coating on surfaces of biomaterials.

Chapter 4: Interaction of the antimicrobial peptide melimine with bacterial membranes

4.1 Introduction

Cationic antimicrobial peptides (AMPs) are a key component of the innate immune response (Gordon *et al*, 2005) and hundreds of such peptides have been identified (Wang and Wang, 2004). AMPs were originally thought to act solely by permeabilisation of bacterial cell membranes (Zhang *et al*, 2001), however there is an increasing body of evidence suggesting that some AMPs may also act through alternative mechanisms or on multiple targets, similarly to a disinfectant rather than a traditional antibiotic (Jenssen *et al*, 2006). AMPs are of particular interest as candidates for the development of new antimicrobial agents because of their potential for broad-spectrum activity, their rapid action and low levels of induced resistance (Gordon *et al*, 2005).

We have recently described a novel AMP, melimine, which meets these criteria and has the advantage of being of low toxicity to mammalian erythrocytes and retaining activity following sterilisation (Willcox *et al*, 2008). In addition, melimine retains activity when bound to surfaces (Chen *et al*, 2009), suggesting that cell surface interactions may be of primary importance in its activity. Melimine is derived from portions of melittin and protamine and has the following amino acid sequence: TLISWIKNKRKQRPRVSRRRRRRGGRRRR (Willcox *et al*, 2008). Melittin has been

extensively studied and has been shown to adopt a disordered structure in solution that converts to an α-helical structure in lipid membranes (Lam *et al*, 2001). The mechanism by which melittin acts is thought to be membrane disruption, via a short-lived channel that disintegrates, allowing a small proportion of melittin to translocate across the membrane (Matsuzaki *et al*, 1997). The membrane potential can supply sufficient energy to allow melittin to span the membrane and so may drive this process (Kempf *et al*, 1982). The resulting translocation may then allow interaction of the peptide with bacterial cytoplasmic proteins. Such interactions have been suggested for melittin and mammalian cytoplasmic proteins (Mousli *et al*, 1990). Although less well characterised, protamine has been shown to adopt an extended random coil conformation in aqueous solution but possesses the ability to form a partially ordered conformation containing the α-helix structure under particular circumstances (Ebert *et al*, 1990). Protamine is thought to act in an antimicrobial capacity by disruption of cellular energy transduction and nutrient uptake by bacteria via cytoplasmic membrane disruption (Aspedon and Groisman, 1996).

However, is it difficult to predict empirically the spectrum of activity and mechanism of action of a peptide, as peptides with similar secondary structures or small changes in the primary sequence can have quite different antimicrobial activities (Abrunhosa, 2005). This unpredictability may reflect a delicate balance between hydrophobicity and charge. In addition, the differing membrane potentials of bacterial cells (Ruhr and Sahl, 1985) are likely to be important and so it is probable that the mode of action may vary according to the target cell.

In this study, we have investigated the interaction of melimine with the bacterial membranes of *P. aeruginosa* and *S. aureus* to gain an understanding of the differing mechanisms by

which a single peptide may interact with these clinically important pathogens. This information will enhance understanding in the further development of this novel and promising class of antimicrobial compounds, the cationic peptides.

4.2 Materials and Methods

4.2.1 Synthesis of melimine

Melimine was synthesised by conventional solid-phase peptide synthesis protocols and was obtained from the American Peptide Company, Inc. (Sunnyvale, CA) at a purity of >90%.

4.2.2 Spectroscopic analysis

Intrinsic fluorescence spectra of melimine were recorded using a Hitachi F-2500 fluorescence spectrophotometer (Schaumberg, Illinois, USA) with an excitation wavelength of 295 nm and an emission range of 300–400 nm. The concentration in each case was 265 μM. Phosphate-buffered saline (PBS) (pH 7.4) was the aqueous medium used. Effects of the presence of the anionic surfactant sodium dodecyl sulphate (SDS) and the zwitterionic surfactant 3-[(3-cholamidopropyl) dimethylammonio] propanesulphonic acid (CHAPS) were also examined. Circular dichroism (CD) spectra were recorded with a Jasco J-715 CD spectropolarimeter (Great Dunmow, Essex, UK) at room temperature (ca. 25 °C). Melimine concentrations of 265 μM were used for recording the far-ultraviolet CD spectra using 0.1-cm path length quartz cells. The effect of pH on the spectrum (and thus the secondary structure) was evaluated under acidic (pH 4.5) and basic (pH 9.4) conditions. In order to analyse the effect of solvent polarity on the secondary structure of the peptide, the water-soluble organic

solvent trifluoroethanol (TFE) was used, and CD spectra of the peptide (265 μM) were recorded at 40% (v/v) TFE at several pH values. The CD spectrum of melimine was also recorded in the presence of SDS (20 mM). The excitation and emission slits were set at 2.5 nm. Three scans of each spectrum were averaged, smoothed and baselines (of buffer alone) subtracted. The intensity of fluorescence is expressed in arbitrary units (AU). Values are expressed as mean residue molar ellipticity, in degrees, assuming the conventionally accepted mean residue molecular weight of 115 Da.

4.2.3 Bacterial strains

Pseudomonas aeruginosa 6294 and *S. aureus* 38 used in this study are clinical isolates. For Atomic force Microscopy, *Pseudomonas aeruginosa* strain Paer 23 (isolated from corneal ulcer at Manchester Royal infirmary; Taylor et al, 1998) and *Staphylococcus aureus* ATCC 12600 (NCTC 8532) were used. Bacterial strains unless otherwise stated were prepared by growing overnight in Mueller–Hinton broth (MBH) (Oxoid, Basingstoke, UK) and 200 μL of culture was inoculated into fresh medium and incubated at 37 °C with shaking to exponential phase. Bacteria were collected by centrifugation, washed twice and re-suspended in MHB. The bacterial concentration was adjusted turbidimetrically and the numbers were confirmed retrospectively by dilution and plate count.

4.2.4 Determination of minimum inhibitory concentration (MIC) and bactericidal concentration (MBC)

MICs of melimine were determined using the modified broth microdilution assay described by Friedrich *et al*, (2000). Briefly, serial dilutions of the peptide were made in 0.2% bovine

serum albumin and 0.1% acetic acid in a 96-well microtitre plate (Nunc, Roskilde, Denmark). MHB was used as it contains high concentrations of anionic species (Turner *et al*, 1998). The MIC was taken as the lowest peptide concentration at which growth was inhibited after 24 h incubation at 37 °C.

MBCs of melimine against the bacterial strains were determined in PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L and KH_2PO_4 0.2 g/L) as previously described by Bucki *et al*, (2004). Cells were grown to exponential phase in MHB, collected by centrifugation and washed three times in PBS. Bacteria [5×10^5 colony-forming units (CFU)] were then incubated at 37 °C for 30 min in PBS containing serial dilutions of melimine. Viable counts were then performed. The MBC of melimine was taken as the concentration at which no colonies grew following overnight incubation at 37 °C.

4.2.5 Fluorescence and scanning electron microscopy

For electron microscopy, bacterial samples were prepared as described above to a concentration of 5×10^7 CFU/mL. Bacteria were incubated with melimine at the MBC for 30 min before being allowed to interact with glass slides for 15 min, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 h and washed three times with 0.1 M phosphate buffer prior to dehydration through a graded series of ethanols. After critical point drying, samples were mounted on 13 mm stubs and were chromium-coated using an EmiTech K575x high-resolution sputter coater (East Grinstead, West Sussex, UK), with an exposure time of 30 s and 125 mA current, and were observed with an FEI Quanta 200 ESEM

(Hillsboro, Oregon, USA) under high vacuum, with a 10 kV beam and a spot size of 4. Images were obtained from at least five representative areas of each specimen.

Samples were stained with LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR). Samples were observed with a Leitz Diaplan fluorescent microscope (Leica Microsystems, Deerfield, IL) and the numbers of bacteria staining green (intact membrane) and red (damaged membrane) were examined.

4.2.6 Effect of Mg^{2+} on antimicrobial activity

To examine the ability of melimine to destabilise bacterial membranes by competing for the divalent cation binding sites, the effect of Mg^{2+} on the activity of melimine was determined by an assay described by Friedrich *et al*, (1999). *Pseudomonas aeruginosa* and *S. aureus* cultures were prepared as described above at a concentration of 5×10^7 CFU/mL in PBS containing 20 mM $MgSO_4$ and melimine at 7.8 μM or 250 μM (four-fold below the MBC) for *P. aeruginosa* and *S. aureus*, respectively. Controls were bacteria in the same buffer without melimine. Bacteria were incubated at 37 °C for 30 min and viable bacterial numbers were determined by dilution and plate count. Results are expressed as a percentage of the controls.

The assay was repeated twice and the mean colony counts obtained for cells exposed to melimine were compared with those for cells exposed to melimine in the presence of Mg^{2+} using a two-tailed Student *t*-test ($P < 0.05$).

4.2.7 Ability of melimine to disrupt the outer membrane of *Pseudomonas aeruginosa*

The ability of melimine to alter the permeability of the outer membrane of *P. aeruginosa* was examined using the method described by Bengoechea *et al*, (1996). Briefly, exponentially growing cells were pelleted and re-suspended in PBS to a final concentration of 10^9 CFU/mL. Cells were then exposed to 7.8 μM melimine for 10 min at 37 °C. Following centrifugation, bacterial cells were re-suspended in PBS containing 0.5% sodium deoxycholate (Sigma-Aldrich, St Louis, MO). The suspension was incubated for an additional 10 min at 37 °C and cell lysis was measured as a decrease in optical density at 450 nm (OD_{450}). Results were expressed as the percentage of OD_{450} of controls (cells not exposed to melimine). Polymyxin B (50 μg/mL) (Fluka Biochemika, Buchs, Switzerland) was used as a positive control. The assay was repeated twice and the data were examined for significance using a two-tailed Student's *t*-test ($P < 0.05$).

4.2.8 Effect of melimine on cytoplasmic membrane permeability

The ability of melimine to alter cytoplasmic membrane permeability was determined using the membrane potential-sensitive cyanine dye diSC_3-5 (Sigma-Aldrich) based on the method of Wu *et al*, (1999 b) with some modifications. Briefly, *S. aureus* strain 38 and *P. aeruginosa* 6294 were grown to mid-log phase in MHB, pelleted, washed in 5 mM HEPES and 20 mM glucose (pH 7.2) and re-suspended in the same buffer to an OD_{600} of 0.05. For *P. aeruginosa*, ethylene diamine tetra-acetic acid (EDTA) was added to a final concentration of 0.5 mM. This concentration of EDTA was used to facilitate the permeabilization of the dye through outer membrane. Preliminary experiments showed that at that concentration no change in MIC to melimine was observed (data not shown). The dye diSC_3-5 was added to the resulting

bacterial suspensions to a final concentration of 4 μM and bacteria were allowed to take up the dye for 60 min. To equilibrate the cytoplasmic and external [K^+], KCl was added to a final concentration of 100 mM and the fluorescence intensity was measured using a Perkin Elmer Luminescence Spectrometer (Waltham, MA) with excitation and emission wavelengths of 622 nm and 670 nm, respectively. Melimine was added to final concentrations of 1000, 125, 31.25, 1.9 and 0.48 μM for *P. aeruginosa* and 15.6, 3.9, 1.9 and 0.97 μM for *S. aureus.* The fluorescence intensity was recorded at intervals for 3 min. Corresponding aliquots were removed and viable bacteria were enumerated. The assay was repeated twice and the data were examined for significance using a two-tailed Student's *t*-test ($P < 0.05$).

4.2.9 Release of 260-nm absorbing material from bacterial cells

The assay was performed as described previously by Carson *et al*, (2002). Both *P. aeruginosa* strain 6294 and *S. aureus* strain 38 cells were grown to stationary phase in Muller Hinton broth, centrifuged at 3000 rpm for 10 minutes, washed twice in PBS and resuspended in the same buffer. Bacterial suspensions were adjusted so that OD_{620} of 1:100 dilutions was 0.3 which equates to approximately 3×10^{10} cfu/ml. The bacterial suspension was exposed to melimine at MBC levels (or remained in PBS as control) and incubated at 37^{o}C. Samples were removed at 0 minute, 30 minutes and 60 minutes intervals, diluted 100 times, filtered through 0.22 μm pore size filter (Millipore Corp., Bedford, Mass.) and OD_{260} was recorded. The results were expressed as proportion of initial OD taken at 0 minute. The assay was repeated twice and the differences of means were compared using a two-tailed Student *t* test ($P < 0.05$).

4.2.10 Atomic Force Microscopy

Pseudomonas aeruginosa strain Paer 23 and *Staphylococcus aureus* ATCC 12600 (NCTC 8532) were grown overnight in Tryptone Soy broth (Oxoid, Basingstoke, UK) at 37^{o}C with shaking. Cells were harvested by centrifugation (3200 rpm for 10 minutes), washed twice and resuspended in sterile distilled water (SDW) to an OD_{660} of 0.1 that corresponds to 1×10^{8} colony forming units per ml of suspension. Melimine was added to final concentrations of 1 ×, and 8 × MIC. In controls, melimine was replaced by SDW. Samples were incubated at 37^{o}C for 30 minutes. Aliquots of samples were taken at 0 minutes and 30 minutes time interval, diluted, and plated on nutrient agar to determine bacterial viability.

Preparation and examination of samples by AFM

Samples treated with various concentrations of melimine and their respective controls were placed on silicon wafers in 10 µl quantity and air dried in fume hood. AFM imaging for bacterial morphology was carried by lateral/phase mode using an Explorer AFM (Veeco Instruments, Cambridge, UK) with silicon nitride pyramidal shaped tips. Three independent samples (produced on three different days) were scanned at 10 × 10 μm^{3}. Various areas were used for imaging however, only representative images are shown.

4.3 Results

4.3.1 Spectroscopic analysis

The high abundance of cationic arginine (R) residues in the melimine sequence suggests high water solubility and also possible pH dependence in its chain conformation similar to that

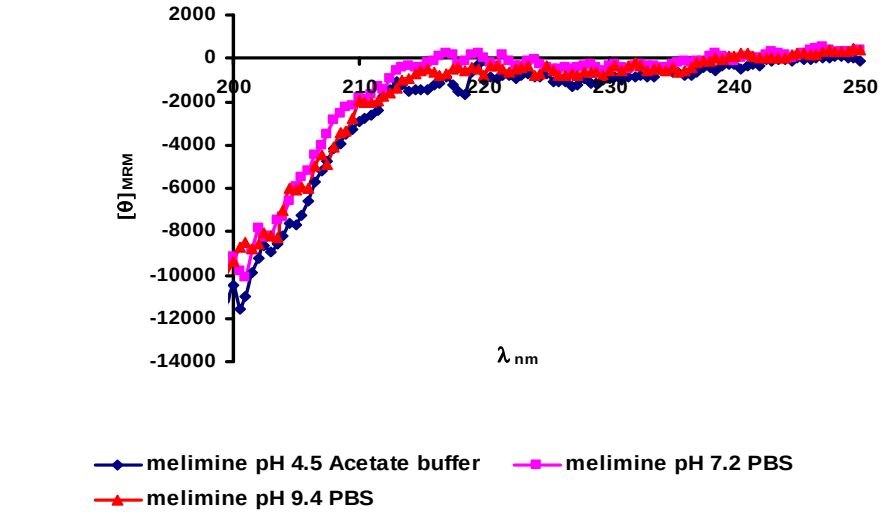
which is seen for poly-L-lysine and poly-L-arginine (Rifkind, 1969). However, the CD spectra in Fig. 4.1A shows that melimine is essentially in a random coil conformation over the pH range studied (pH 4.5–9.4). The predominance of hydrophilic residues over hydrophobic ones (15:9), plus the random coil structure, suggests that most of the hydrophobic residues are exposed to the aqueous medium. This is supported by the fluorescence spectrum of the tryptophan (W) residue in these solutions (data not shown). The emission maximum occurs at 348 nm, with a relative intensity of 700 AU; these are typical of a W residue that is fully exposed to water.

Addition of organic solvents to the aqueous solutions of melimine alters the chain conformation towards adopting a helical fold (Fig. 4.1B). The presence of ellipticity minima around 225 nm and 208 nm suggests the conformation to be the classical right-handed α-helix. The maximum effect is seen upon addition of 40% (v/v) TFE and is independent of pH (Fig. 4.1B). Estimates based on the values seen in 100% helical polypeptides (ca. –33 000 at 222 nm and ca. –27 000 at 208 nm) suggest that the chain folds to no more than 10% helical conformation, with the rest remaining an unstructured random coil.

Melimine shows some surface activity and is solubilised in surfactant micelles. In the zwitterionic micelles of CHAPS, it is included but does not change its chain conformation and the CD spectrum is the same as that in aqueous medium (data not shown). The fluorescence of the tryptophan residue also remains the same, with a maximum at 348 nm and an intensity of ca. 700 AU. However, when solubilised in SDS, the α-helical content increases to ca. 35–40% (Fig. 4.2A). The tryptophan fluorescence is also blue-shifted (to 333 nm) and lowered in intensity (to ca. 280 AU), indicating that it is in a somewhat more apolar

microenvironment (Fig. 4.2A). The AMP appears to prefer the anionic micellar environment of SDS over CHAPS and is also able to fold into a somewhat more compact helical conformation (Fig. 4.2A). Fig. 4.2B shows a hypothetical helical fold for melimine; as can be seen, segregation of non-polar and polar side chains does not occur. Given the 40S helicity, and assuming the first half of the molecule to be helical, the first three or four turns of the helix at least seem to cluster the side chains of S4, N8, T1, Q12 and W5 on one side to yield a quasi-non-polar surface (this feature may cause the W emission to occur at 333 nm in SDS). When the latter half of the molecule is assumed to be helical, a clustering of charged residues all over the helical cylinder is seen—R18, R19, R22, R15, R26, R29 and R23 on one side, and R20, R21, R23, R27, R28 and G24 cluster on the other side. This part of the cylinder is thus mostly ion-decorated.

