Activity and biocompatibility of antimicrobial contact lenses

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Activity and Biocompatibility of Antimicrobial Contact Lenses

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B. Optometry

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Dedication

আমার বাবা রাধেশ্যাম দত্ত, মা অজন্তা দত্ত
এবং
মৌ - এর জন্য

To my dad Radheshyam Dutta,

Mum Ajanta Dutta

&

Mou

Abstract

Contact lens related infection and inflammation are a major problem for contact lens wear for wearers and practitioners alike. Colonisation by a variety of microorganisms such as bacteria, fungi and *Acanthamoeba* on contact lenses is implicated in the development of these adverse events. A contact lens with high antimicrobial activity may inhibit the ability of microorganisms to grow on the surface and consequently reduce these contact lens related adverse events.

Antimicrobial peptides (AMPs) are small proteins which have been shown to have activity against a wide spectrum of microorganisms. This study investigated the spectrum of antimicrobial activity of an AMP covalently attached to contact lenses and its subsequent safety and biocompatibility in animal and human studies.

The AMPs melimine and LL-37 were covalently attached onto contact lenses via EDC coupling. This attachment method was able to bind high concentrations of melimine but not LL-37. Therefore, melimine-coated lenses were further investigated and demonstrated high bactericidal activity including activity against multidrug-resistant strains. The coated antimicrobial lenses also reduced viability of adherent fungal and *Acanthamoeba* strains. However, the lenses were associated with apparently high levels of dead bacterial cells (as evidenced by no differences in radio-labelled RNA or bacterial endotoxin from Gram negative bacteria on melimine-coated and uncoated contact lenses). Despite this, previous studies had shown that the presence of these bacterial components on lenses did not induce a keratitis response in animal models. The melimine coating was readily heat sterilisable, non-toxic to mammalian cells *in vitro*, did not change contact lens parameters, and formed a wettable lens surface. Antimicrobial activity was stable following 30 days incubation of melimine-coated

lenses in saline. The melimine coating was compatible with the lens care disinfectants Biotrue™ and RevitaLens OcuTec®. Further *in vivo* investigation of the safety of the melimine coating in a rabbit model of contact lens wear following ISO guidelines revealed that these lenses were not associated with any toxic or hypersensitive responses. A one day clinical trial showed that melimine-coated lenses could be safely worn by humans without any major side effects and any delayed toxic reactions. However, melimine-coated lenses were less preferred over control lenses and were associated with higher corneal straining. This corneal staining was similar to solution induced corneal staining seen when certain lens types are used with certain multipurpose disinfecting solutions containing (most commonly) polyhexamethyl biguanide. *Ex vivo* investigation showed that the lenses retained more than 1.5 log inhibition against *Pseudomonas aeruginosa* and *Staphylococcus aureus* after wear.

Melimine coatings on contact lenses have excellent potential for further development of biocompatible and effective broad spectrum antimicrobial contact lenses.

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Terminology and Abbreviations

AFM	atomic force microscopy
AMP	antimicrobial peptides
amu	atomic mass unit
ANOVA	analysis of variance
ANZCTR	Australian New Zealand Clinical Trials Registry
ATCC	American Type Culture Collection
Baseline	before lens wear
$C_2H_3NaO_2$	sodium acetate
°C	degree celsius
CFU	colony forming unit
CI	confidence interval
CLARE	contact lens-induced acute red eye
CLPU	contact lens peripheral ulcer
cm	centimetre(s)
Cu	copper
D	dioptre
Da	dalton
Dc	dioptre cylindrical
Dk	oxygen transmissibility
Dk/t	oxygen permeability
DNA	deoxyribonucleic acid

dpm	disintegrations per minute
Ds	dioptre spherical
DW	daily wear
e.g.	example
EDC	1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride
EW	extended wear
eV	electronvolt
FDA	Food and Drug Administration
g	gram(s)
HEMA	poly-2-hydroxyethylene methacrylates
HIV	human immunodeficiency virus
hr	hour(s)
i.e.	that is
IK	infiltrative keratitis
K_2HPO_4	dipotassium phosphate
kg	kilogram(s)
KH_2PO_4	monopotassium phosphate
km	kilometre(s)
Kα	K-alpha (emission lines)
l	litre(s)
LASIK	laser-assisted in situ keratomileusis
M	molar
mbar	millibar

µg	microgram
µm	micrometre
µmol	micromole
MBC	minimum bactericidal concentration
mg	milligram
$MgSO_4$	magnesium sulphate
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
min	minute(s)
MK	microbial keratitis
ml	millilitre
mm	millimetre
mmol	millimole
mol	mole
MRSA	methicillin-resistant *Staphylococcus aureus*
MW	molecular weight
N/A	not applicable
NaCl	sodium chloride
ng	nanogram
nmol	nanomole
OD	optical density
PBS	phosphate buffered saline
pH	potential hydrogen