A



B

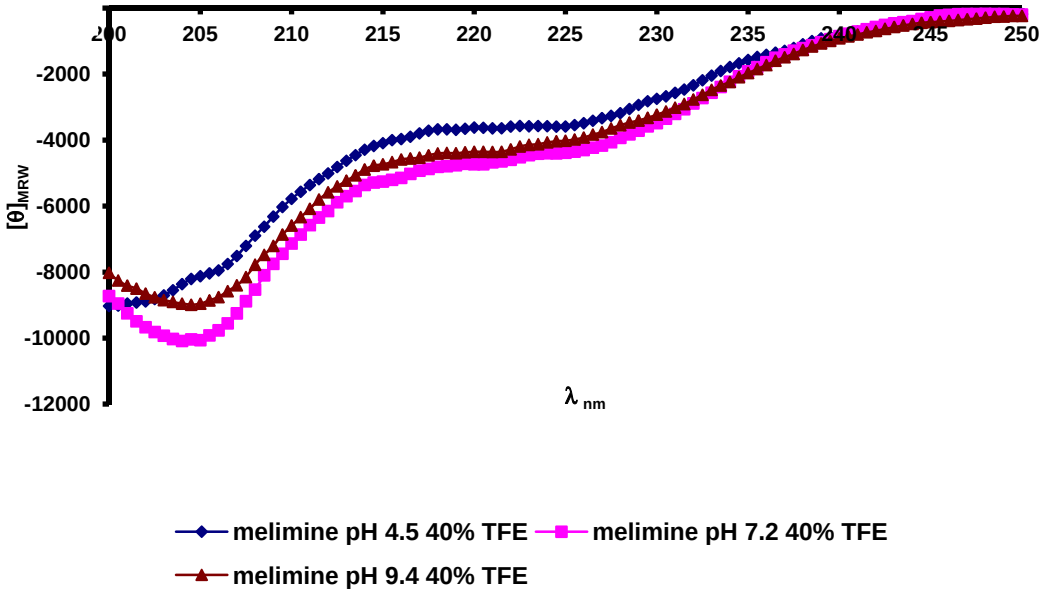
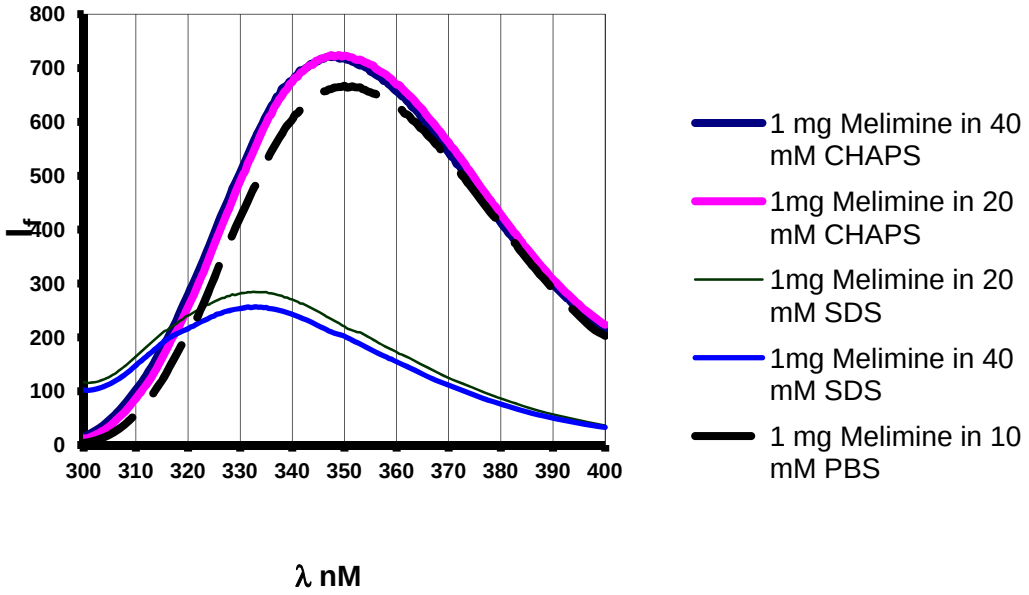


Figure 4.1: Circular dichroism spectra of melimine (265 μM): (A) effect of pH on the conformation of melimine in aqueous media under acidic and basic conditions, indicating a random coil conformation over the pH range examined; and (B) effect of pH on the conformation of melimine in 40% trifluoroethanol (TFE) under acidic and basic conditions, indicating a helical fold in the presence of this solvent and the pH range examined.

A



B

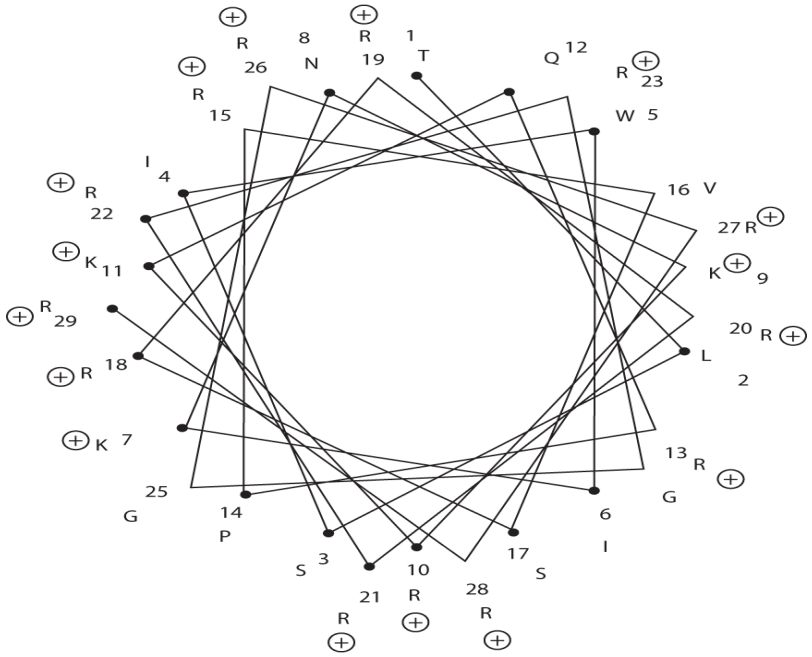


Figure 4.2: (A) Fluorescence spectra of melimine in sodium dodecyl sulphate (SDS) or 3-[(3-cholamidopropyl) dimethylammonio] propanesulphonic acid (CHAPS), exhibiting an increase in helical content. (B) Representation of the sequence of melimine according to the Schiffer–Edmundson wheel projection, showing no segregation of polar and non-polar side chains.

4.3.2 Determination of the minimal inhibitory concentration and bactericidal concentration of melimine

For *P. aeruginosa* 6294, the MIC was found to be 125 μM, whilst for *S. aureus* the MIC was 3.9 μM. The MBCs of melimine for these strains of bacteria in PBS for 30 min were 31.25 μM for *P. aeruginosa* and 1000 μM for *S. aureus*.

The MICs for *P. aeruginosa* Paer023 and *S. aureus* ATCC 12600 against melimine were found to be 125 μM and 62.5 μM respectively.

4.3.3 Fluorescence and scanning electron microscopy

In the absence of melimine, *P. aeruginosa* showed a smooth bright surface (Fig. 4.3A). Exposure of the bacteria to 31.25 μM melimine for 30 min resulted in a marked change in the appearance of the cells. Many cells appeared shortened compared with those in the control groups, with membranes showing some evidence of blebbing and roughening (Fig. 4.3B).

Compared with the control (Fig. 4.3C), *S. aureus* cells treated with 1 mM melimine showed marked structural changes (Fig. 4.3D). Cells appeared to be distorted, bleb formation was evident and cell debris could be observed. Melimine killing of bacteria in this experiment was confirmed by live/dead staining of the cells. Both *P. aeruginosa* and *S. aureus* stained red, indicating loss of viability and membrane damage (data not shown).

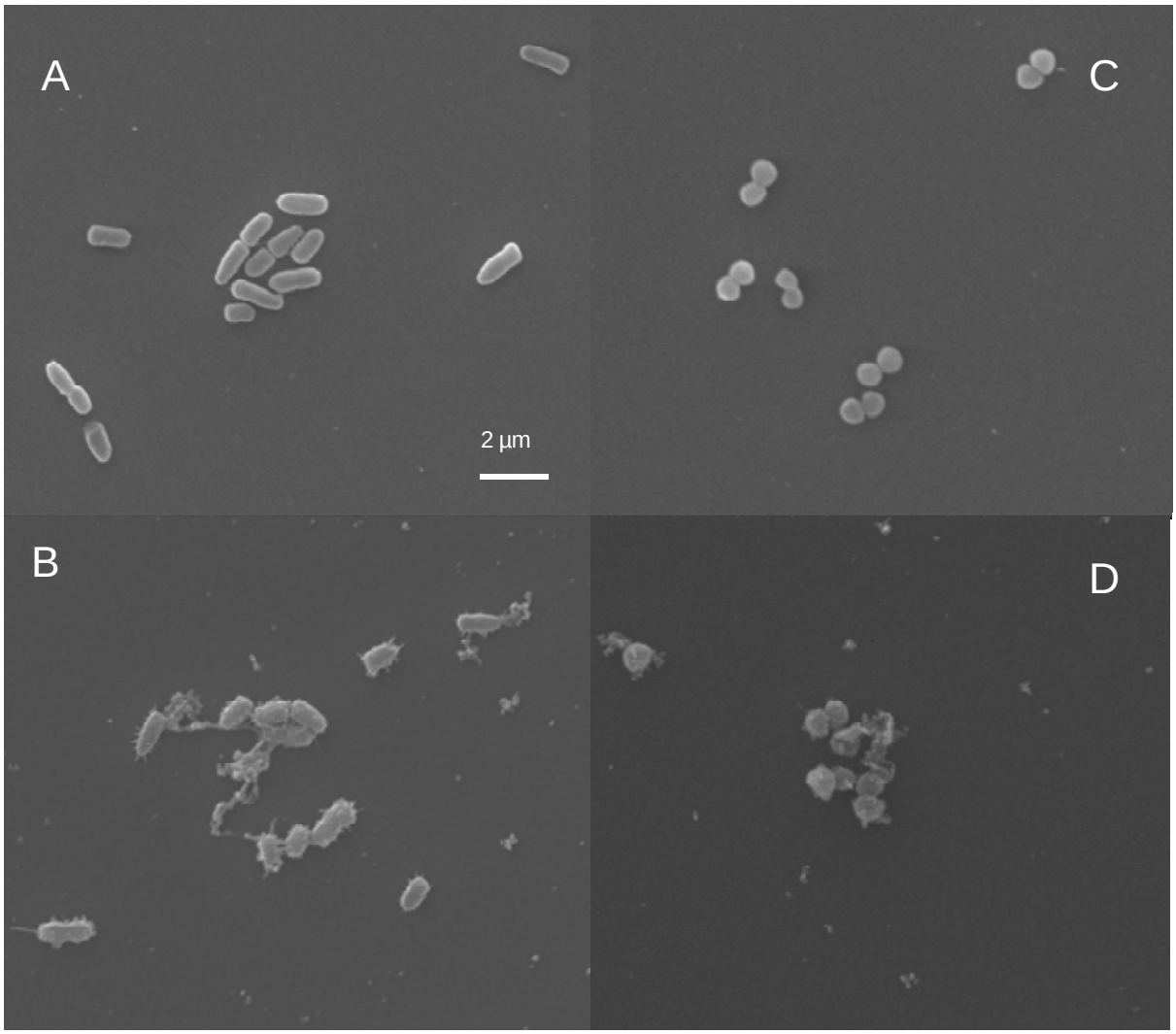


Figure 4.3: Scanning electron micrographs of *Pseudomonas aeruginosa* and *Staphylococcus aureus* treated with melimine: (A) *P. aeruginosa* in the absence of melimine; (B) *P. aeruginosa* after 30 min exposure to melimine; (C) *S. aureus* in the absence of melimine; and (D) *S. aureus* after 30 min exposure to melimine.

4.3.4 Effect of Mg^{2+} on the antimicrobial activity of melimine

Addition of melimine to cultures of *P. aeruginosa* and *S. aureus* resulted in a 98 ± 2% and 50 ± 13% reduction in bacterial numbers, respectively, over the time of the assay (Fig. 4.4) These reductions were significantly different from their controls ($P < 0.01$). Addition of 20 mM $MgSO_4$ to the medium negated the effects of melimine on bacterial viability for both strains of bacteria.

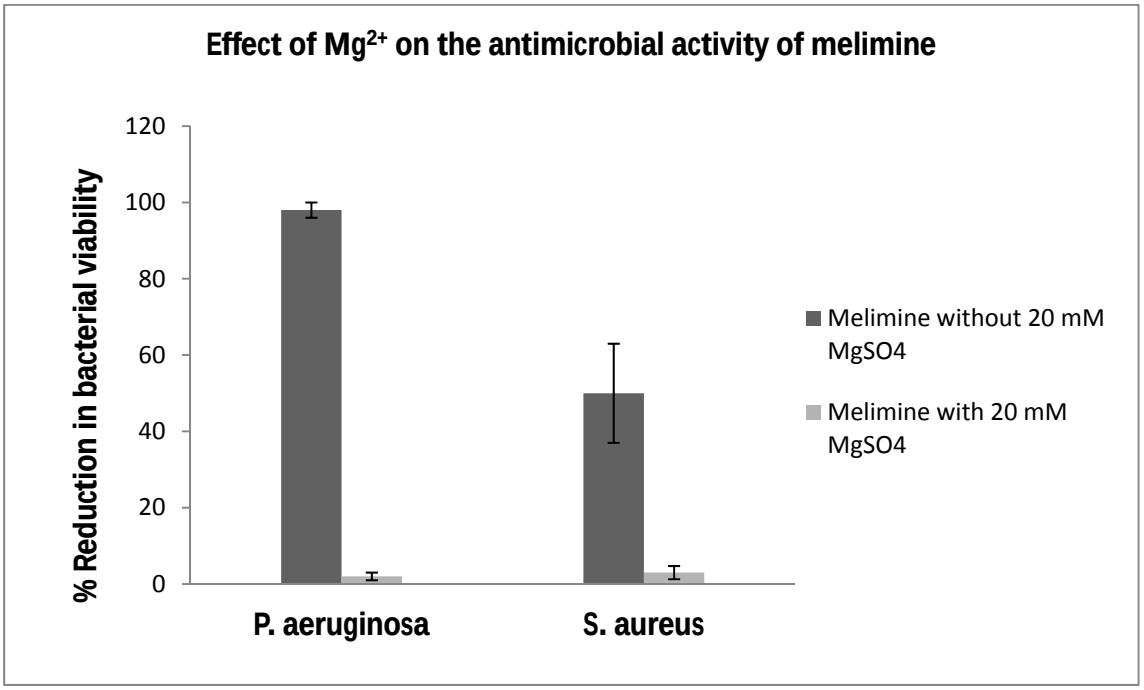


Figure 4.4: Effect of Mg^{2+} on antimicrobial activity of melimine. Standard deviation is shown by the vertical bars (n = 9).

4.3.5 Ability of melimine to disrupt the outer membrane of *Pseudomonas aeruginosa*

The ability of melimine to induce sensitivity of *P. aeruginosa* to deoxycholate was examined. Exposure of *P. aeruginosa* to 0.5% deoxycholate in the absence of melimine did not result in

cell lysis and no change in optical density was recorded. Exposure of the bacteria to the antibiotic polymyxin B, which disrupts the outer membrane (Vaara, 1992), prior to addition of deoxycholate resulted in lysis of 8 ± 7% of cells, and addition of deoxycholate resulted in a significant increase in the percentage of lysed cells to ca. 45 ± 4% (P = 0.0004). Similarly, melimine alone resulted in lysis of 4 ± 2% of cells and addition of deoxycholate resulted in lysis of 48 ± 5% (P = 0.00003) of cells. Lysis of cells by deoxycholate was not significantly different following polymyxin B or melimine (P = 0.44), suggesting similar levels of outer membrane disruption between the two compounds.

4.3.6 Effect of melimine on cytoplasmic membrane permeability

Melimine was able to cause rapid depolarisation of the cytoplasmic membrane of *P. aeruginosa* at concentrations >0.48 μM (Fig. 4.5A), a concentration that did not result in loss of bacterial viability (Fig. 4.5B). Above this concentration of melimine, the levels of dye release were concentration-dependent, with only a 1.5-fold increase in dye release over a 500-fold increase in melimine concentration observed. Loss of bacterial viability was concentration-dependent above 0.48 μM melimine, however there was no further increase in loss of bacterial viability above 125 μM melimine where an ca. 4 log reduction in bacterial numbers was observed after 5 min (Fig. 4.5B).

Melimine was also able to cause rapid depolarisation of the cytoplasmic membrane of *S. aureus* 38 (Fig. 4.6A). In contrast to *P. aeruginosa*, release of $diSC_3$-5 was not dependent on the concentration of melimine. Mean values of fluorescence intensity obtained for 15.6 μM melimine was not statistically significant compared with those for all other concentrations

tested ($P > 0.05$) (Fig. 4.6A). In contrast to the results for *P. aeruginosa*, there was a <1 log reduction in bacterial numbers at all concentrations tested after 5 min (Fig. 4.6B).

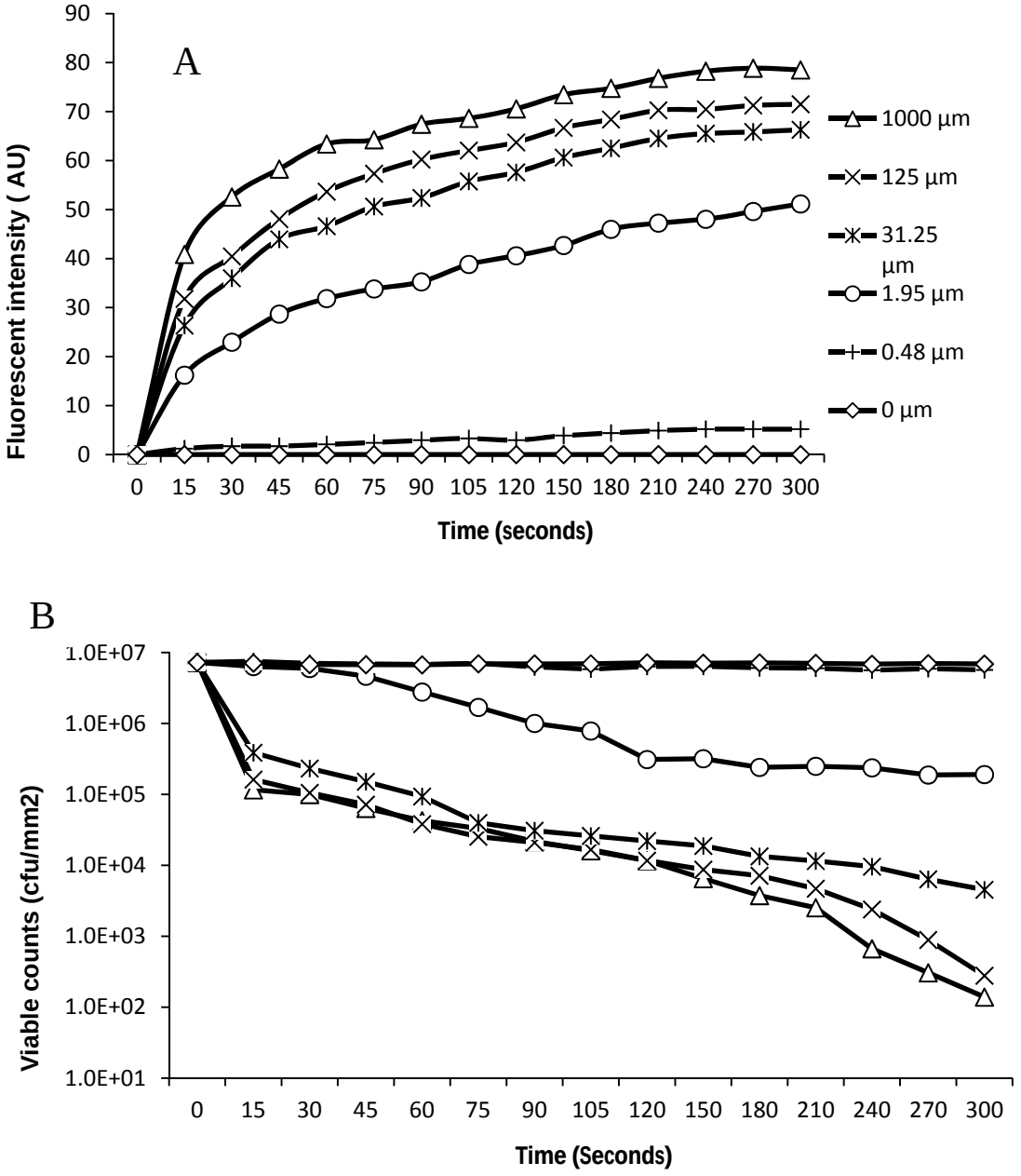


Figure 4.5: (A) Cytoplasmic membrane permeabilisation of *Pseudomonas aeruginosa* by melimine at >0.48 µM, as assessed by release of the membrane potential-sensitive dye diSC$_3$-5. (B) Corresponding bacterial survival as determined by plate counts and expressed as colony-forming units (CFU). (n = 9).

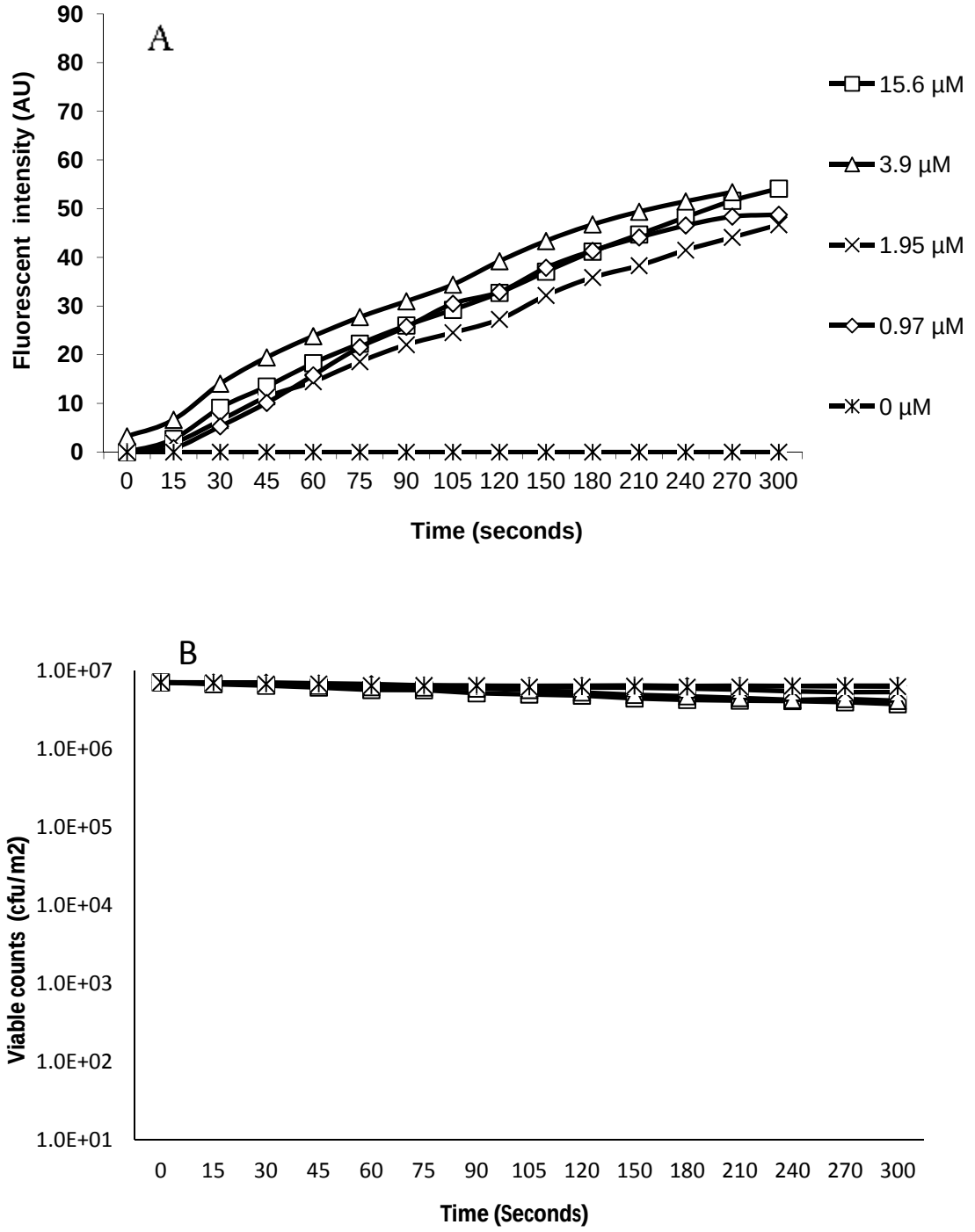
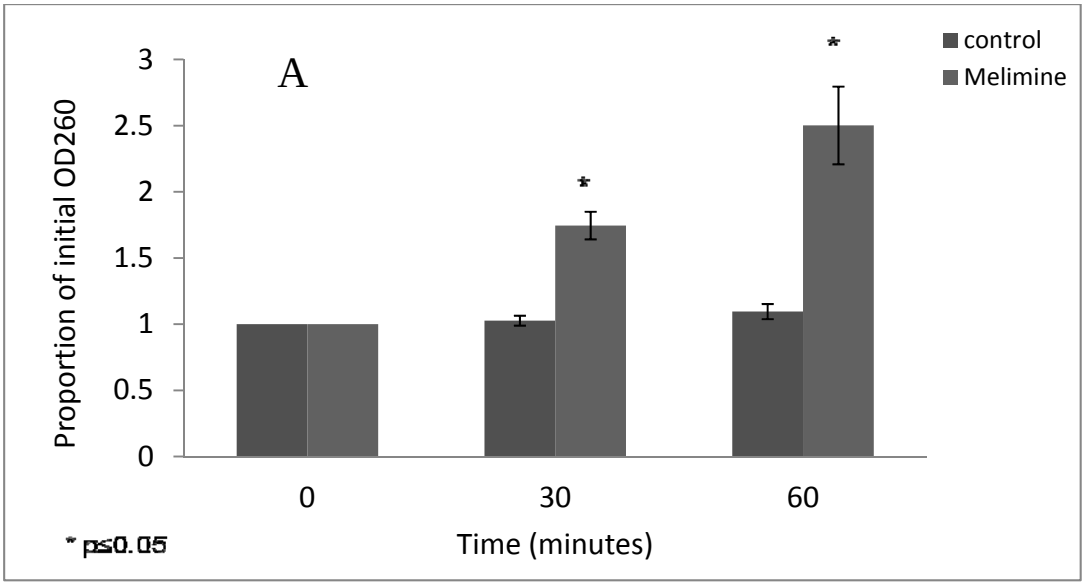


Figure 4.6: (A) Rapid depolarisation and permeabilisation of the cytoplasmic membrane of *Staphylococcus aureus* by melimine, exhibiting independence of melimine concentration, as determined by release of the membrane potential-sensitive dye diSC$_3$-5. (B) Corresponding bacterial survival as determined by plate counts and expressed as colony-forming units (CFU). (n = 9).

4.3.7 Loss of 260-nm absorbing material from bacterial cells

Compared to controls, exposure of *P. aeruginosa* to MBC levels of melimine resulted in 73 % increase in the amount of 260-nm absorbing material from the cells after 30 minutes ($P < 0.01$; fig. 4.7A). A further 73% increase in the amount of 260-nm absorbing material was observed after 60 minutes ($P < 0.01$). The increase in 260-nm absorbing material at 60 minutes was statistically different to that observed at 30 minutes time interval ($P < 0.01$; fig. 4.7A).

For *S. aureus,* melimine caused 66% leakage of 260-nm absorbing material in the cell filtrates at 30 minutes time interval ($P < 0.01$; fig. 4.7B). However, no further leakage of cytoplasmic material was observed at 60 minutes time interval ($P = 0.522$; fig. 4.7B).



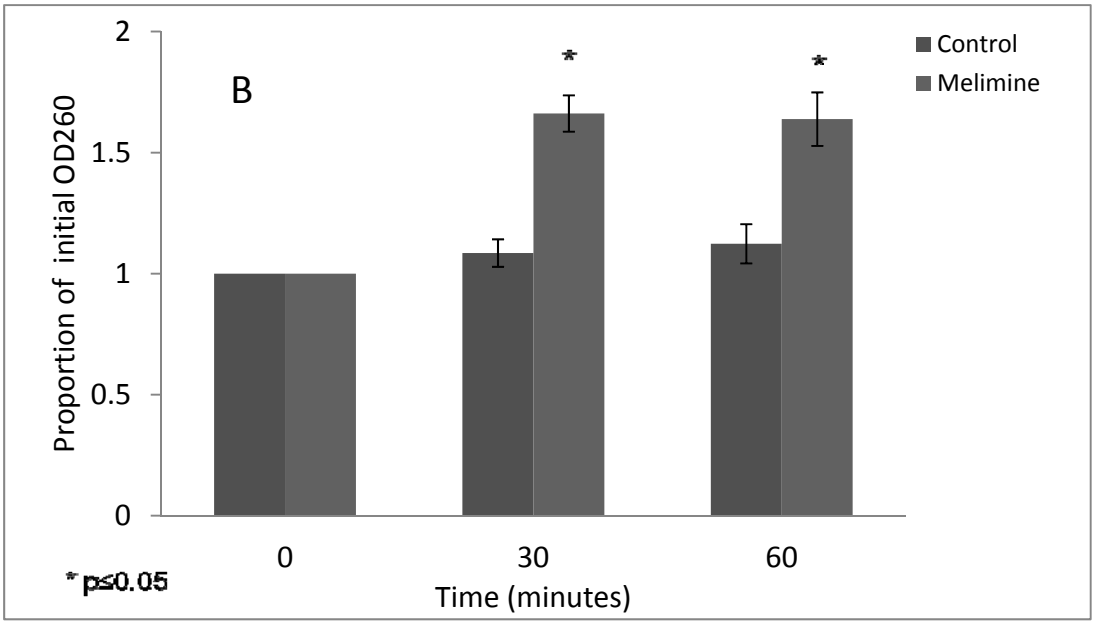


Figure 4.7: (A) Loss of 260-nm absorbing material after exposure of *P. aeruginosa* to MBC levels of melimine; * p<0.05 compared to control and standard deviation is shown by the vertical bars. Also, incubation for 60 mins resulted in a significant increase in material adsorbing at 260nm compared to 30 mins. (B)Loss of 260-nm absorbing material after exposure of *S. aureus* to MBC levels of melimine. * p<0.05 compared to control and standard deviation is shown by the vertical bars (n = 9).

4.3.8 Atomic Force Microscopy

In the absence of melimine, *P. aeruginosa* strain 023 appeared smooth, mostly in aggregates, with well defined margins and flagella clearly attached to the cells (Fig. 4.8A). When cells were exposed to melimine at 1× MIC, they appeared to be distorted, cellular debris was observed and the flagella found detached from the cells (Fig. 4.8B; arrow). Exposure of cells to 8× MIC resulted in complete collapse of the cells and, compared to 1× MIC, cellular debris was more pronounced (Fig. 4.8B).

There was a three-log reduction in viability of *P. aeruginosa* observed when cells were exposed to melimine for 30 minutes at 1× MIC (Fig. 4.9A). However, at 8× MIC a complete loss in viability was observed for P. aeruginosa (Fig. 4.9A).

S. aureus ATCC 12600 showed typical Staphylococcal morphology in the absence of melimine with cells appearing spherical, mostly in clusters and with well defined cell wall (Fig. 4.8D; arrow). No obvious changes were observed in morphology when cells were treated with melimine at 1× MIC for 30 minutes (Fig. 4.8E), although there did appear to be more debris around the cells. However, gross cellular damage was observed at 8× MIC of melimine (Fig. 4.8F). Cells appeared to be collapsed, the cell walls were collapsed/ damaged (as shown by an arrow) and cellular debris was clearly visible (Fig. 4.8F).

There was only one-log reduction in viability for *S. aureus* after 30 minutes of exposure to melimine at 1× MIC (Fig. 4.9B). However *S. aureus* cells were exposed to melimine at 8× MIC for 30 minutes, a three-log reduction in viability was observed (Fig. 4.9B).

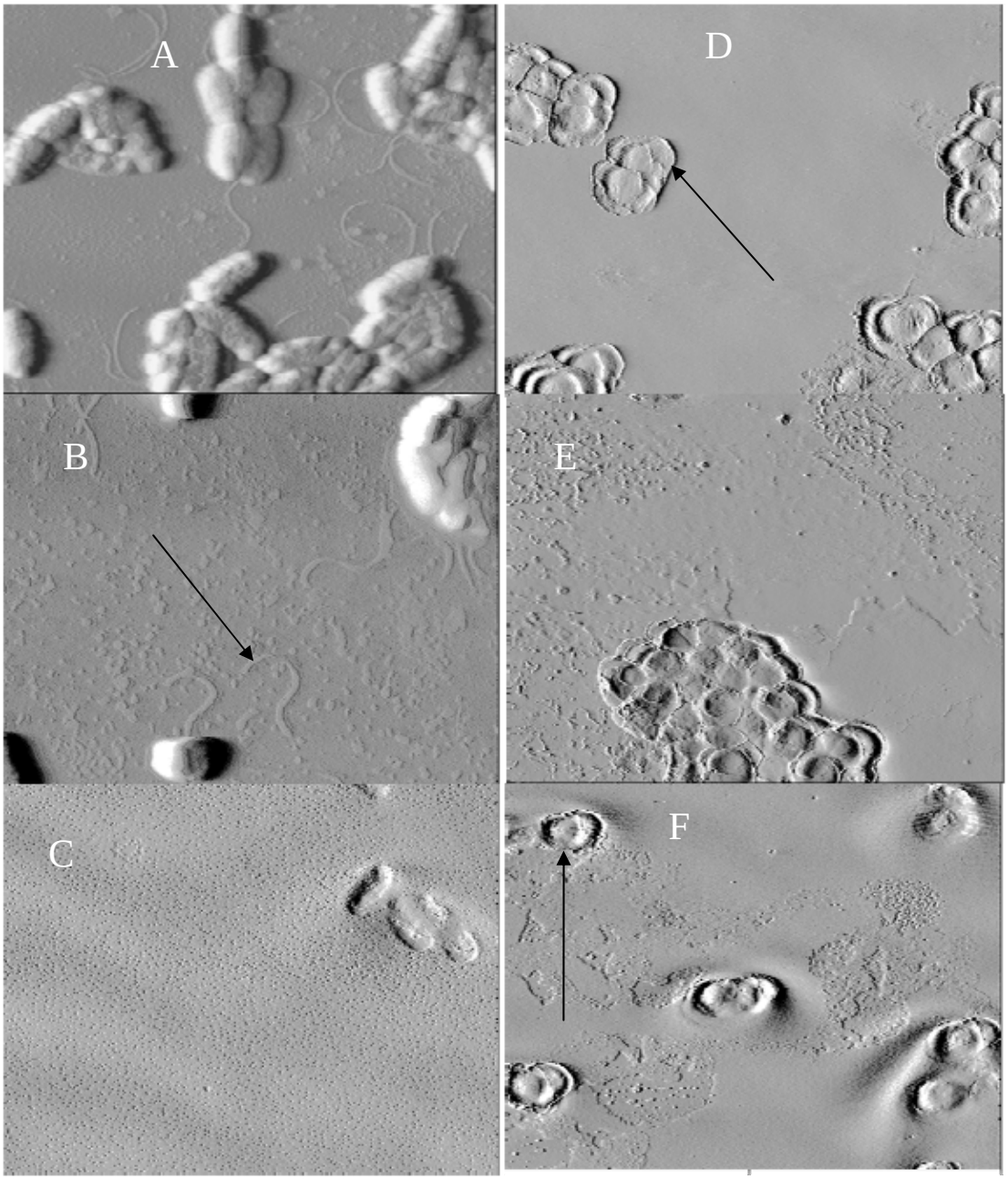
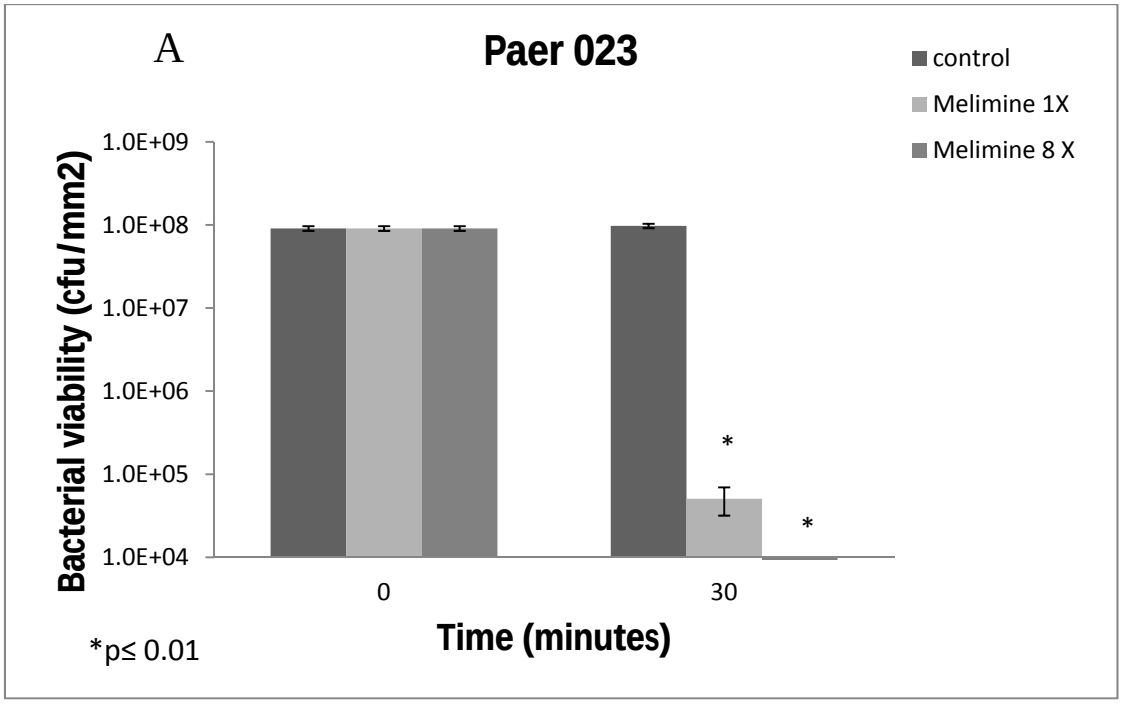


Figure 4.8: Atomic microscopy images of *P. aeruginosa* (A. B, C) and *S. aureus* (D, E, F) treated with 1× (B, E) and 8× (C, F) concentrations of melimine or with sterile distilled water (A, D). Arrow in B shows detached flagella. Arrow in D points to cell wall of the cell. Arrow in F indicates a collapsed cell wall compared to the well defined cell walls in D.