%	percentage
pmol	picomole
RGP	rigid gas permeable lenses
RNA	ribonucleic acid
rpm	revolutions per minute
sec	seconds
SD	standard deviation
SEM	scanning electron microscopy
tRNA	transfer-ribonucleic acid
TRIS	trisaminomethane, $(HOCH_2)_3CNH_2$
UNSW	University of New South Wales
W	watts
w/v	mass per volume
XPS	X-ray photoelectron spectroscopy

Chapter 1: Introduction and Literature Review

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1.1 Contact Lenses

Contact lenses are small, bowl-shaped glass or plastic lenses that rest on the eye, in contact with the cornea or the sclera or both, serving as a new anterior surface of the eye (Mandell, 1971). Generally they are used to correct refractive error. Contact lenses are one of the most widely used biomedical devices in the world (Weissman *et al.*, 2006; Brennan *et al.*, 2000) with an estimated 140 million wearers world-wide (Subbaraman *et al.*, 2013). Soft contact lenses (SCL) for cosmetic purposes to correct refractive errors were approved for use on a daily wear (DW) basis in 1971 (Napier *et al.*, 2011). Extended wear (EW; 7 days & nights) SCLs for aphakia were approved in 1979 and for myopia in 1981 (Taddeo *et al.*, 2000). Presently, contact lens materials can broadly be classified into two types based on modulus and elasticity (a) water-containing soft and (b) non-water containing rigid gas-permeable (RGP) materials. Based on the polymer materials, soft contact lenses can be either of hydrogel or silicone hydrogel type.

1.1.1 Conventional Hydrogels

Hydrogels are cross-linked hydrophilic polymer structures that can imbibe large amounts of water or biological fluids. The first material of this type (poly-2-hydroxyethyl methacrylates [polyHEMA]) was developed by Wichterle *et al.* (1960) as a general purpose surgical material. At present biomedical and pharmaceutical applications of this material include a very wide range of systems and processes that include various molecular designs (Peppas *et al.*, 2000). In addition to HEMA, other hydrophilic monomers used to attract water include N-vinyl pyrrolidone and methacrylic acid. Methacrylic acid is an important monomer that is the base material for commercial hydrogel lenses such at Acuvue® 2® (etafilcon A).

One of the important properties of contact lenses is oxygen transmissibility (DK/t) which depends on oxygen permeability (DK) of the material and the thickness of the lens (t) (Peppas *et al.*, 2000). Diffusion coefficient (D) defines the speed of movement of the gas molecules within the material and solubility coefficient (K) governs the amount oxygen that the material can dissolve (Fatt, 1991). Incorporating water into the polymer provides a medium in which the oxygen can dissolve but water has a limited ability to dissolve and transport oxygen, with approximate oxygen permeability (Dk) of around 80 Dk units (Gurland, 1979). Therefore, in the case of conventional hydrogels the more water a polymer contains, the more oxygen that will be dissolved and higher the resultant oxygen permeability (Holden *et al.*, 1984). The United States Food and Drug Administration (FDA) currently classifies contact lens materials into four groups, depending upon their charge and water content (Table 1.1).

Table 1.1: FDA classification of hydrogel contact lenses.

FDA classification	Group I	Group II	Group III	Group IV
Water content	< 50% water (Low)	> 50% water (High)	< 50% water (Low)	> 50% water (High)
Charge	Non-Ionic	Non-Ionic	Ionic	Ionic

1.1.2 Silicone Hydrogels

Years of research into the effect of contact lens wear on the ocular surface has clearly demonstrated that corneal hypoxia is implicated in the aetiology of many complications observed during contact lens wear (Bruce *et al.*, 1990). Oxygen is more soluble in silicone than in water. The major challenge in designing HEMA and silicone polymers such as TRIS copolymers (Tighe, 2004) was to combine the hydrophobic silicone macromers with the hydrophilic monomers. To address this issue, the most widely used approach was introduction of hydrophilic groups into the section of the

TRIS molecule (indicated by the arrow in Figure 1-1) to increase its compatibility with hydrophilic monomers (Tighe, 2004).

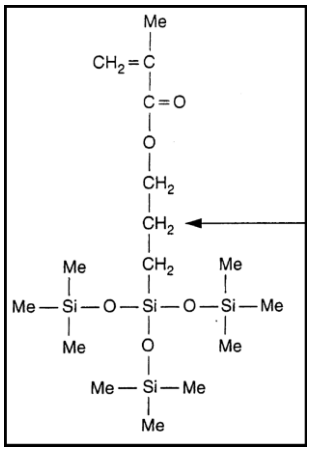

Figure 1-1: Modification site of TRIS by the introduction of hydrophilic groups.

To increase oxygen transmissibility, silicone hydrogel contact lens materials are made of various cross-linked polymers, combined with hydrophobic siloxanes. First generation silicone hydrogels required a surface treatment, such as plasma coating to mask their hydrophobic surface. Second generation silicone hydrogels used HEMA and siloxane macromer as the back-bone and incorporated internal wetting agents (Tighe, 2004). Recently, third generation silicone hydrogel polymers (such as comfilcon A or enfilcon A) have not used TRIS-based derivatives and do not require either plasma coating or an internal wetting agent (Szczotka-Flynn, 2008). Third generation materials use variably sized of siloxy macromers, which are naturally wettable. Table 1.2 summarises the silicone hydrogel contact lenses available in Australia, 2010 - 2013.

Table 1.2: Characteristics of presently available silicone hydrogel contact lenses in Australia in 2010-2013.

Generations	First			Second		Third				
Proprietary name	**AIR OPTIX® NIGHT& DAY® AQUA**	**AIR OPTIX™ AQUA**	**PureVision ™**	**Acuvue® Advance™**	**Acuvue® OASYS™**	**Biofinity®**	**Menicon® PremiO™**	**Avaira™**	**1-DAY Acuvue® TruEye™**	**Clarity™ 1 day**
USAN*	lotrafilcon A	lotrafilcon B	balafilcon A	galyfilcon A	senofilcon A	comfilconA	asmofilcon A	enfilcon A	narafilcon A	filcon II 3
Manufacturer	Ciba Vision	Ciba Vision	Bausch & Lomb	Johnson & Johnson	Johnson & Johnson	Cooper Vision	Menicon	Cooper Vision	Johnson & Johnson	Sauflon
Water content (%)	24	33	36	47	38	48	40	46	46	56
Oxygen permeability (Dk)†	140	110	91	60	103	128	129	100	100	60
Centre thickness (mm) @ -3.00D	0.08	0.08	0.09	0.07	0.07	0.08	0.08	0.08	0.08	0.07
Oxygen transmissibility (Dk/t) †† at 35°C	175	138	101	86	147	160	161	125	118	86
FDA group	1	1	3	1	1	1	1	1	1	2
Modulus (Mpa)	1.5	1	1.1	0.4	0.72	0.75	0.9	0.5	0.66	0.5
Contact angle(°)†††	42	35	84	102	93	31	96	unpublished	unpublished	unpublished

Generations	First			Second		Third				
Proprietary name	**AIR OPTIX® NIGHT& DAY® AQUA**	**AIR OPTIX™ AQUA**	**PureVision ™**	**Acuvue® Advance™**	**Acuvue® OASYS™**	**Biofinity®**	**Menicon® PremiO™**	**Avaira™**	**1-DAY Acuvue® TruEye™**	**Clarity™ 1 day**
Surface treatment	25-nm plasma coating with high refractive index	25-nm plasma coating with high refractive index	Plasma oxidation	No surface treatment, internal wetting agent (PVP)	No surface treatment, internal wetting agent (PVP)	No surface treatment	Nanogloss ™	No surface treatment	No surface treatment, internal wetting agent (PVP)	No surface treatment, internal wetting agent (AquaGen™)
Principal monomers	DMA + TRIS + siloxane monomer	DMA + TRIS + siloxane monomer	NVP + TPVC + NVA + PBVC	mPDMS+DMA+ HEMA+ siloxane macromer+ PVP+ TEGDMA	mPDMS+ DMA+ HEMA+ siloxane macromer +PVP+ TEGDMA	FM0411M + HOB + IBM + M3U + NVPTAIC + VMA	unpublished	unpublished	unpublished	filcon II 3

DMA, N,N-dimethylacrylamide; EGDMA, ethylene glycol dimethacrylate; FM0411M, methacryloyloxyethyl iminocarboxyethyloxypropylpoly (dimethylsiloxy)-butyldimethylsilane; HOB, 2-hydroxybutyl 6ethacrylates; IBM, isobornyl 6ethacrylates; M3U, bis(methacryloyloxyethyl iminocarboxy ethyloxypropyl)-poly(dimethylsiloxane) poly(trifluoropropylmethylsiloxane)-poly(methoxy-poly(ethyleneglycol)propylmethylsiloxane); MA, methacrylic acid; mPDMS, monofunctional polydimethylsiloxane; NVA, N-vinyl aminobutyric acid; NVP, N-vinyl pyrrolidone; PBVC, poly(dimethysiloxy) di silylbutanol bis vinyl carbamate); PC, phosphorylcholine; PVP, polyvinyl pyrrolidone; TAIC, 1,3,5-Triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione; TEGDMA, tetraethyleneglycol dimethacrylate; TPVC, tris-(trimethylsiloxysilyl) propylvinyl carbamate; TRIS, trimethylsiloxy silane; VMA, N-Vinyl-N-methylacetamide.

**United States adopted name.*

† The oxygen permeability of a material is referred to as the Dk. The units of 10 -11 cm^2/s ml O_2/ml X mm Hg are often omitted for convenience. Dk value is a physical property of a contact lens material and describes its intrinsic ability to transport oxygen. "D" is the diffusion coefficient – a measure of how fast dissolved molecules of oxygen move within the material and "k" is a constant representing the solubility coefficient or the number of oxygen molecules dissolved in the material.

†† Oxygen transmissibility is referred to as Dk/t, with units of 10 -9 cm/s ml O_2/ml X mm Hg. Here "t" is the thickness of the lens or sample of the material, and "D" and "k" are as defined above.

††† Sessile drop technique (Jones et al., 2010).