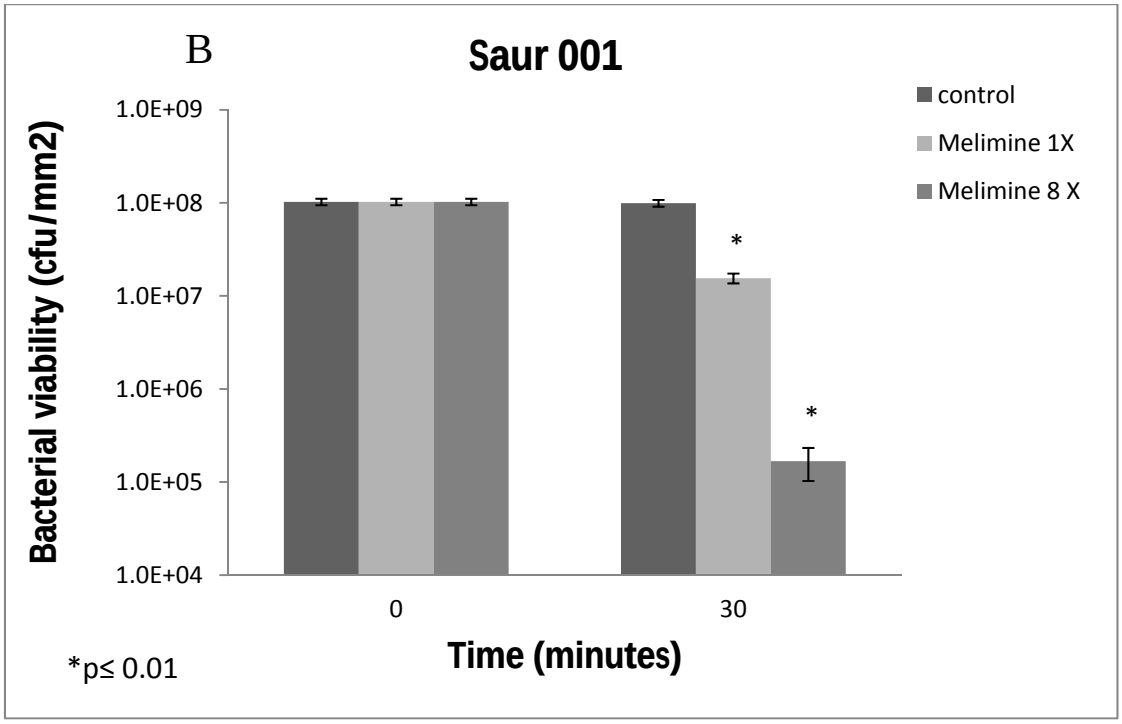


Figure 4.9: Bacterial viability after 30 minutes exposure to 1× and 8× MIC levels of melimine; (A) *P. aeruginosa* and (B) *S. aureus*. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.01 (n = 9).

4.4 Discussion

In common with findings for melittin and other amphipathic cationic peptides, melimine adopts a more helical structure in membrane-mimetic environments. In solution, the random coil structure found is unlikely to be influenced by concentration of either peptide or physiologically relevant salt concentrations (Rifkind, 1969). That its preference for interaction with, and to be solubilised in, SDS over CHAPS is most likely to be reflective of its charge interactions with the anionic detergent, particularly for *P. aeruginosa*, is supported by our finding that the presence of divalent cations reduces its effects on cell viability. However, its inherent surface activity may also play a role. This preference for interaction with SDS over CHAPS also explains its low haemolytic activity (Willcox *et al*, 2008) while retaining strong antimicrobial characteristics.

Melimine does not appear to be as surface active as melittin or other classical surface-active peptides such as δ-haemolysin or E4orf6 protein, which adopt the amphipathic α-helical fold in micellar media (Lee *et al*, 1987; Orlando and Ornelles, 1999; Raghuraman and Chattopadhyay, 2004). In these amphipathic helices, the non-polar side chains neatly segregate onto one side of the helical cylinder, whilst the polar and charged residue side chains cluster onto the other. For melimine, this division of polarity is not so clear cut. Considering the spectroscopic data together, melimine is not as good an amphiphile as melittin or δ-haemolysin, which are able to generate channels and rafts in membranes (Verdon *et al*, 2009), and so its mode of action would be expected to be different from that of these classical surface-active peptides.

Investigation of the interaction of melimine with *P. aeruginosa* and *S. aureus* showed that the initial interaction of the peptide with both types of bacteria was predominantly governed by electrostatic forces, a finding consistent with that for many cationic peptides (Aspedon and Groisman, 1996; Groisman, 1996; Zhang *et al*, 2000). We have also demonstrated that for *P. aeruginosa*, disruption of the outer membrane occurs rapidly and at well below the concentration required to kill the bacteria in the same medium. Furthermore, our data indicate that a major effect of melimine is on the integrity of the cytoplasmic membrane both for *P. aeruginosa* and *S. aureus*, however it was of particular interest that there were differences in the relationship between depolarisation of the cytoplasmic membrane and the kinetics of loss of viability of the bacteria. For *P. aeruginosa* the rapid loss of cytoplasmic membrane integrity demonstrated by release of diSC_3-5 correlated directly with loss of cell viability (Fig. 4), whilst for *S. aureus* maximal dye release was obtained at concentrations where there was no significant loss of viability (Fig. 5). This suggests that either the effects of depolarisation of the cytoplasmic membrane were delayed in *S. aureus* or that a secondary process affecting metabolic activity in the cell may occur in this case. However, when melimine is attached to a surface and it is likely that such secondary processes are precluded, surface-adherent *S. aureus* showed a higher proportion of cells with loss of viability demonstrated using fluorescent staining than adherent *P. aeruginosa* (Chen *et al*, 2009). This suggests that the processes involving loss of integrity of the cell membrane are of primary importance. However, at least in solution, other intracellular processes cannot be ruled out, including macromolecular synthesis and reversible phosphorylation of proteins (Jia *et al*, 1997) or other energy transducing processes.

The release of 260-nm absorbing material from the cell serves as a good indicator of a compromised membrane (Chen and Cooper, 2002). Both *P. aeruginosa* and *S. aureus* showed

marked leakage of cytoplasmic material after 30 minutes exposure to MBC concentrations of melimine. It is important to note that due to very high inoculum size used in this experiment the actual number of cells could be higher than that used to determine MBC. However, at 60 minutes, further leakage of cytoplasmic material continued only for *P. aeruginosa* and not for *S. aureus*. These results are in contrast to Carson *et al,* (2002) who while exploring the mechanism of action of Tea tree oil on *S. aureus* found no release of cytoplasmic material at MIC after 30 minutes exposure, yet a significant increase in OD_{260} was observed after 60 minutes time interval. The difference could be attributed to the difference in nature of the two antimicrobials as well as the concentration used in the study; MBC levels were used in the present study as opposed to MIC used by Carson *et al* (2002). However, in another study Oliva *et al,* (2004) reported approximately 8-fold increase in OD_{260} of *S. aureus* filtrates after 10 minutes exposure to 4 × MIC of synthetic phenazine drug clofazimine suggesting a rapid release of cytoplasmic material. Minahk *et al,* (2000) using Enterocin CRL35, an AMP that belongs to class II bacteriocins, demonstrated that the release of 260 nm absorbing material from cells of *Listeria monocytogenes* was time and peptide concentration dependant. Between time intervals of 30 minutes and 60 minutes, they reported approximately 2.5 time increase in $\%OD_{260}$ at high concentrations. Their findings correlated somewhat with the results obtained for *P. aeruginosa* in the present study where leakage of cytoplasmic material was found to be time-dependant.

AFM images also revealed a distinct effect of melimine on the Gram-negative bacterium *P. aeruginosa* as compared to the Gram-positive bacterium *S. aureus*. At 1× MIC the cells of *P. aeruginosa* appeared shorter as compared to controls and the viability counts determined at the same time interval showed a three-log reduction. This suggests a rapid killing induced by melimine with accompanying cellular damage. Mortensen *et al,* (2009) in a study on effect

of colistin on ultrastructures of *P. aeruginosa* also found that at low concentration of the peptide (10 µg/ml: concentration where lethal effects of colistin just started to appear) the cell size appeared to reduce significantly suggesting that the bacterial division was halted immediately after the formation of septum. At 8 × MIC of melimine, cells of *P. aeruginosa* were completely collapsed, surrounded by cell debris and viability was completely lost. Rossetto *et al*, (2007) exploring effects of a novel antimicrobial multimeric peptide SB006 on *P. aeruginosa* reached similar conclusions. They reported collapse of bacterial cells started at the MIC level after 30 minutes of treatment with the peptide; however a complete collapse and loss of smoothness of bacteria occurred at 4 × MIC.

S. aureus cells after 30 minutes of exposure at 1× MIC of melimine showed no morphological changes and the viability count at the same time period showed only one-log reduction suggesting slow killing without any apparent morphological changes. At 8 × MIC, *S. aureus* cells showed gross morphological alterations, manifested by a partial or complete loss of peptidoglycan (appeared as concentric rings around the untreated control cells; Turner *et al*, 2010). The peptidoglycan, a major component of cell wall of *S. aureus* is approximately 20 – 30 nm thick that serves not only as a permeability barrier, but is also important in providing structural stability to bacteria (Rogers *et al*, 1980; Giesbrecht *et al*, 1998). The latter could account for the observed collapsed cells seen after melimine treatment. Furthermore, only three-log reduction in viability counts at 8× MIC, where most cells appeared to be deformed, suggest that the killing mechanism in *S. aureus* was delayed.

It is imperative that new treatments for bacterial infection are developed, as currently ca. 70% of all *S. aureus* strains found in hospitals are multidrug-resistant meticillin-resistant *S. aureus*

(MRSA), with more than 19 000 MRSA-related fatalities per annum in the USA alone (Taubes, 2008). The AMP melimine shows potential for development as an adjunct therapy to conventional antibiotic treatments (Willcox *et al*, 2008) as it has low cytotoxicity for mammalian membranes while retaining excellent antimicrobial activity even in the presence of high concentrations of anionic peptides. Further, it is readily sterilisable (Willcox *et al*, 2008). Here we have shown that this peptide may act predominantly by disruption of the membrane for the Gram-negative bacterium *P. aeruginosa*, whilst its interactions with *S. aureus* may be more complex and potentially involve interaction with a metabolic pathway that is yet to be identified. Interestingly, this interaction results in a lower MIC for the Gram-positive organism. There have been few studies to date investigating differences in the action of cationic peptides towards Gram-positive and Gram-negative bacteria, thus further investigation of these mechanistic differences may allow more refined targeting of these increasingly difficult-to-treat bacterial infections and/or further inform design of novel peptides with improved broad-spectrum activity.

Chapter 5: Mechanism of action of melimine and melimine 4 attached to surfaces

5.1 Introduction

Antimicrobial peptides (AMPs) in covalently-attached form represent a promising way to prevent biomaterial-associated infections (Hilpert *et al*, 2009; Humblot *et al*, 2009). Some of the advantages they offer over conventional antibiotics include broad spectrum antimicrobial activity, rapid killing of microorganisms at a very low concentration, relatively non-specific mechanism of action with likelihood of multiple targets and very little chance of bacteria to acquire resistance (Toke, 2005; Marr *et* al, 2006; Humblot *et al*, 2009). Covalent attachment of these peptides to surfaces not only improves stability for longer periods, but also results in lower toxicity as compared to release-based systems (Venter, 1982).

A number of recent studies have clearly demonstrated the ability of covalently-bound AMPs to reduce adhesion and/or killing of bacteria on surfaces (Humblot *et al*, 2009; Chen *et al*, 2009; Hilpert *et al*, 2009; Cole *et al*, 2010). However, the exact mechanism of action by which AMPs act while attached to surfaces is yet to be elucidated. LaPorte *et al*, (1977) in their study on Polymyxin B covalently attached to agarose beads, suggested that the peptide mediated perturbation of the outer membrane of *E. coli*, caused a subsequent alteration in permeability of the cytoplasmic membrane and hence the killing of the bacteria. Haynie *et al* (1995), using covalently-bound magainin, reached a similar conclusion. They found that the

covalent attachment of the peptide to the surface resulted in restricted penetration depth and subsequent inability to reach the cytoplasmic membrane. Hence, bacterial killing could be attributed to interaction of the peptide with the outer membrane of the cell (Haynie *et al,* 1995).

Hilpert *et al,* (2009) hypothesised that bacterial killing by covalently-bound peptides was independent of the linkers through which they are attached to the surface and bacterial killing was not dependent on actual penetration of peptides into bacterial membranes. Moreover, very short peptides that are composed of only 9 amino acid residues, yet active while attached to surface, further strengthened their hypothesis (Hilpert *et al*, 2009).

However, a completely different view was presented by Gabriel *et al* (2006). In their study on titanium surfaces coated with LL-37, they found that LL-37 covalently-bound to titanium surfaces via long and flexible linkers i.e. functionalized poly (ethylene glycol) (PEG) molecules, resulted in bactericidal activity towards *E. coli* while no bactericidal activity was observed when LL-37 was covalently-bound to titanium in the absence of PEGs. They concluded that for LL-37 to be active in bound form it must reach the bacterial membrane and long linkers such as PEGs facilitate that interaction (Gabriel *et al*, 2006).

The results from chapter 2 suggested that melimine in covalently-bound form is effective in reducing bacterial viability on biomaterial surfaces and is an excellent candidate to develop further as coating on biomaterial surfaces. In chapter 3, various sequences of melimine were tested to determine the shortest possible peptide and melimine 4 with 17 amino acids residues

as compared to 29-residue parent peptide was found to be effective in reducing bacterial adhesion. Mechanism of action of melimine in solution was studied in chapter 4. The current study will explore how the parent peptide melimine and its shortest derivative melimine 4 in covalently-bound form interact with bacteria. Importantly, some of the techniques used for determining mechanism of action of melimine in solution will be used with modifications to assess whether or not they can be used effectively with covalently-bound peptides.

The specific aims of the study include:

- To determine the viability of *P. aeruginosa* and *S. aureus* attached to surfaces coated with melimine and melimine 4 at one hour and five hour time intervals
- To examine the effect of excess divalent cations in culture medium on adhesion of bacteria to surfaces covalently-bound with peptides.
- To assess the membrane damage caused by the covalently bound peptides by Live-Dead staining.

5.2 Materials and Methods

5.2.1 Synthesis of Peptides

Melimine (T L I S W I K N K R K Q R P R V S R R R R R R R G G R R R R-NH2) and its variant: melimine 4 (KNKRKRRRRRRGGRRRR) were synthesized by conventional solid-phase peptide synthesis protocols and were purchased from the American peptide company, Inc (Sunnyvale, CA, USA) at a purity of >90%.

5.2.2 Coating of surfaces

Both melimine and melimine 4 were attached on glass coverslips via 4-azidobenzoic acid (ABA) linkers. Glass was chosen because it has surface chemistry analogous to other biomaterial surfaces such as silicone rubber and plastics and hence results from glass surfaces could be easily related to other biomaterials in general (Chen *et al*, 2009).

5.2.3 Attachment of peptides to surfaces via 4-azidobenzoic acid (ABA)

Attachment of peptides was carried out using a method described by Chen *et al* (2009). Briefly, glass coverslips (No. 1, diameter 13 mm, D263 M glass, ProSciTech, Australia) were ultrasonically cleaned in dichloromethane and ethanol for 15 minutes followed by immersion in methanol solution of ABA (TCI Organic Chemicals, OR, USA; >98% purity). After air drying, coverslips were irradiated for 20 minutes under UV light at 320 nm in a CL-1000 Crosslinker (Ultra-Violet Products Ltd, Upland, CA, USA). Surfaces were functionalized with ABA by washing three times in methanol followed by air drying and treatment with hydrochloride (EDC; Alfa Aesar, 65 mM; 27 mg ml71) in water for 1 h at room temperature. In the final step, the surfaces functionalised with ABA–EDC were reacted with either melimine or melimine 4 (2 mg ml^{-1} in PBS) for 24 h in a humidified chamber (Figure 5. 1).

5.2.4 Estimation of peptide concentrations on the modified surfaces

The recently published method of Chen *et al* (2009) adapted from the direct dye binding method of Bonde *et al*, (1992) was employed to quantify the amount of peptide attached to the surfaces. Briefly, coated and uncoated coverslips were immersed in Bradford reagent

(Biorad, CA, USA) and mixed by inverting for 30 minutes. The absorbance of the supernatant was recorded at 465 nm to determine the levels of unbound dye. A standard curve was constructed from from soluble protein according to the manufacturer's directions but measured at 465 nm instead of 595 nm in order to determine the levels of remaining unbound dye at each concentration.

5.2.5 Bacterial strains and growth conditions

P. aeruginosa strain 6294 serogroup 06, isolated from a human corneal ulcer, was obtained from Dr. Suzanne Fleiszig, School of Optometry, University of California, Berkeley, USA (Fleiszig et al, 1997). While *S. aureus* strain 38 was a clinical isolate from human corneal ulcer (Hume et al, 2005). Bacterial strains, unless otherwise stated, were prepared by growing overnight in Mueller Hinton broth (MHB; Oxoid, Basingstoke, UK) and 200 µl was inoculated in the fresh medium and incubated at 37^{o}C with shaking at 120 rpm to exponential phase. Bacteria were collected by centrifugation, washed twice and resuspended in MHB. Bacterial concentration was adjusted turbidimetrically and the numbers confirmed retrospectively by dilution and plate count.

5.2.6 Assessment of viable bacteria adherent to glass surfaces

Overnight cultures of *P. aeruginosa* and *S. aureus* were centrifuged, washed in PBS and resuspended to a concentration of 10^5 cfu/ml in PBS containing MHB (1 part of MHB in 100 parts of PBS for *P. aeruginosa* and 1 part of MHB in 50 parts of PBS for *S. aureus*). Coated surfaces were immersed in 1 ml of bacterial suspension and incubated at 37^{o}C with shaking. Coverslips were removed at one hour and 5 hour time intervals, washed three times with

PBS, homogenised and viable bacterial numbers were determined by dilution and plate count. Results are expressed as a colony forming units per square millimetre of glass surfaces (cfu/mm^2). The assay was repeated twice. A two-way mixed ANOVA was used to compare the differences between the groups with $P \leq 0.05$ considered as statistically significant.

5.2.7 Effect of excess Mg^{2+} on viability of bacteria adhered to coated surfaces

In solution studies, the presence of magnesium in the form of 20 mM $MgSO_4$ in assay medium resulted in significantly reduced activity of melimine against *P. aeruginosa* as well as *S. aureus* (See chapter 4, section 4.3.4, page 154). To test whether magnesium has similar role against surface-bound melimine and its derivative, melimine 4, the same assay as described above was used with the inclusion of assay medium supplemented with 20 mM $MgSO_4$. Coated surfaces were immersed in 1 ml of bacterial suspension and incubated at 37^{o}C with shaking. Bacterial viability was determined at 1 hour and 5 hour time intervals in presence or absence of 20 mM $MgSO_4$ in the assay medium and results were expressed as a colony forming units per square millimetre of glass surfaces (cfu/mm^2). The assay was repeated twice. A two-way mixed ANOVA was used to compare the differences between the groups with $P \leq 0.05$ considered as statistically significant.

5.2.8 Live-Dead Staining

To assess whether coated peptides caused damage to the cytoplasmic membranes of *P. aeruginosa* and *S. aureus*, samples taken at 1 hour and 5 hour time intervals with or without 20 mM $MgSO_4$ in the assay medium were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR). Samples were observed with a Leitz Diaplan fluorescent microscope (Leica Microsystems, Deerfield, IL) and the numbers of bacteria staining green (intact membrane) and red (damaged membrane) were examined. Due to very low attachment for both *P. aeruginosa* and *S. aureus* at 1 hour interval, cells were manually counted and expressed per field of view and no image analysis was performed. Ten representative images at 5 hour time interval were analysed using ImageJ software (Rasband 1997–2010; http://rsb.info.nih.gov/ij/index.html). The average surface area occupied by live and dead cells per field of view was measured and expressed in mm^2. Further, the average number of cells per field of view was calculated and expressed as percentage of live (green colour) and dead (red colour) cells per field of view. A two-way mixed ANOVA was used to compare the differences between the groups with $P \leq 0.05$ considered as statistically significant.