1.1.3 Contact lens Wear Schedules

Contact lenses are broadly used in three different wearing modalities. These are daily disposable wear, daily wear and extended wear. The daily wear modality has been widely used where daily insertion and removal of the same lenses are required. Extended wear (sometimes called continuous wear) defines lenses worn overnight. However, there is a clear link between extended contact lens wear and corneal infection and inflammation (Stapleton *et al.*, 2008; Keay *et al.*, 2007; Weissman *et al.*, 2003; Stapleton, 2003).

1.1.4 Lens Care System

Reusable contact lenses used on a daily wear basis, require an efficient lens care system for sterilisation and removal of deposits from the contact lens surface when they are not worn. The options for a daily wear lens disinfection system include multipurpose solutions, one-step hydrogen peroxide, two-step hydrogen peroxide, chlorine and heat (Morgan *et al.*, 2006). Similarly to the advances in materials, multipurpose solutions have evolved over the years to more complex combinations of cleaning and disinfecting agents. These solutions perform multiple functions such as providing initial comfort, moisturising the lens, preventing build-up of tear components on the lens and providing protection from contaminating microorganisms. Although these solutions contain disinfectants, contact lens related bacterial biofilm can be resistant to the antimicrobial activity of these care solutions (Szczotka-Flynn *et al.*, 2009c).

1.2 Contact Lens Related Corneal Infection and Inflammation

Adhesion and colonisation by microorganisms, particularly bacteria to contact lenses, continues to be implicated in a number of adverse events. These include microbial keratitis (MK) (Willcox *et al.*, 2001b) and various inflammatory responses such as contact lens-induced acute red eye (CLARE) (Sankaridurg *et al.*, 1996a), contact lens peripheral ulcer (CLPU) (Wu *et al.*, 2003), and infiltrative keratitis (IK) (Willcox *et al.*, 2011).

1.2.1 Microbial Keratitis (MK)

MK is defined as corneal inflammation in response to replicating microorganisms at the ocular surface (Willcox *et al.*, 2004). MK is a rare but serious complication of contact lens wear and can result in vision loss as a consequence of corneal scarring (Edwards *et al.*, 2007; Dart *et al.*, 1991; Ormerod *et al.*, 1986). Contact lens wear is the most commonly identified risk factor for development of MK (Otri *et al.*, 2012; Green *et al.*, 2008b; Keay *et al.*, 2006b). Depending on the study design and location, contact lens wear accounts for approximately 12% to 66% of all MK events (Keay *et al.*, 2006b; Schein *et al.*, 2005; Fong *et al.*, 2004; Bourcier *et al.*, 2003; Mela *et al.*, 2003; Wong *et al.*, 2003; Rattanatam *et al.*, 2001; Gebauer *et al.*, 1996). Various studies have reported that the risk of developing MK is 9 to 15 times higher with overnight wear compared to daily wear (Dart *et al.*, 2008; Edwards *et al.*, 2007; Schein *et al.*, 2005; Schein *et al.*, 1989). The annualised incidence of MK ranges between 9.3 to 20.9 during overnight wear of lenses and 2.2 to 3.5 per 10,000 wearers during daily wear of lenses (Lam *et al.*, 2002; Cheng *et al.*, 1999; Seal *et al.*, 1999; Poggio *et al.*, 1989). Although introduction of silicone hydrogel lenses has dramatically reduced

corneal hypoxia-related changes, recent studies have indicated that the incidence of MK has not been reduced during use of these lenses (Szczotka-Flynn *et al.*, 2009a; Stapleton *et al.*, 2008; Dart *et al.*, 2008; Lee *et al.*, 2003).

Bacteria are the predominant causative agents in contact lens related-MK (Lam *et al.*, 2002; Houang *et al.*, 2001; Cheng *et al.*, 1999). Fungi (Yildiz *et al.*, 2010; Tuli *et al.*, 2007) and *Acanthamoeba* (Yoder *et al.*, 2012; Anger *et al.*, 2008) are the other pathogenic microorganisms responsible for MK (Otri *et al.*, 2012; Por *et al.*, 2009; Tuli *et al.*, 2007). Table 1.3 shows types of microorganism that have been isolated from lens-related MK.

Table 1.3: Microorganisms isolated from MK induced by contact lens wear (Adapted and updated from Willcox *et al.* (2001b)).