5.3 Results

5.3.1 Quantification of peptide concentrations on the surfaces modified by ABA linkers

The peptides melimine and melimine 4 were quantified on glass surfaces by using a direct dye binding method (Bonde *et al*, 1992). The estimated amount of melimine attached to glass

surfaces was found to be 4.3×10^{-9} moles cm^{-2}, while that of melimine 4 was 1.26×10^{-8} moles cm^{-2}.

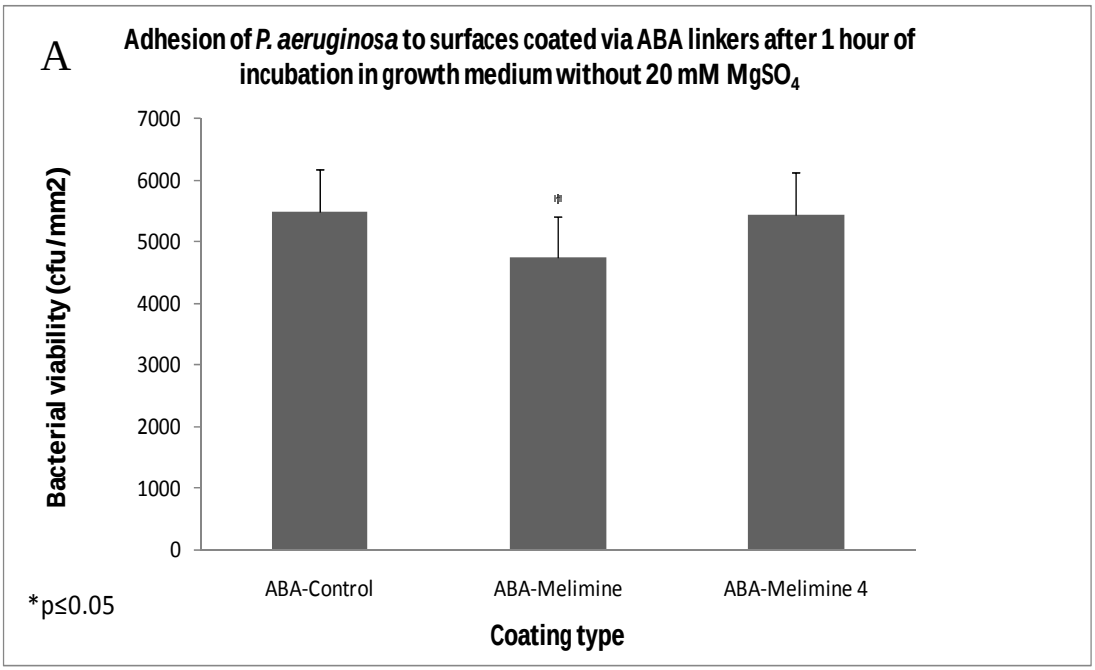
5.3.2 *P. aeruginosa* at 1 hour interval

5.3.2.1 Viability of bacteria adhered to coated surfaces in the absence of Mg^{2+}

Compared to process control (ABA without any peptide), glass surfaces coated with melimine after 1 hour adhesion resulted in significant reduction in viability of *P. aeruginosa* ($P = 0.039$; Fig. 5.1 A) however no change in viable counts was observed for melimine 4. The reduction in viability caused by melimine was also statistically significant ($P = 0.018$) when compared to melimine 4 (Fig. 5.1 A).

5.3.2.2 Effect of excess Mg^{2+} on viability of bacteria adhered to coated surfaces

Compared to process controls no significant reduction in bacterial viability was observed for either melimine or melimine 4 when assay medium was supplemented with 20 mM $MgSO_4$ (Fig. 5.1 B). Additionally, viable counts obtained for process control, melimine and melimine 4 obtained at 1 hour interval were not statistically different in presence or absence of 20 mM $MgSO_4$ (Fig. 5.1 A and B).



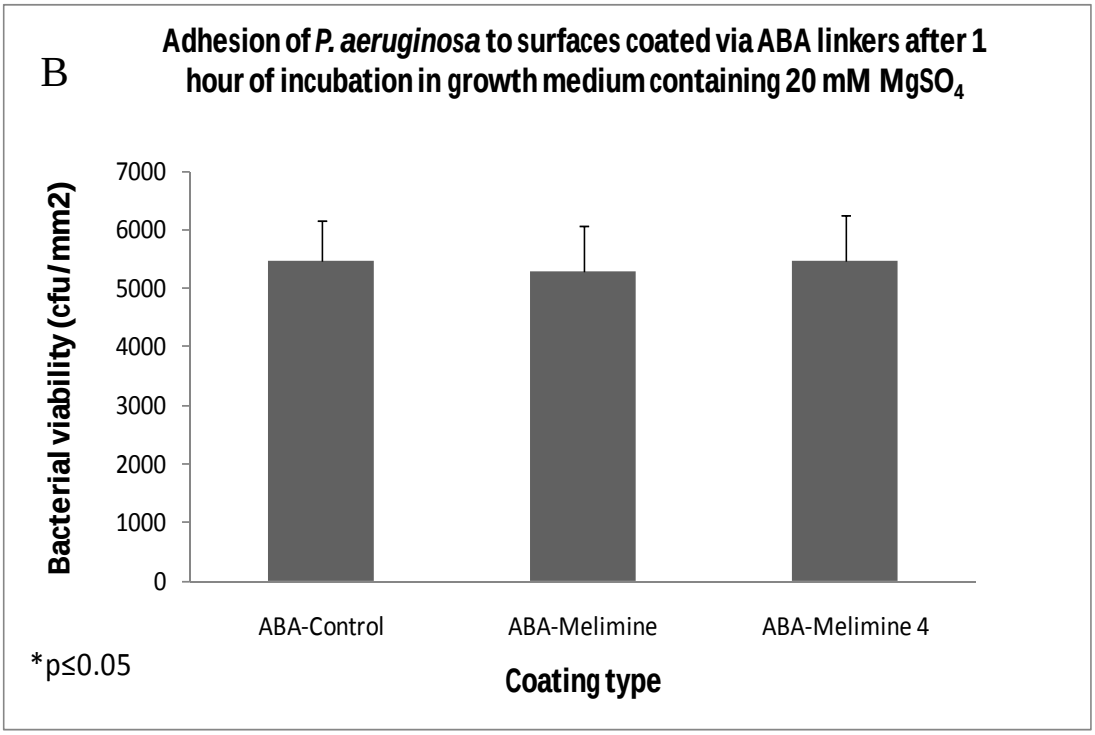


Figure 5.1: Effect of coated surfaces on viability of *P. aeruginosa* cells after 1 hour incubation in growth medium with or without 20 mM $MgSO_4$. (A) Viability of bacterial cells adhered to coated surfaces in growth medium without 20 mM $MgSO_4$, (B) Viability of bacterial cells adhered to coated surfaces in growth medium that contains 20 mM $MgSO_4$. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.05 (n = 9).

5.3.2.3 Live-Dead staining

Live dead staining of bacteria attached to surfaces after one hour of incubation in growth medium with or without 20 mM $MgSO_4$ showed no statistical difference in total, live or dead cells for any of the group tested (table 5.1).

Table 5.1 Live-Dead staining of *P. aeruginosa* adhered to coated surfaces after 1 hour of incubation in growth medium with or without 20 mM $MgSO_4$

Type of Coating	**20 mM $MgSO_4$ in assay medium**	**Total cells per field of view**	**% Live cells per field of view**	**% Dead cells per field of view**
ABA	absent	8 ± 1	97 ± 7	2.2 ± 7
ABA-Melimine	absent	13 ± 4	67 ± 38	32 ± 38
ABA-Melimine 4	absent	9 ± 2	96 ± 7	3 ± 7
ABA	present	8 ± 2	100	0
ABA-Melimine	present	9 ± 3	95 ± 6	4 ± 6
ABA-Melimine 4	present	10 ± 2	98 ± 3	1 ± 3

Fig. 5.2 shows representative fields of adhered cells of process control, melimine and melimine 4 in the presence or absence of 20 mM $MgSO_4$. Very few attached cells were seen after 1 hour with most cells appearing green (intact membrane) with the exception of melimine (Fig. 5.2 B) where most cells appeared red (damaged membrane).

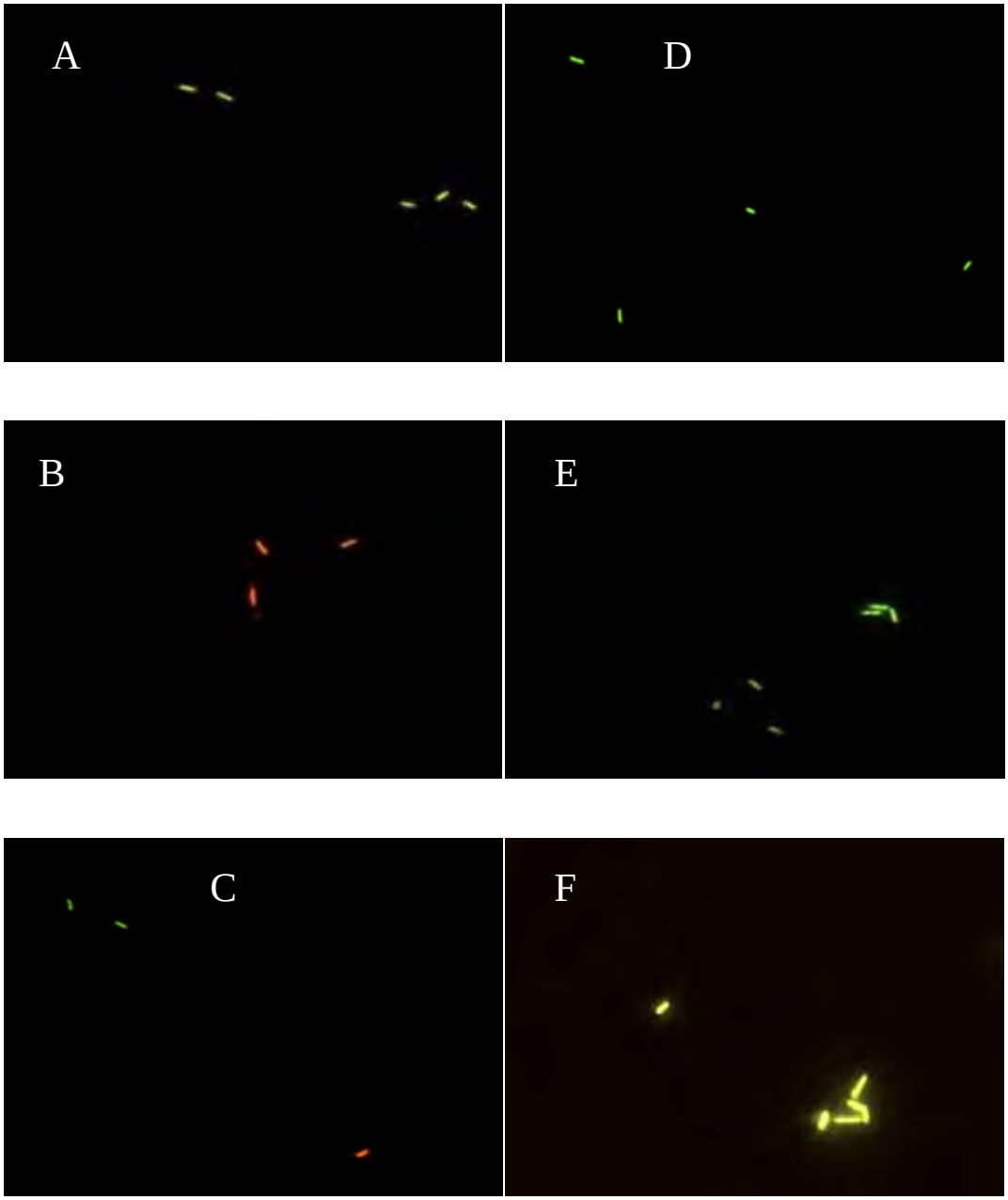


Figure 5.2: Live-Dead staining of *P. aeruginosa* cells attached to surfaces coated with peptides via ABA linkers after 1 hour incubation in assay medium with or without 20 mM $MgSO_4$. Cells stained green indicate an intact membrane while cells stained red indicate damaged membrane. (A) Process control in PBS, (B) ABA-Melimine in PBS, (C) ABA-Melimine 4 in PBS, (D) Process control in PBS containing 20 mM $MgSO_4$, (E) ABA-Melimine in PBS containing 20 mM $MgSO_4$ and (F) ABA-Melimine 4 in PBS containing 20 mM $MgSO_4$

5.3.3 *P. aeruginosa* at 5 hour interval

5.3.3.1 Viability of bacteria adhered to coated surfaces in the absence of Mg^{2+}

Compared to process controls, glass surfaces coated with melimine or melimine 4 after 5 hour adhesion resulted in significant reduction in viability of *P. aeruginosa* (approximately 2-fold reduction; $P \leq 0.01$ for melimine and approximately 1.5-fold reduction; $P = 0.005$ for melimine 4; Fig. 5.3 A). The reduction in viability caused by melimine was also statistically significant (approximately 1.3-fold reduction; $P = < 0.01$) when compared to melimine 4 (Fig. 5.3 A).

5.3.3.2 Effect of excess Mg^{2+} on viability of bacteria adhered to coated surfaces

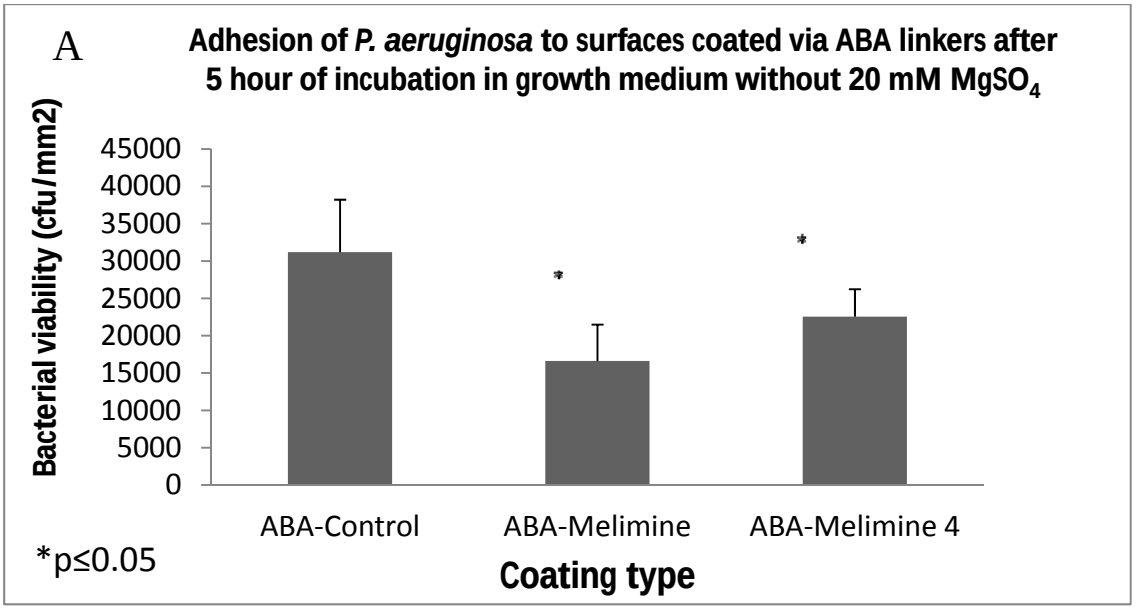
Compared to process controls no significant reduction in the adhesion of viable bacteria was observed for either melimine or melimine 4 treated surfaces when assay medium was supplemented with 20 mM $MgSO_4$ (Fig. 5.3 B). However, for all groups (control, melimine and melimine 4), a significantly higher number of viable adherent bacterial was observed in growth medium supplemented with 20 mM $MgSO_4$ compared to growth medium without added 20 mM $MgSO_4$ (Fig. 5.3 A and Fig. 5.3 B).

5.3.3.3 Image analysis

In the absence of 20 mM $MgSO_4$ no significant difference was observed in surface area covered by bacteria for melimine and melimine 4 when compared to process control (Fig. 5.3C). Compared to controls, surfaces coated with melimine, caused an approximate 5-fold reduction ($P < 0.01$) in live cells (bacteria with intact membranes) and an approximate 24-fold increase ($P < 0.01$) in dead cells (bacteria with damaged membranes). Surfaces coated with melimine 4 resulted in approximately 6-fold ($P < 0.01$) reduction in live cells and approximately 13-fold increase in dead cells ($P < 0.01$. No significant difference was observed for live or dead cell counts between melimine and melimine 4 (Fig. 5.3C).

When assay medium was added with 20 mM $MgSO_4$, compared to process controls, both melimine and melimine 4 showed a reduction in surface area coverage (approximately 3-fold for melimine; $P = 0.042$ and approximately 4-fold for melimine 4; $P = 0.015$) however, no significant reduction in Live or dead-cell count was observed for melimine as well as melimine 4 when compared to process controls (5.3 D).

The presence of 20 mM $MgSO_4$ in the assay medium also resulted in significantly higher surface area coverage for process controls (approximately 8-fold increase; $P < 0.01$), melimine (approximately 2.5-fold increase; $P < 0.01$) and melimine 4 (approximately 2.5-fold increase; $P < 0.03$) when compared to respective surfaces in the absence of 20 mM $MgSO_4$ (Fig. 5.3 C and 5.3D).



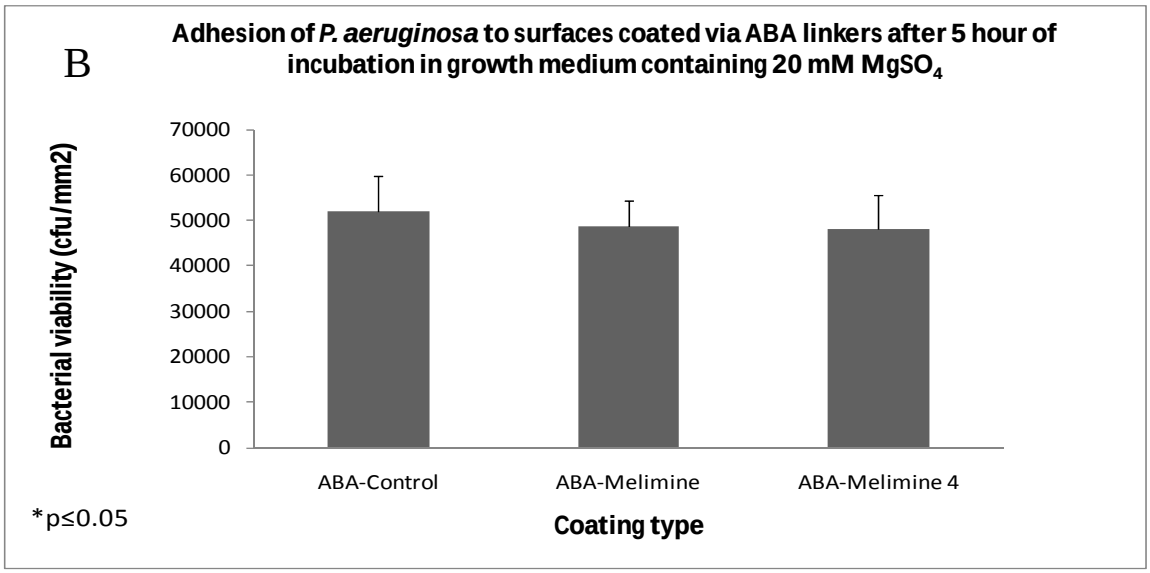
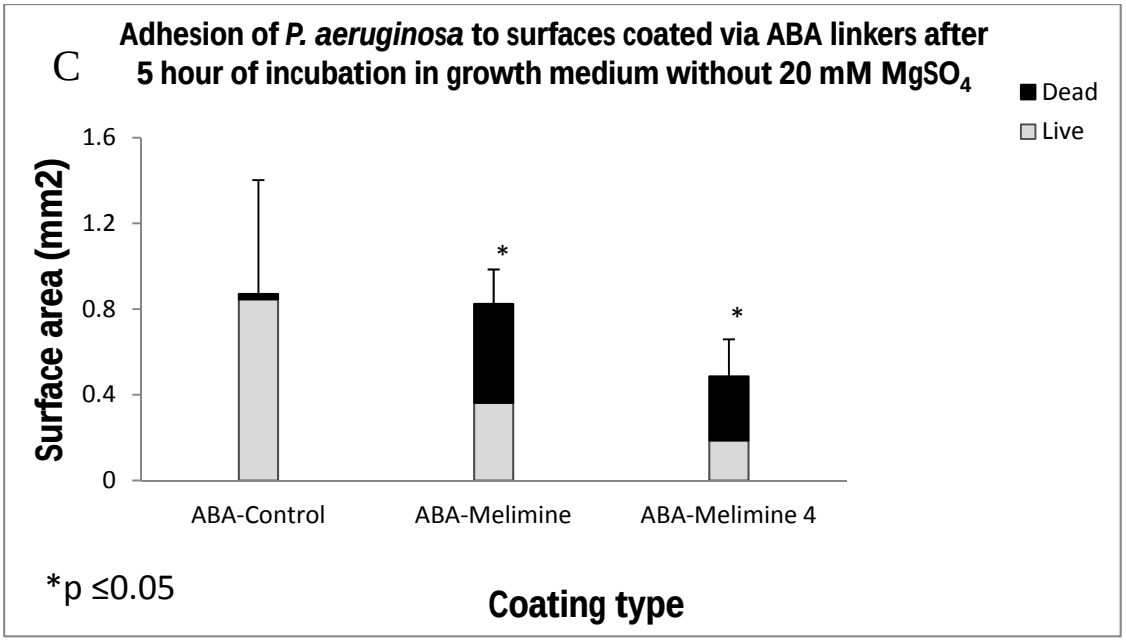


Figure 5.3: Effect of coated surfaces on viability of *P. aeruginosa* cells after 5 hour incubation in growth medium with or without 20 mM $MgSO_4$. (A) Viability of bacterial cells adhered to coated surfaces in growth medium without 20 mM $MgSO_4$, (B) Viability of bacterial cells adhered to coated surfaces in growth medium that contains 20 mM $MgSO_4$. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.05 (n = 9).



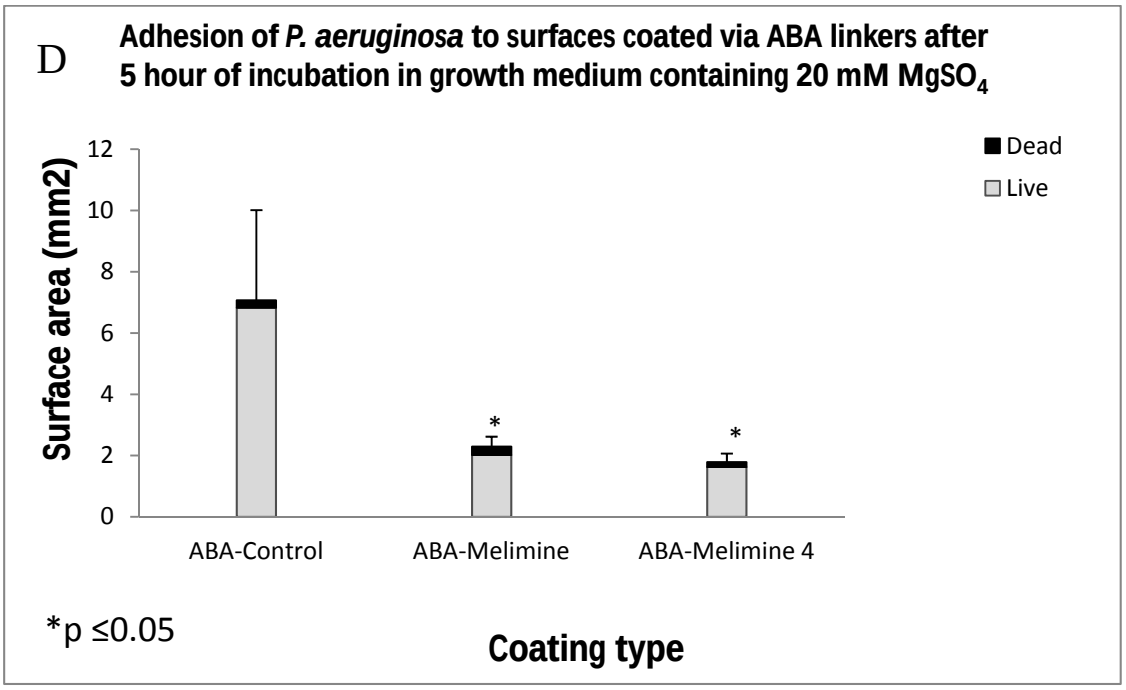


Figure 5.3: Effect of coated surfaces on viability of *P. aeruginosa* cells after 5 hour incubation in growth medium with or without 20 mM $MgSO_4$. (C) Live-dead count and surface area coverage of *P. aeruginosa* on coated surfaces in growth medium without 20 mM $MgSO_4$ and (D) Live-dead count and surface area coverage of *P. aeruginosa* on coated surfaces in growth medium containing 20 mM $MgSO_4$. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.05 (n = 9).

Fig. 5.4 shows the representative fields of adhered cells after 5 hours of adhesion of process control, melimine and melimine 4 in the presence or absence of 20 mM $MgSO_4$. Significantly higher numbers of cells were found to be attached to surfaces when compared to their respective groups at 1 hour interval ($P < 0.01$). Process control in the absence 20 mM MgSO4 of showed >97% of green cells with (intact membranes; Fig. 5.4 A). Surfaces coated with melimine and melimine 4 (Fig 5.4 B and 5.4 C), in the absence of 20 mM $MgSO_4$, resulted in cells with damaged cell membrane (red cells). A comparatively higher number of cells were found to be attached to surfaces in medium supplemented with 20 mM $MgSO_4$, (Fig 5.4 D, E and F) however, surfaces coated with both melimine (Fig. 5.4 E) and melimine 4 (Fig. 5.4 F) shows less surface area coverage ($P = 0.042$ for melimine and $P = 0.015$ for melimine 4) as compared to process controls. However, no significant difference was observed for live-dead cell counts

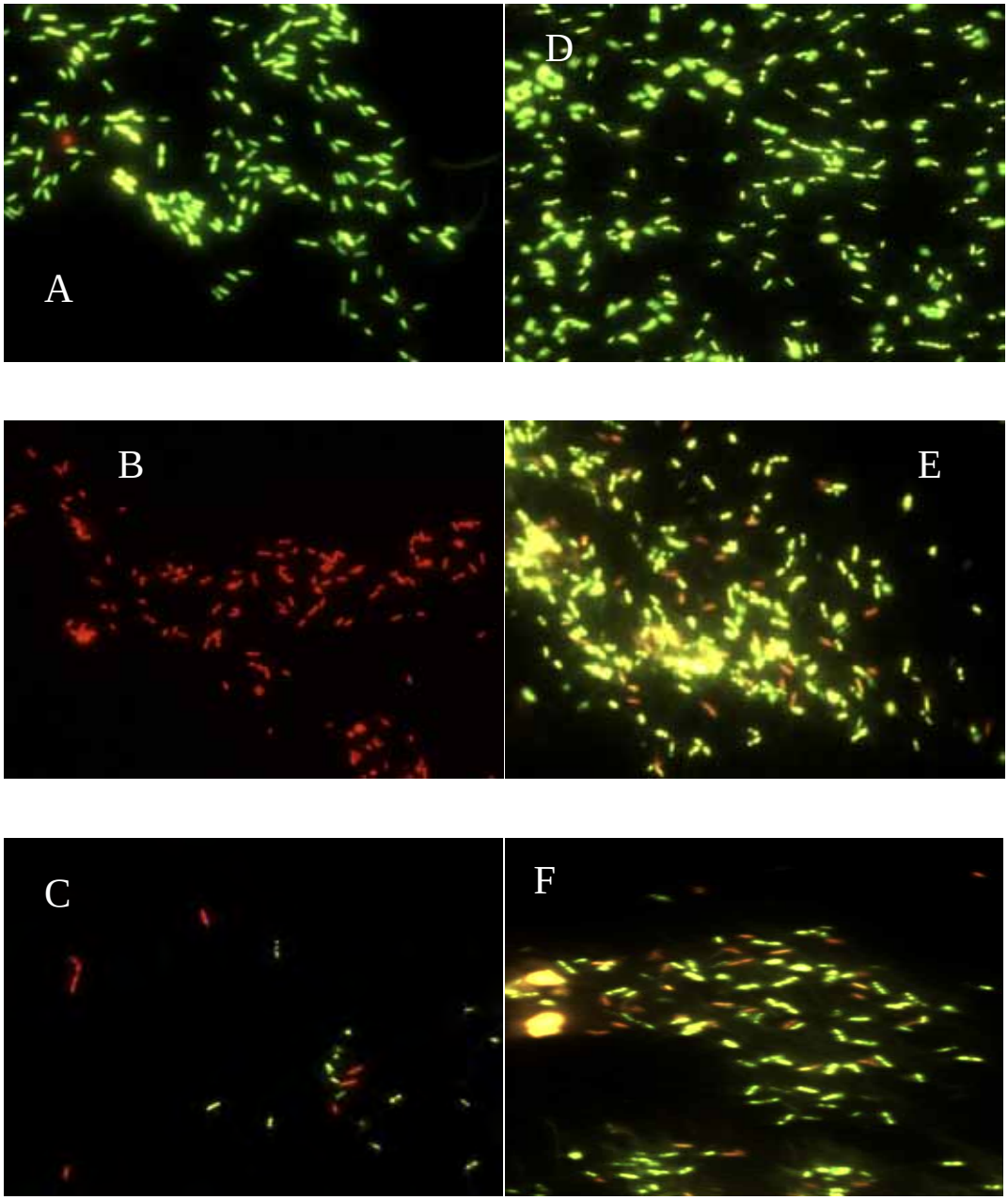


Figure 5.4: Live-Dead staining of *P. aeruginosa* cells attached to surfaces coated with peptides via ABA linkers after 5 hour incubation in assay medium with or without 20 mM $MgSO_4$. Cells stained green indicate an intact membrane while cells stained red indicate damaged membrane. (A) Process control in PBS, (B) ABA-Melimine in PBS, (C) ABA-Melimine 4 in PBS, (D) Process control in PBS containing 20 mM $MgSO_4$, (E) ABA-Melimine in PBS containing 20 mM $MgSO_4$ and (F) ABA-Melimine 4 in PBS containing 20 mM $MgSO_4$

5.3.4 *S. aureus* at 1 hour interval

5.3.4.1 Viability of bacteria adhered to coated surfaces in the absence of Mg^{2+}

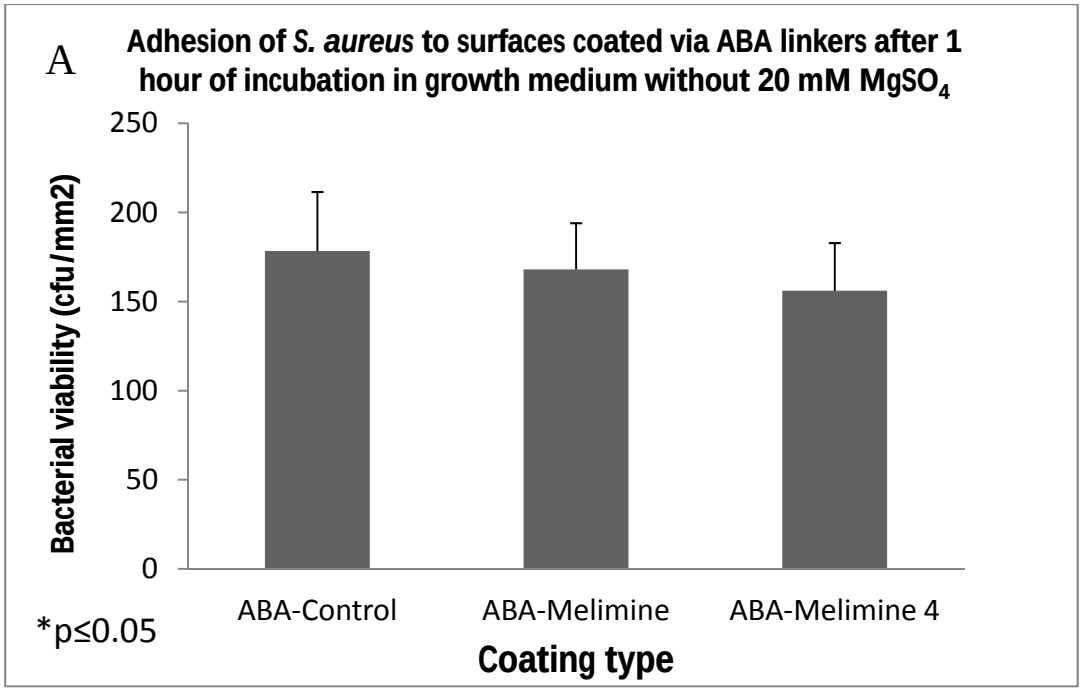
In the absence of 20 mM $MgSO_4$ in assay medium, compared to process controls, no significant reduction in numbers of viable adherent bacteria or bacterial viability was observed for surfaces coated with either melimine or melimine 4 (Fig. 5.5 A).

5.3.4.2 Effect of excess Mg^{2+} on viability of bacteria adhered to coated surfaces

In the presence of 20 mM $MgSO_4$ in assay medium, compared to process controls, no significant reduction in bacterial viability was observed for surfaces coated with either melimine or melimine 4 (Fig. 5.5 B). Furthermore, no significant difference was observed for bacterial viability in presence or absence of 20 mM $MgSO_4$ in assay medium.

5.3.4.3 Live-Dead staining

Live dead staining of bacteria attached to surfaces after one hour of incubation in growth medium with or without 20 mM $MgSO_4$ showed very little adhesion. The results are summarized in table 5.2 with no statistical difference found in total, live or dead cells for any of the group tested).



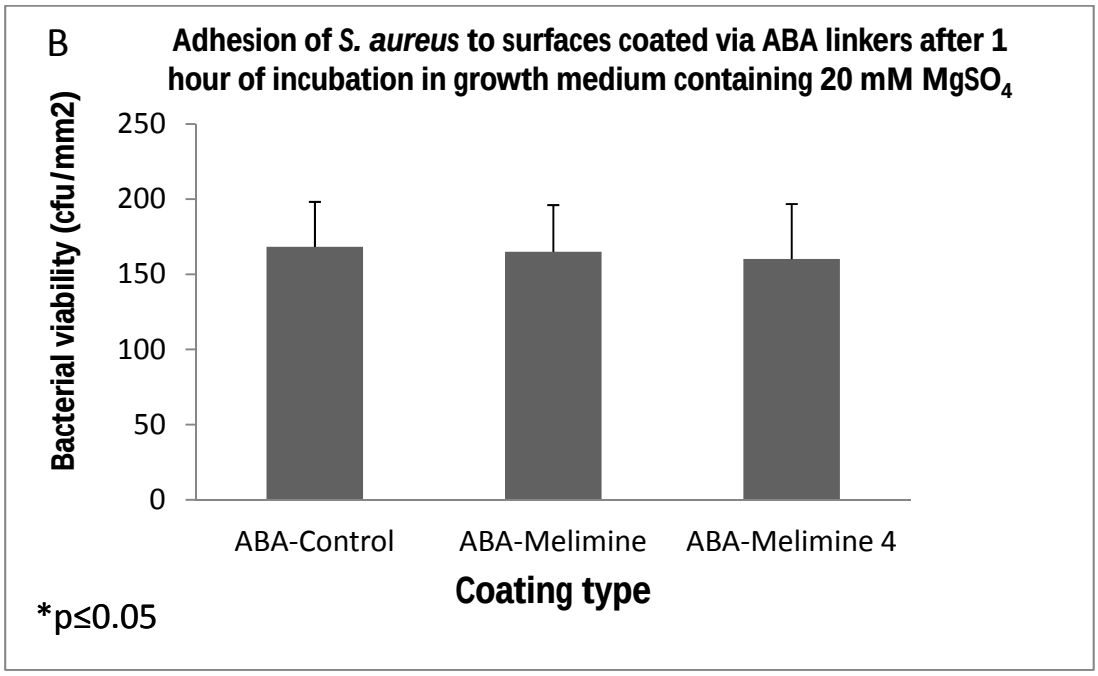


Figure 5.5: Effect of coated surfaces on viability of *S. aureus* cells after 1 hour incubation in growth medium with or without 20 mM $MgSO_4$. (A) Viability of bacterial cells adhered to coated surfaces in growth medium without 20 mM $MgSO_4$, (B) Viability of bacterial cells adhered to coated surfaces in growth medium that contains 20 mM $MgSO_4$. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.05 (n = 9).

Table 5.2: Live-Dead staining of *S. aureus* adhered to coated surfaces after 1 hour of incubation in growth medium with or without 20 mM $MgSO_4$

Type of Coating	20 mM $MgSO_4$ in assay medium	Total cells per field of view	% Live cells per field of view	% Dead cells per field of view
ABA	absent	6 ± 2	100	0
ABA-Melimine	absent	5 ± 2	91 ± 25	8 ± 25
ABA-Melimine 4	absent	5 ± 1	100	0
ABA	present	5 ± 2	97 ± 7	3 ± 7
ABA-Melimine	present	4 ± 1	100	0
ABA-Melimine 4	present	5 ± 1	100	0

Fig. 5.6 shows the representative fields of the cells attached to various glass surfaces. Very little adhesion was observed for process control, melimine and melimine 4 in the presence or absence of 20 mM $MgSO_4$. All the cells appeared green (intact membrane).