Microorganisms reported	Authors
Bacteria (Gram negative)	
Acinetobacter calcoaceticus	Cheng *et al*. (1999), Lemp *et al*.(1984)
Acinetobacter spp.	Houang *et al.* (2001), Bennett *et al.* (1998), Ormerod *et al.* (1986)
Enterobacter aerogenes	Cooper *et al.* (1977)
Enterobacter spp.	Cheng *et al*. (1999)
Escherichia coli	Houang *et al.* (2001), Cooper *et al.* (1977), Lemp *et al.* (1984), Weissman *et al.* (1984)
Haemophilus influenzae	Al-Yousuf (2009), Mondino *et al*.(1986)
Kingella kingae	Otri *et al.* (2012)
Klebsiella oxytoca	Dart (1988), Lemp *et al.* (1984)
Klebsiella pneumoniae	Lemp *et al.* (1984)
Klebsiella spp.	Cheng *et al.* (1999)
Moraxella lacunata	Dart (1988)
Moraxella spp.	Stapleton *et al.* (1993b)
Morganella morganii	Mondino *et al.* (1986)
Proteus mirabilis	Alfonso *et al.* (1986)
Proteus morganii	Mondino *et al.* (1986)
Proteus vulgaris	Patrinely *et al.* (1985)
Pseudomonas cepacia	Patrinely *et al.* (1985)
Pseudomonas aeruginosa	Otri *et al.* (2012), Al-Yousuf (2009), Green *et al.* (2008a), Green *et al.* (2008b), Yu *et al.* (2007), Houang *et al.* (2001), Ormerod *et al.* (1986), Lam *et al.* (2002), Stapleton *et al.* (1995), Cooper *et al.* (1977), Ormerod *et al*.(1986), Bennett *et al.* (1998)
Pseudomonas fluorescens	Stapleton *et al.* (1995)
Pseudomonas spp.	Sharma *et al*. (2003), Houang *et al.* (2001), Stapleton *et al*. (1993b), Alfonso *et al*. (1986)
Serratia liquefaciens	Cooper *et al.* (1977)
Serratia marcescens	Yu *et al.* (2007), Alfonso *et al.* (1986), Cheng *et al.* (1999), Cohen *et al.* (1987), Dart (1988), Lemp *et al.*(1984)
Serratia spp.	Al-Yousuf (2009), Houang *et al.* (2001), Ormerod *et al.* (1986)
Stenotrophomonas maltophilia	Houang *et al.* (2001), Cheng *et al.* (1999), Lemp *et al.* (1984)
Bacteria (Gram positive)	
Aerobic spore-forming bacilli	Cheng *et al.* (1999)
Alpha-haemolytic *streptococci*	Sharma *et al.* (2003), Dart (1988), Ormerod *et al.* (1986), Bennett *et al*. (1998)
Bacillus cereus	Patrinely *et al*. (1985)
Bacillus spp.	Ormerod *et al*. (1986)
Coagulase-negative *Staphylococcus*	Green *et al.* (2008b), Houang *et al.* (2001), Bennett *et al.* (1998)
Corynebacterium diphtheriae	Cheng *et al.* (1999)

Microorganisms reported	Authors
Bacteria (Gram positive; Cont.)	
Diphtheroids	Houang *et al.* (2001), Cohen *et al.* (1987), Dunn *et al.* (1989)
Micrococcus spp.	Ormerod *et al.* (1986)
Nocardia spp.	Houang *et al.* (2001), Weissman *et al*. (1984)
Propionibacterium acnes	Mondino *et al*. (1986), Weissman *et a*l. (1984), Dunn *et al*. (1989)
Staphylococcus aureus	Otri *et al.* (2012), Green *et al.* (2008a), Green *et al.* (2008b), Ormerod *et al*. (1986), Bennett *et al*. (1998), Cohen *et al*. (1987), Mondino *et al*. (1986), Patrinely *et al*. (1985), Sharma *et al*. (2003), Weissman *et al*. (1984)
Staphylococcus epidermidis	Ormerod *et al*. (1986), Cohen *et al*. (1987), Dart (1988), Mondino *et al*. (1986), Patrinely *et al*. (1985), Sharma *et al*. (2003)
Staphylococcus spp.	Al-Yousuf (2009), Cheng *et al.* (1999), Cohen *et al*. (1987), Stapleton *et al*. (1993b)
Streptococcus pneumoniae	Green *et al.* (2008a), Green *et al.* (2008b), Bennett *et al*. (1998), Dart (1988)
Streptococcus spp.	Al-Yousuf (2009), Alfonso *et al.* (1986), Cheng *et al*. (1999)
Streptococcus viridans	Bennett *et al*. (1998), Dart (1988)
Fungi	
Aspergillus flavus	Wong *et al.* (1997)
Arthographis karlae	Perlman *et al*. (1997)
Aspergillus wentii	Wilhelmus *et al*. (1988)
Candida parapsilosis	Wilhelmus *et al*. (1988)
Candida spp.	Patel *et al.* (2008), Wilhelmus *et al*. (1988)
Candida tropicalis	Wilhelmus *et al*. (1988)
Cephalosporium spp.	Wilhelmus *et al*. (1988)
Fusarium solani	Patel *et al.* (2008), Green *et al.* (2008a), Green *et al.* (2008b), Wilhelmus *et al*. (1988)
Fusarium spp.	Tu *et al.* (2010), Patel *et al.* (2008), Rao *et al.* (2007), Gorscak *et al.* (2007), Green *et al.* (2008b), Alfonso *et al*. (1986)
Paecilomyces spp.	Wilhelmus *et al*. (1988)
Penicillium spp.	Ormerod *et al*. (1986)
Protozoa	
Acanthamoeba spp.	Tu *et al.* (2010), Otri *et al.* (2012), Yoder *et al.* (2012), Al-Yousuf (2009), Houang *et al.* (2001), Bennett *et al*. (1998), Cohen *et al*. (1987), Sharma *et al.* (2003), Stapleton *et al*. (1993b)
Vahlkampfia	Bennett *et al*. (1998)

1.2.1.1 Bacterial Keratitis

Gram negative bacteria are the leading pathogenic microorganisms in contact lens-related MK, with the *Pseudomonas* species being the most commonly isolated microorganism (Lam *et al.*, 2002; Houang *et al.*, 2001; Cheng *et al.*, 1999; Schein *et al.*, 1990). The range of organisms associated with contact lens MK may show regional variation (Lam *et al.*, 2002), with Gram negative bacteria being more common in tropical climates. *Pseudomonas* keratitis is characterised by rapid, suppurative stromal infiltrates with tissue necrosis and excessive muco-purulent discharge (Sankaridurg *et al.*, 2004). *P. aeruginosa* is a ubiquitous environmental Gram negative bacterium, with a complex genetic makeup enabling its survival in a wide variety of nutritional environments. A significant proportion of ocular infections caused by *P. aeruginosa* also have been associated with antibiotic resistant strains (Willcox, 2011). *S. marcescens,* coagulase-negative staphylococci and *S. aureus* are often the next most commonly identified causative microorganisms (Szczotka-Flynn *et al.*, 2010a; Green *et al.*, 2008b; Houang *et al.*, 2001; Alexandrakis *et al.*, 2000). Although, while *S. aureus* has been the most commonly isolated organism found in some studies (as many as 45% of all cases bacterial keratitis) (Otri *et al.*, 2012; Nayak *et al.*, 2007; Nayak *et al.*, 2000), recently an increasing incidence of MK by methicillin-resistant *S. aureus* (MRSA) and coagulase-negative staphylococci is being reported (Haas *et al.*, 2011; Willcox, 2011; Green *et al.*, 2008b). Rates of *S. aureus* resistance in Florida to ciprofloxacin, a first line monotherapy for MK have increased from 3–8% in the early 1990s to 27–40% in 2000–2001, mainly due to frequent isolation of MRSA strains, which have 30–97% resistance to ciprofloxacin (Marangon *et al.*, 2004). Contact lens-related bacterial keratitis associated with drug resistant strains can increase morbidity and treatment cost, and have a poor prognosis (French, 2005).

1.2.1.2 Fungal Keratitis

Fungal keratitis is characterised by 'fluffy' or branched infiltrate margins and satellite lesions (Sankaridurg *et al.*, 2004). *Fusarium* is the most commonly isolated fungal species (Tu *et al.*, 2010; Iyer *et al.*, 2006) followed by *Aspergillus* spp. (Iyer *et al.*, 2006; Wong *et al.*, 1997) and *Candida* (Iyer *et al.*, 2006) from MK. Fungal keratitis is generally highly symptomatic and associated with prolonged treatment and poor outcome with 34% of fungal keratitis patients in the United States requiring corneal transplantation (Alfonso *et al.*, 2006; Chang *et al.*, 2006a). Fungal keratitis has received significant attention in the last decade because of an upswing in the incidence associated with a specific type of multipurpose disinfection solution (MPDS), ReNu® MiostureLoc™ (Bausch & Lomb, Rochester, NY);(Keay *et al.*, 2011; Imamura *et al.*, 2008; Chang *et al.*, 2006a; Khor *et al.*, 2006; Alfonso *et al.*, 2006). The outbreak revealed a 5–13 times increased risk of *Fusarium* keratitis associated with the use of ReNu® MiostureLoc™ compared to the use of ReNu® Multiplus®. Prior to 2005, contact lens wear was rarely associated with fungal keratitis (Patel *et al.*, 2008; Tuli *et al.*, 2007; Wong *et al.*, 1997) and neither were fungi commonly cultured from contact lens related MK (Mah-Sadorra *et al.*, 2005; Watt *et al.*, 2005; Mela *et al.*, 2003; Sharma *et al.*, 2003; Koidou-Tsiligianni *et al.*, 1989). When checked retrospectively, the unopened ReNu® MoistureLoc™ bottles were not contaminated with fungus growth. This solution contained alexidine, a disinfectant which can be substantially taken up by hydrogel group IV lenses during overnight storage, resulting in a decrease in the residual antifungal activity in the lens storage solution (Rosenthal *et al.*, 2006). In addition, high molecular weight polyquaternium−10 was present in this formulation that may have contributed to the loss of antifungal activity upon drying and/or being concentrated (Levy *et al.*, 2006; Zhang *et al.*, 2006).