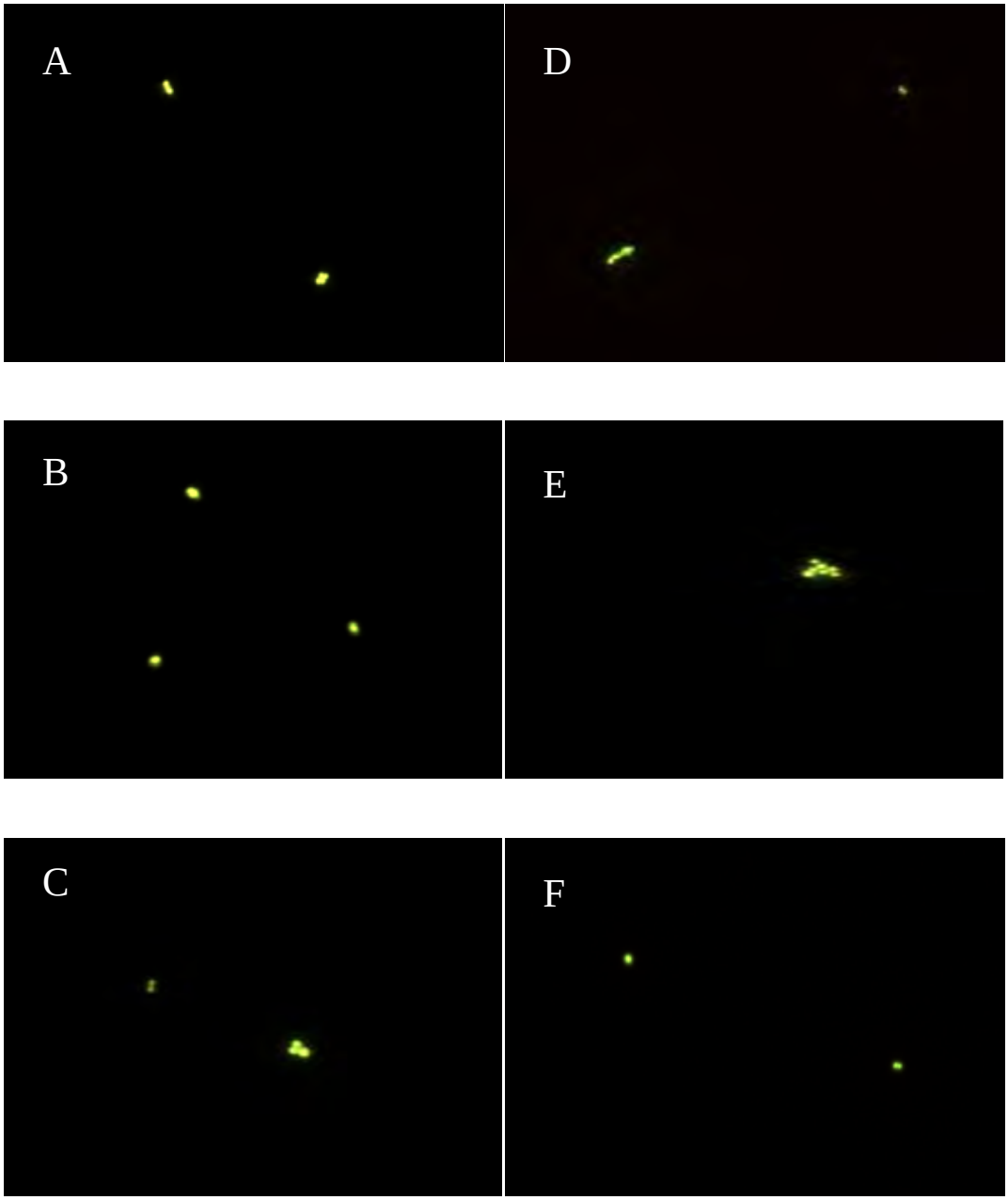


Figure 5.6: Live-Dead staining of *S. aureus* cells attached to surfaces coated with peptides via ABA linkers after 1 hour incubation in assay medium with or without 20 mM $MgSO_4$. Cells stained green indicate an intact membrane while cells stained red indicate damaged membrane. (A) Process control in PBS, (B) ABA-Melimine in PBS, (C) ABA-Melimine 4 in PBS, (D) Process control in PBS containing 20 mM $MgSO_4$, (E) ABA-Melimine in PBS containing 20 mM $MgSO_4$ and (F) ABA-Melimine 4 in PBS containing 20 mM $MgSO_4$

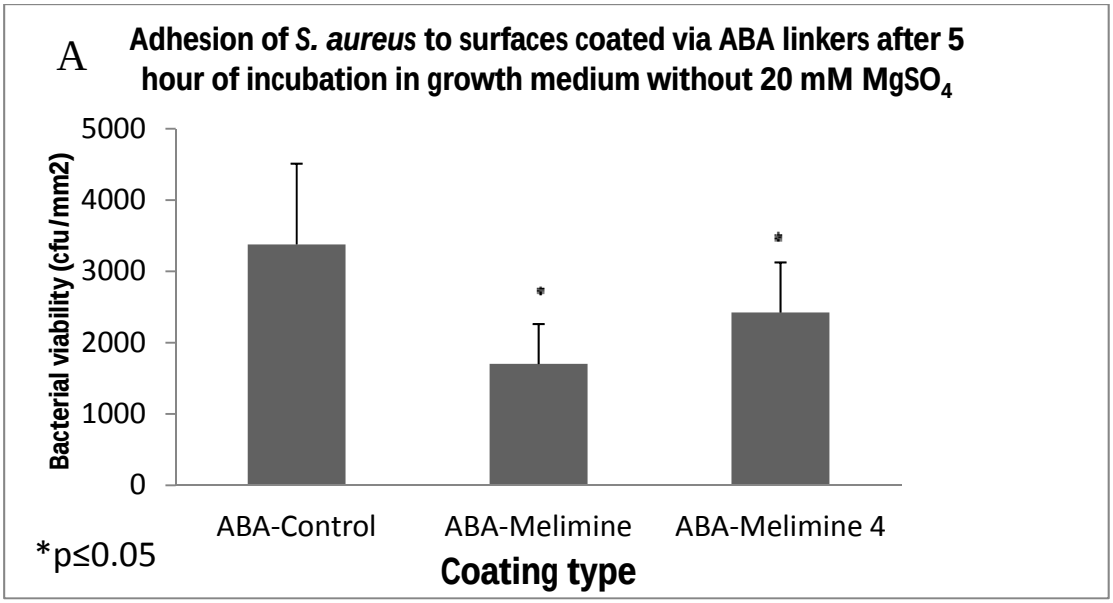
5.3.5 *S. aureus* at 5 hour interval

5.3.5.1 Viability of bacteria adhered to coated surfaces in the absence of Mg^{2+}

In the absence of 20 mM $MgSO_4$ in assay medium, compared to process controls, significant reductions in the numbers of viable adherent bacteria was observed for surfaces coated with either melimine (approximately 2-fold reduction; $P < 0.01$) or melimine 4 (approximately 1.4-fold reduction; $P = 0.02$; Fig. 5.7 A). Melimine coated surfaces resulted in higher reductions (approximately 1.4-fold reduction; $P = 0.02$) in bacterial viability as compared to melimine 4 coated surfaces ($P < 0.01$; Fig. 5.7 A).

5.3.5.2 Effect of excess Mg^{2+} on viability of bacteria adhered to coated surfaces

In the presence of 20 mM $MgSO_4$ in assay medium, compared to process controls, no significant reduction in bacterial viability was observed for surfaces coated with either melimine or melimine 4 (Fig. 5.7 B) however, significantly higher bacterial viability was observed in process controls (2-fold; $P = 0.005$), melimine (approximately 4-fold; $P < 0.001$) and melimine 4 (approximately 2.7-fold; $P = 0.002$) in assay medium supplemented with 20 mM $MgSO_4$ as compared to assay medium without added 20 mM $MgSO_4$ (Fig. 5.7 A, 5.7B).



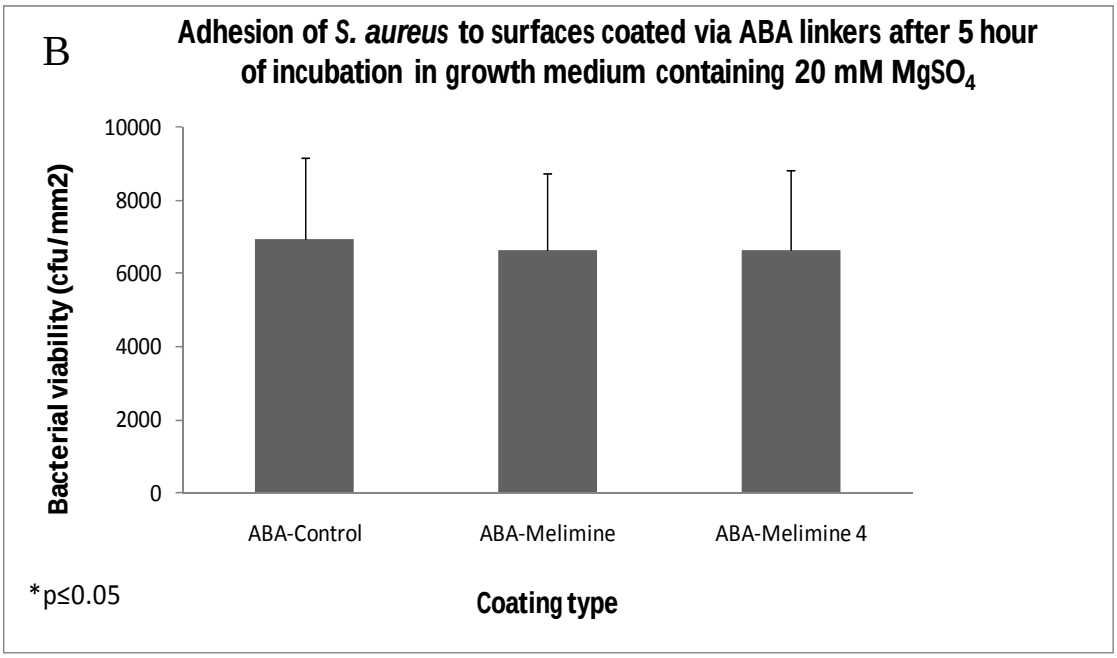


Figure 5.7: Effect of coated surfaces on viability of *S. aureus* cells after 5 hour incubation in growth medium with or without 20 mM $MgSO_4$. (A) Viability of bacterial cells adhered to coated surfaces in growth medium without 20 mM $MgSO_4$, (B) Viability of bacterial cells adhered to coated surfaces in growth medium that contains 20 mM $MgSO_4$. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.05 (n = 9).

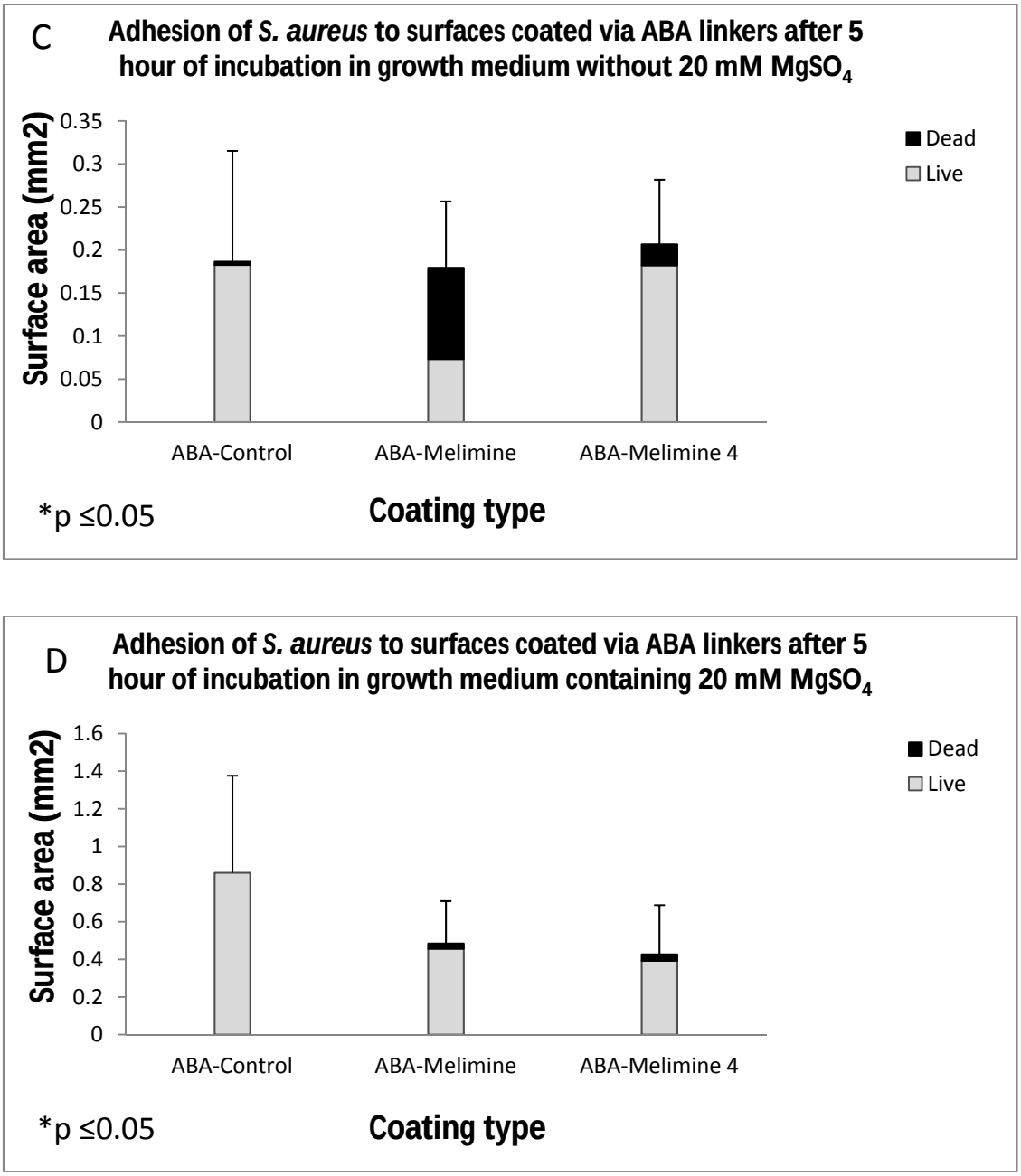


Figure 5.7: Effect of coated surfaces on viability of *S. aureus* cells after 5 hour incubation in growth medium with or without 20 mM $MgSO_4$. (C) Live-dead count and surface area coverage of *S. aureus* on coated surfaces in growth medium without 20 mM $MgSO_4$ and (D) Live-dead count and surface area coverage of *S. aureus* on coated surfaces in growth medium containing 20 mM $MgSO_4$. Standard deviation is shown by the vertical bars and * denotes statistical significance of ≤ 0.05 (n = 9).

5.3.5.3 Image analysis

Within the same group, compared to controls, no significant difference was observed in surface area coverage by cells on surfaces coated with either melimine or melimine 4 in presence or absence of 20 mM $MgSO_4$ (Fig. 5.7 C, Fig. 5.7 D). When surface area was compared between groups, a significantly higher (4.5-fold; $P = 0.03$) surface area was found to be covered by process control in assay medium that contained 20 mM $MgSO_4$ as compared to the medium without 20 mM $MgSO_4$, however this increase was not observed for melimine or melimine 4 in presence of 20 mM $MgSO_4$ in the assay medium (Fig. 5.7 C, Fig. 5.7 D).

No statistical difference was observed in live and dead cell counts for all groups in the presence or absence of 20 mM $MgSO_4$ in assay medium (Fig. 5.7 C, Fig. 5.7 D).

Fig. 5.8 shows the representative fields of the cells attached to various glass surfaces. Bacterial adhesion observed for process controls, melimine and melimine 4 modified surfaces increased considerably at 5 hour interval as compared to the 1 hour interval. Some dead cells could be observed for surfaces coated with melimine (Fig. 5.8 B) and melimine 4 (Fig. 5.8 C).

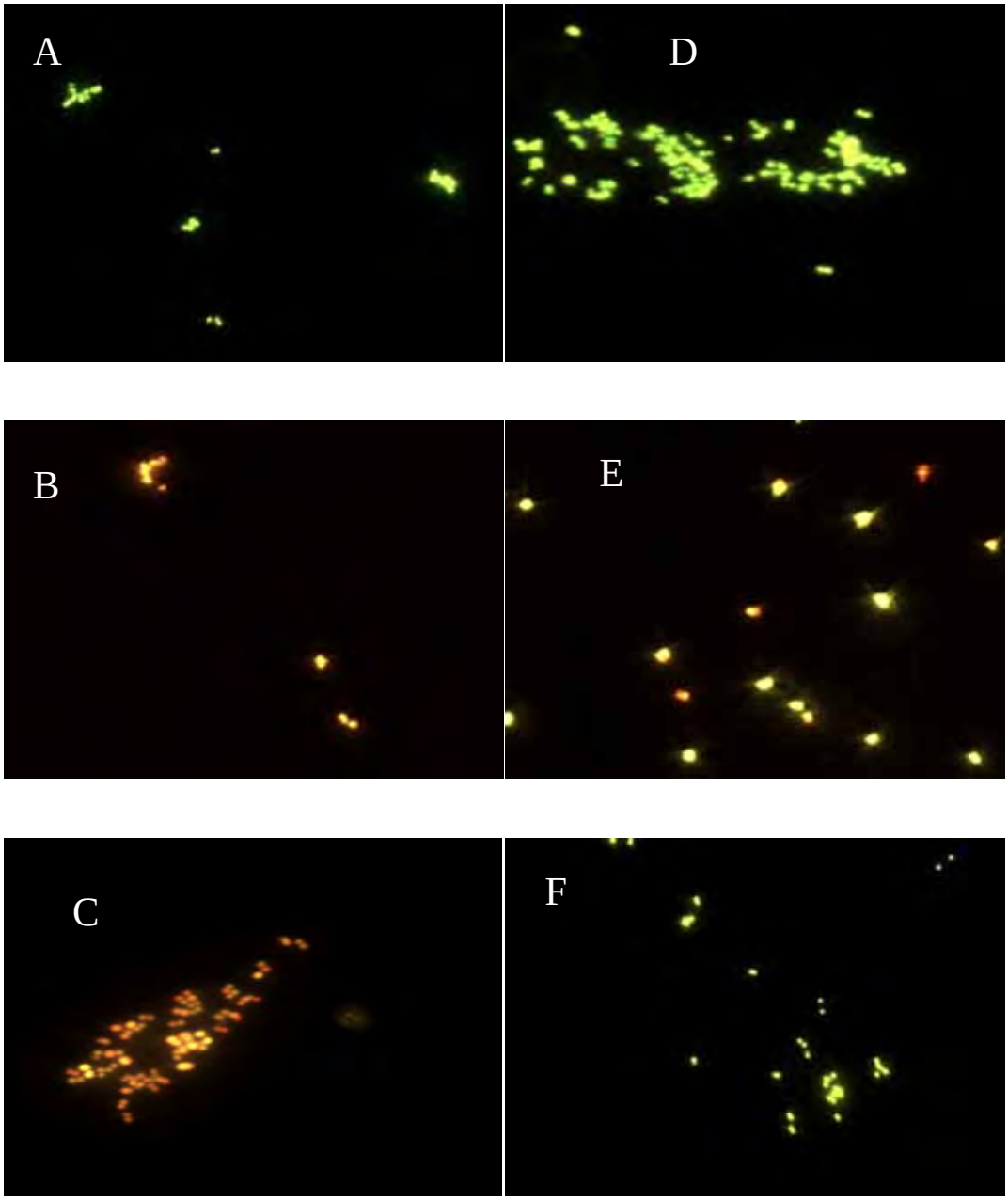


Figure 5.8: Live-Dead staining of *S. aureus* cells attached to surfaces coated with peptides via ABA linkers after 5 hour incubation in assay medium with or without 20 mM $MgSO_4$. Cells stained green indicate an intact membrane while cells stained red indicate damaged membrane. (A) Process control in PBS, (B) ABA-Melimine in PBS, (C) ABA-Melimine 4 in PBS, (D) Process control in PBS containing 20 mM $MgSO_4$, (E) ABA-Melimine in PBS containing 20 mM $MgSO_4$ and (F) ABA-Melimine 4 in PBS containing 20 mM $MgSO_4$

5.4 Discussion

The current study aimed at the exploring mechanisms of action of the antimicrobial peptide melimine and its shorter derivative melimine 4 when covalently attached to surfaces. Although no conclusive evidence was found about the exact mechanism of action of these peptides, some of the observations made led to a better understanding of the peptide's interactions with bacteria and provide a platform for future work.

For surfaces coated with melimine, the membrane damage for *P. aeruginosa*, as assessed by Live-Dead staining was swift as a significant proportion of bacteria appeared red after one hour of incubation in the assay medium without excessive magnesium. The membrane damage correlated well with loss of viability as bacterial viability data at corresponding time interval showed a significant reduction in viable counts when compared to process controls. This data also correlated well with the mechanistic studies carried out in solution with melimine (see chapter 4) where membrane depolarisation of *P. aeruginosa* was accompanied by a loss of viability. Similar trend was observed at five hour interval. However, surfaces coated with melimine 4 at one hour time interval showed no appreciable bacterial cells with damaged membranes. Additionally, no significant reduction in viable counts after one hour indicated that the antimicrobial action of melimine 4 in covalently bound to glass surfaces were not initiated at one hour time interval. However, after 5 hours of incubation a 13-fold increase in number of cells with damaged membranes could be visualized on surfaces coated with melimine 4 along with 1.5-fold reduction in viability. The antimicrobial action of melimine, however, was significantly superior to that of melimine 4 at 5 hour time interval. The delayed and comparatively inferior antimicrobial activity of melimine 4 could be

attributed to its shorter length (17 residues as compared to 29 residues for melimine), low amphipathicity (2% as compared to 25% for melimine) and lack of hydrophobicity (0% as compared to 17% in melimine) (calculations based on a peptide sequence analysis tool set up by Tossi *et al*, (2002)). These results suggest that while disruption of the cytoplasmic membrane appeared to induce loss of viability in *P. aeruginosa*, melimine 4 acts by binding to negatively charged surfaces of the bacterial cells without any penetration in the hydrophobic core that requires the amphipathic structures and its mode of action may be similar to another hydrophilic, non-amphipathic AMP, Androctonin (Hetru *et al*, 2000).

The mode of action of covalently bound peptides used in the study appeared to be distinct for *S. aureus*. Unlike *P. aeruginosa*, loss of cell viability was not accompanied by membrane damage. Additionally, antimicrobial activity was only evident at 5 hour interval. This discrepancy could be attributed to the presence of bacterial cells in intermediate states (Berney *et al*, 2007) referred to in the BacLight Bacterial Viability Kit manufacture material as 'unknown' (Molecular Probes, Inc, Eugene, OR, 2004). Daptomycin, a lipopetide, was also shown to reduce the viability of *S. aureus* by 99% within 10 minutes at 4 × MIC without any apparent damage to membrane as assessed by live-dead staining (Hobbs *et al*, 2008). Chen *et al*, (2009) demonstrated a higher proportion of surface-adherent *S. aureus* cells with damaged membrane as compared to the current study. However, their experimental conditions differed to those in the present study. That study examined the effects of melimine coating on biofilm formation in high nutrient conditions whereas the present study examined the effects on initial adhesion and colonisation of the surface under low nutrient conditions.

Addition of excess Mg^{2+} in the assay medium resulted in negating the effects of both peptides as reduction in adhesion by coated surfaces was not found to be statistically significant to controls. The antagonistic nature of Mg^{2+} has been reported before and reflects the competition between the Mg^{2+} and the peptides for the available binding sites to the phosphate groups within the lipopolysaccharides of Gram-negative bacteria or lipotechoic acid in case of gram-positive bacteria (Aspedon and Groisman, 1996; Jenssen *et al*, 2006; Sanchez-Gomez *et al*, 2008).

Adhesion of both *P. aeruginosa* and *S. aureus* to surfaces significantly increased in the presence of 20 mM $MgSO_4$. Adhesion of bacteria has been known to be influenced by Mg^{2+} through its direct effects on electrostatic interactions promoting an increase in attached cells without having an influence on growth of planktonic cells (Song and Leff, 2006). Additionally, Mg^{2+} also acts indirectly as a vital cellular cation and enzyme cofactor (Fletcher, 1988). Notably, the observed increase in adhesion of both organisms was not a direct consequence of higher cationicity of either of the two peptides (melimine and melimine 4 has a net positive charge of +16 and 14 respectively), as assay medium without excess Mg^{2+} failed to increase adhesion compared to medium supplemented with 20 mM $MgSO_4$. Previous published reports suggested an increased adhesion of bacteria on high-density positive charge surfaces such as polylysine layers (Richert *et al*, 2002) and on surfaces modified by magainins (Humblot *et al*, 2009).

Finally, adhesion of *P. aeruginosa* was significantly higher than *S. aureus* on glass surfaces. The results from chapter 2 demonstrated a similar pattern of adhesion on contact lenses. Although adhesion of bacteria to biomaterial surfaces has been shown to be strain dependent (Borazjani *et al*, 2004), there is a general agreement that *P. aeruginosa*, by means of

producing greater quantities of extracellular polymeric substances, shows a superior tendency to adhere to hydrophilic glass surfaces than *S. aureus* (Bruinsma *et al*, 2001; Mitik-Dineva *et al*, 2009).

Unlike mechanism of action studies in solution that rely primarily on direct observations, most studies conducted thus far on attached peptides have relied on hypothesis or indirect evidence. For example, one of the earliest studies conducted on surface bound peptides by LaPorte (1977), attributed the killing of *P. aeruginosa* and *E. coli* by covalently attached polymyxin B to the disruption of their outer membranes. They proposed that disruption of outer membrane indirectly affected the selective permeability of the cytoplasmic membrane that resulted in bacterial killing. Similar conclusions were made by Haynie *et al*, (1995). They used short linkers (two or six carbon atoms in length) to attach magainin to surfaces and suggested that the antimicrobial activity of the attached magainin, with limited ability to penetrate inside the cytoplasmic membrane, could be a consequence of interaction with outer membrane of Gram-negative bacteria. More recently, Hilpert *et al*, (2009), used some direct techniques to elucidate the mechanism of action of peptides bound to resin. These techniques included determination of ATP following bacterial contact with the bound peptide to assess change in cytoplasmic membrane permeability which under normal conditions is impermeable to ATP release; and cytoplasmic membrane depolarisation assays to assess the effect of bound peptide on membrane potential of bacteria. They concluded that the attached peptides changed the cytoplasmic membrane permeability and caused a leakage of ATP.

In conclusion, the data from this study demonstrates that both melimine and its derivative melimine 4 in covalently bound form, reduce adhesion of bacteria however, the parent

peptide melimine appeared to be superior in bound form than the shorter derivative melimine 4 at concentration of only 4.3×10^{-9} moles cm $^{-2}$ as compared 1.26×10^{-8} of concentration melimine 4 which is approximately three times as high as that of melimine. Although more work is still required to fully understand the mechanism of action, the data suggest an initial electrostatic interaction of these peptides with the polyanionic surfaces of bacteria causing a displacement of cations on bacterial surfaces. This process may lead to ionic imbalance across the cytoplasmic membrane and potentially initiate a chain of events such as activation of autolytic enzymes (Hilpert *et al*, 2009).

Chapter 6: Summary and Future Perspectives

6.1 Summary

The extensive use of biomaterials has resulted in significant improvement in longevity and quality of life for patients. However, infections associated with the use of biomaterials remain a major concern. Despite advancement in biomaterial manufacturing technology coupled with improvements in minimising risk factors such as contamination during implantation, antibiotic prophylaxis and prompt treatment of peripheral infections, biomaterial infections are still on the rise (Campoccia *et* al, 2006; Qiu *et al*, 2007). For example, Darouiche *et al*, (2007) suggested that of the two million nosocomial infections that occur in the United States (US) annually, almost half were device-related. Additionally, the cost to the community of BAI is high, with total additional expenses exceeding $US11 billion per annum for biomaterial infections (Henderson and Levy, 1997). These figures consider only the direct medical costs to the community and not costs associated with reduced work productivity or quality of life.

The current preventive measures, alone or in various combinations, are not enough to prevent BAI as even the most carefully implanted devices are contaminated by small numbers of bacteria (Schierholz, and Beuth, 2001). There is a growing need to find suitable alternatives for the prevention of BAI. One emerging area of research to address this need is the use of antimicrobial peptides: a new class of therapeutic antibiotics.

In this study, a synthetic antimicrobial peptide melimine was attached to contact lens surfaces in soaked as well as covalently-bound form to assess its ability to reduce adhesion of bacteria commonly associated with lens-related infections i.e. *P. aeruginosa* and *S. aureus*. Additionally, multi-drug resistant strains of both organisms including methicillin-resistant *S. aureus* strains (MRSA) were also used in the study. The results suggested that melimine was effective in reducing adhesion of bacteria both in soaked as well covalently-bound form. Further, melimine was found to be equally effective against multi-drug resistant strains.

Once melimine's ability to reduce bacterial adhesion on contact lens surfaces was established, the next step involved in the development of melimine was to explore whether comparable antibacterial activity could be achieved by shortening the sequence length of peptide. Parent peptide melimine (TLISWIKNKRKQRPRVSRRRRRRGGRRRR) is composed of a portion of protamine and a peptide based on melittin (Willcox et al. 2008). Four sequences of melimine of varying length, amphipathicity and hydrophobicity were synthesised by removal of amino acids from various positions in the protamine and melittin moiety of the peptide and adhesion assays were carried out. One of the short sequences of melimine, melimine 4, that was composed of 17 amino acids (KNKRKRRRRRRGGRRRR) as compared to 29 amino acids of the parent peptide melimine, caused a reduction in adhesion of bacteria in both soaked and covalently-bound form. The results were comparable to parent peptide melimine. Melimine 1 (TLISWIQRPRVS) was found to be ineffective in reducing adhesion of both *P. aeruginosa* and *S. aureus* while melimine 2 (TLISWIKNKRKQRPRVS) was only found to be effective against *S. aureus*. Both melimine 3 (TLISWIQRPRVSRRRRRRGGRRRR) and melimine 4 (KNKRKRRRRRRGGRRRR) were effective in reducing adhesion of *S. aureus* as well as *P. aeruginosa*. Of the four

sequences tested, melimine 4 was found to be the most efficacious with the least number of amino acids residues. Additionally, the efficacy of melimine 4 was comparable to that of parent peptide melimine. The results from the these experiments suggest that the *N*- terminal region composed primarily of poly-arginine (RRRRRRGGRRRR) is vital to melimine activity as this region was retained in both melimine 3 (TLISWIQRPRVSRRRRRRGGRRRR) and melimine 4 (KNKRKRRRRRRGGRRRR).

The parent peptide melimine was further investigated to determine its mechanism of action in solution. Spectroscopic analysis of melimine suggested that it adopts a more helical structure in bacterial membrane-mimetic environments, but not those mimicking mammalian membranes. Investigation of the interaction of melimine with *P. aeruginosa* and *S. aureus* showed that the initial interaction of the peptide with both types of bacteria was predominantly governed by electrostatic forces, a finding consistent with that for many cationic peptides (Aspedon and Groisman, 1996; Groisman, 1996; Zhang *et al*, 2000). The study also demonstrated that for *P. aeruginosa*, disruption of the outer membrane occurs rapidly and at well below the concentration required to kill the bacteria in the same medium. The data also suggested that a major effect of melimine is on the integrity of the cytoplasmic membrane both for *P. aeruginosa* and *S. aureus*, however differences in the relationship were observed between depolarisation of the cytoplasmic membrane and the kinetics of loss of viability of the bacteria. For *P. aeruginosa* the rapid loss of cytoplasmic membrane integrity correlated directly with loss of cell viability, whilst for *S. aureus* depolarisation of cytoplasmic membrane occurred at concentrations where there was no significant loss of viability. This suggests that either the effects of depolarisation of the cytoplasmic membrane were delayed in *S. aureus* or that a secondary process affecting metabolic activity in the cell

may occur in this case. Therefore, other intracellular processes cannot be ruled out, including macromolecular synthesis and reversible phosphorylation of proteins (Jia, 1997) or other energy transducing processes.

The next step was to study mechanism of action of parent peptide melimine and its shorter derivative melimine 4 while covalently-bound to surfaces. The data suggested an initial electrostatic interaction of these peptides with the polyanionic surfaces of bacteria causing a displacement of cations on bacterial surfaces. This process may lead to ionic imbalance across the cytoplasmic membrane and potentially initiate a chain of events such as activation of autolytic enzymes (Hilpert *et al*, 2009). For *P. aeruginosa*, membrane damage on melimine coated surfaces, as assessed by Live-Dead staining was evident after one hour and correlated well with loss of viability at this time. However, the results from surfaces coated with melimine 4 indicated that the antimicrobial effects of melimine 4 were not initiated at one hour time interval. Nevertheless, after five hours melimine 4 appeared to cause damage to membranes with a corresponding loss of bacterial viability. The mode of action of covalently bound peptides used in the study appeared to be distinct for *S. aureus*. Unlike *P. aeruginosa*, loss of cell viability was not accompanied by evidence of membrane damage evaluated by live-dead staining. Additionally, antimicrobial activity was only evident at 5 hour interval. This discrepancy could be attributed to the presence of bacterial cells in intermediate states (Berney *et al*, 2007) referred to in the BacLight Bacterial Viability Kit manufacture material as ‘unknown’ (Molecular Probes, Inc, Eugene, OR, 2004) where they lost the ability to produce viable colonies.

In conclusion, the parent peptide melimine and its shorter derivative melimine 4 appeared to be excellent candidates for further development as antimicrobial peptides covalently-bound to surfaces. Melimine appeared to cause a disruption of cytoplasmic permeability of *S. aureus* and *P. aeruginosa* in solution that correlates well with the loss of viability in case of *P. aeruginosa*. However, for *S. aureus* there appeared to be a lag between disruption of cytoplasmic permeability and loss of viability. Membrane damage as assessed by live-dead staining also appeared to account for the loss of viability as assessed by viable counts for *P. aeruginosa* when both peptides were covalently-bound to surfaces. However, melimine, as compared to melimine 4, caused a rapid disruption of cytoplasmic membrane permeability with accompanying loss of viability for *P. aeruginosa*. For *S. aureus*, no significant membrane damage as assessed by live-dead staining occurred in presence of covalently-bound melimine and melimine 4. Nevertheless, a loss of viability as assessed by viable counts indicated that presumably the bacterial cells were in intermediate states and lacked the ability to produce viable colonies.

6.2 Future Perspectives

The results from current study have clearly demonstrated the effectiveness of the parent peptide melimine and its shorter derivative melimine 4 as coatings on biomaterial surfaces to prevent adhesion of bacteria. Additionally, recent in-vivo studies by Cole *et al*, (2010) showed effectiveness of melimine-coated contact lenses for reductions in ocular symptom scores and in the degree of corneal infiltration. However the current findings suggest a considerably lower antibacterial activity of peptides in covalently-bound form as compared to their antimicrobial activity in solution. Bagheri *et al*, (2009) also found that immobilization of peptide resulted in reduced activity without any influence upon the activity spectrum.

Further investigations are required to test if that reduction in activity is dependent upon the strategy used for covalent attachment and whether it could be improved by other modifications in the peptide.

Cytotoxicity experiments carried out for melimine showed that it was cytotoxic at concentrations well above the MIC (Willcox *et al*, 2008). Furthermore, Cole *et al*, (2010) demonstrated excellent tolerance for melimine in a 5-day continuous wear of melimine-coated contact lenses in rabbit as well as guinea pig models where no adverse responses were observed including increased redness or chemosis. However, toxicity assays for melimine 4 will be required for its further development as antimicrobial peptide.

In solution, while mechanism of action of melimine elucidated in the present study was sufficient to account for *P. aeruginosa*, the step-by-step detail for *S. aureus* has yet to be fully demonstrated. There appeared to be a lag between cytoplasmic membrane depolarisation and bacterial killing which could be attributed to other intracellular targets. Further, studies that focus on the effects of melimine on autolysis and reversible phosphorylation of proteins (Jia, 1997) or other energy transducing processes could be helpful. Additionally, the differences between mechanisms of action of covalently-bound peptides as compared to their mechanism of action in solution require further investigation.

An important aspect of further development of melimine includes its ability to retain efficacy and maintain stability in different lens care products. Melimine will need to be further tested in contact lens cleaning solutions, contact lens storage solutions and contact lens rinsing

solutions. Additionally, melimine coated surfaces will also be tested *in vivo* to determine the possibility of fouling due to deposition of proteins and cellular debris.

The present study has shown melimine to be active against multi-drug resistant strains of *P. aeruginosa* and MRSA. Melimine could also be tested for efficacy against numerous other medically important bacterial species that are resistant to many conventional antibiotics such as vancomycin resistant *Enterococci* (VRE), coagulase-negative *Staphylococci*. *Acinetobacter* species, multi-drug resistant *Streptococcus pneumoniae* and multi-drug resistant bacteria belong to family Enterobacteriaceae including *E. coli* and K*lebsiella pneumonia*.

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Awards

2007	$10,000 Commercialisation training scholarship, the University of New South Wales
2008 – 2009	$7000 Cornea and Contact lens society of Australia award, two successive years
2009	$3000 UNSW travel grant, the University of New South Wales

Publications

1. **R. Rasul**, N. Cole, D. Balasubramanian, R.Chen, N. Kumar, M.D.P. Willcox (2010). "Interaction of the antimicrobial peptide melimine with bacterial membranes." Int J Antimicrob Agents 35(6): 566-572.
2. R Chen, N Cole, MDP Willcox, J Park, **R Rasul**, E Carter, N Kumar. (2009). "Synthesis, characterization and in vitro activity of a surface-attached antimicrobial cationic peptide." Biofouling 25(6): 517-524

Presentations at Conferences

1. Poster presentation at Federation of European Microbiological Societies (FEMS) conference 2009, Gothenburg, Sweden. **R Rasul**, N Cole, R Chen, N Kumar, MDP Willcox. Novel antimicrobial peptide melimine prevent adhesion of bacterial cells on Contact lens surfaces. FEMS, Gothenburg, June - July, 2009.

2. Poster presentation at Australian Society for Antimicrobials. **R Rasul**, N Cole, R Chen, J Park, N Kumar, MDP Willcox. Efficacy and mechanism of action of the antimicrobial peptide melimine. ASA, Sydney, February, 2008.

3. Poster presentation at Australian Society for Microbiology conference. N.Cole, R Chen, P Sankaridurg, N Kumar, A Vijay, **R Rasul**, M Willcox. The cationic peptide Melimine and its ability to control microbial colonisation of biomaterials. ASM Sydney, May 2010.