1.2.1.3 Acanthamoeba Keratitis

Acanthamoeba keratitis is a serious eye infection that can manifest as a dendritic ulcer or patchy stromal infiltrates (Sankaridurg *et al.*, 2004). Eye lid ptosis, conjunctival redness and lack of discharge is common with this type of keratitis (Niederkorn *et al.*, 1999). The global incidence of *Acanthamoeba* keratitis for contact lens users is poorly defined; however, the estimated annual incidence in developed countries is 17–33 per million depending on the geographical location (Patel *et al.*, 2008; Seal, 2003; Radford *et al.*, 2002; Schaumberg *et al.*, 1998). *Acanthamoeba* keratitis was first reported in the 1970s (Naginton *et al.*, 1974) and outbreaks were associated with the use of homemade saline solutions, lens rinsing with tap water and swimming with contact lenses (Stehr-Green *et al.*, 1987; Moore *et al.*, 1987). *Acanthamoeba* keratitis has also been reported with the use of orthokeratology lenses (Sun *et al.*, 2006; Wilhelmus, 2005; Watt *et al.*, 2005; Xuguang *et al.*, 2003). During 2004–2007, an outbreak of *Acanthamoeba* keratitis occurred (Verani *et al.*, 2009; McAllum *et al.*, 2009; Joslin *et al.*, 2007) and investigations identified a strong association with the use of Advanced Medical Optics Complete® MoisturePlus™ MPDS (AMO, Santa Ana, California, USA) (Verani *et al.*, 2009; Patel *et al.*, 2008; Joslin *et al.*, 2007). In 2007 the Centres for Disease Control and Prevention reported that use of this solution was associated with seven times higher incidence of *Acanthamoeba* keratitis (2007). However, following withdrawal of the contact lens solution in 2007, the reported number of *Acanthamoeba* keratitis cases has remained elevated (Yoder *et al.*, 2012; Tu *et al.*, 2010). Insufficient antimicrobial efficacy of the contact lens solution was hypothesised as a contributing factor (Yoder *et al.*, 2012; Johnston *et al.*, 2009). In addition, the presence of propylene glycol in this formulation was demonstrated to be associated with *Acanthamoeba* encystment (Kilvington *et al.*, 2008). Incubation for 6 to 24 h with *Acanthamoeba* in

MoisturePlus™ MPDS produced immature cysts (Kilvington *et al.*, 2008). The cysts remained viable in MoisturePlus™ and it was thought that at the ocular surface they excysted and caused infection (Kilvington *et al.*, 2008). Contact lens related *Acanthamoeba* keratitis is a severe infection with heightened symptoms, requiring prolonged and aggressive treatment, and resulting in poor visual outcome including complications such as secondary glaucoma (Yoder *et al.*, 2012; Yamazoe *et al.*, 2012; Por *et al.*, 2009; Tu *et al.*, 2008). If not treated promptly, therapeutic corneal transplants may be needed in up to 17% cases (Tu *et al.*, 2008).

1.2.2 Inflammatory Responses

CLARE, CLPU and IK are relatively common inflammatory complications resulting from microbial contamination of lenses (Sankaridurg *et al.*, 2004; Sweeney *et al.*, 2003; Holden *et al.*, 1996).

1.2.2.1 Contact lens–Induced Acute Red Eye (CLARE)

CLARE is an inflammatory reaction characterised by severe conjunctival and limbal hyperaemia, corneal infiltration and pain observed during extended wear of hydrogel or silicone hydrogel contact lenses. CLARE is associated with sleeping in lenses (Sankaridurg *et al.*, 2004; Sweeney *et al.*, 2003; Sankaridurg, 1999; Sankaridurg *et al.*, 1996b; Holden *et al.*, 1996) and is usually unilateral. Microbiological analysis of contact lenses during CLARE has revealed high levels of Gram negative bacteria (Sankaridurg *et al.*, 1996b; Holden *et al.*, 1996) including *Haemophilus influenzae*, *S. marcescens* and *Pseudomonas* spp. (Willcox *et al.*, 2004; Sankaridurg *et al.*, 2004; Estrellas *et al.*, 2000). Occasionally, Gram positive bacteria are associated with CLARE events (Sankaridurg *et al.*, 1999). Table 1.4 details microorganisms that have been found to be associated with CLARE. Gram negative bacteria on lenses, as well as

endotoxin (lipopolysaccharide), bacterial enzymes and other by-products released from bacteria on the lenses are probably the main causes of the cellular infiltration seen in this condition (Holden *et al.*, 1996). A period of eye closure which reduces the flushing mechanism of the tears is reported to trigger this inflammatory response (Thakur *et al.*, 1998; Ramachandran *et al.*, 1995). CLARE was associated with 34% of patients in a study of continuously worn hydrogel lenses over 12 months (Sankaridurg, 1999). However, the incidence of CLARE has been shown more recently, to be less than 1% during overnight wear of silicone hydrogel lenses (Ozkan *et al.*, 2012). High water content contact lenses, tight lens fit, and upper respiratory tract infections have been identified as risk factors (Sankaridurg *et al.*, 1996b).

1.2.2.2 Contact Lens Peripheral Ulcer (CLPU)

CLPU as with CLARE, is generally associated with overnight lens wear (Iruzubieta *et al.*, 2001; Long *et al.*, 2000), but can also occur with daily wear (Grant *et al.*, 1998). Depending on the severity, CLPU is characterised by acute bulbar and limbal hyperaemia with a single, small, circular, well circumscribed, full thickness epithelial lesion in the peripheral cornea associated with stromal infiltration (Sankaridurg *et al.*, 2004; Aasuri *et al.*, 2003; Sankaridurg, 1999; Grant *et al.*, 1998; Suchecki *et al.*, 1996; Donshik *et al.*, 1995). The diameter of the lesions typically ranges between 0.2 to 1.2 mm (Sankaridurg *et al.*, 2004; Holden *et al.*, 1999). Neither the aetiology of CLPU nor the reason of the peripheral location of these events on the cornea is clearly understood (Sankaridurg *et al.*, 2004). Given that both scraping and biopsies from CLPU are sterile, it is not considered an infection (Holden *et al.*, 1999). Depending on the study location, the incidence of CLPU can vary from 0.3% to 13.6% of contact lens wearers (Sankaridurg *et al.*, 2004; Iruzubieta *et al.*, 2001; Long *et al.*, 2000; Sankaridurg, 1999). In Australia, the incidence for extended wearers ranges from 1.6% to 2.9% (Zantos *et*

al., 1978). *S. aureus* has been shown to produce CLPU in animal model studies (Wu *et al.*, 2003), and contact lenses from CLPU patients have been found to be colonised by *S. aureus* or *Streptococcus pneumoniae* (Jalbert *et al.*, 2000; Holden *et al.*, 1999; Sankaridurg *et al.*, 1999; Willcox *et al.*, 1995). Table 1.4 details microorganisms that have been reported associated to be with CLPU.

1.2.2.3 Infiltrative Keratitis (IK)

Any symptomatic corneal infiltrative event that happens during contact lens wear which cannot be categorised as MK, CLARE or CLPU is termed as IK (Sankaridurg *et al.*, 2004). Unlike CLARE, IK is not predicated by sleeping with lenses. The annualised incidence of IK is 2.1% to 9.7% for extended contact lens wear (Sankaridurg *et al.*, 2004). Continuous wear has been associated with even higher infiltrative events, with an incidence rate of 10.3% at the end of 3 years (Szczotka-Flynn *et al.*, 2007). IK can be due to trauma, or a foreign body trapped between eye and CL (Sankaridurg *et al.*, 2004). IK is often associated with bacterial contamination which may be either Gram negative or Gram positive (Table 1.4). Contact lenses contaminated with Gram positive bacteria are approximately eight times more likely to contribute to IK than when colonised with Gram negative bacteria (Willcox *et al.*, 2011). The incidence rate with first generation silicone hydrogel contact lens wear was higher than HEMA-based hydrogel lenses (Sankaridurg *et al.*, 2004; Skotnitsky *et al.*, 2002), suggesting ocular trauma resulting from the higher modulus of elasticity in silicone hydrogels might be a contributing factor. This has been supported by recent reports that overall silicone hydrogel contact lenses are associated with a twofold increased risk of corneal infiltrative events when compared with hydrogel lenses (Szczotka-Flynn *et al.*, 2013b).

Table 1.4: Microorganisms associated with CLARE, IK and CLPU

Micro-organism	Adverse responses*
Gram negatives	
Abiotrophia defectiva	IK
Acinetobacter spp.	CLARE, IK
Aeromonas hydrophilia	CLARE
Alcaligenes xylosoxidans subsp. dentrificans	IK
Branhamella catarrhalis	IK
Enterobacter cloacae	IK
E. coli	CLARE, IK
H. influenzae	CLARE, IK
Haemophillus parainfluenzae	CLARE, IK
K. oxytoca	CLARE, IK
K. pneumoniae	CLARE
Neisseria spp.	IK
P. aeruginosa	CLARE, CLPU
Serratia. liquefaciens	CLPU, IK
S. marcescens	CLARE, IK
S. maltophilia	CLARE
Gram Positives	
Non-haemolytic *Streptococcus* spp.	IK
S. aureus	CLPU, IK
S. pnuemoniae	CLARE, CLPU, IK
S. viridans	CLARE, IK
Fungus	
Yeast	IK
Mould	IK

**Data from (Willcox et al., 2004; Estrellas et al., 2000; Jalbert et al., 2000; Sankaridurg et al., 1999; Sankaridurg et al., 1996b; Holden et al., 1996; Sankaridurg et al., 1996a)*

1.3 Contamination of Contact Lenses

Microbial keratitis in contact lens wearers is often associated with formation of bacterial biofilm on the contact lens surface (McLaughlin-Borlace *et al.*, 1998). Despite good compliance with the care regimen, 30% to 70% contact lenses of all asymptomatic wearers can be contaminated (Willcox *et al.*, 2002; Keay *et al.*, 2001; Gopinathan *et al.*, 1997). During continuous lens wear, lid and conjunctival bioburden is possibly the primary route of lens contamination (with a 2.5-fold and 4-fold higher risk of contact lens contamination respectively) (Szczotka-Flynn *et al.*, 2009b). In addition, more than 64% of contact lens related infiltrative events have been associated with substantial lens contamination compared to 12% during uncomplicated wear (Szczotka-Flynn *et al.*, 2010a). The contact lens may behave as a vector that delivers microorganisms or retains them at the cornea initiating infection or inflammation. Therefore, adhesion of microorganisms to contact lenses is a crucial step in the development of MK or non-infectious keratitis such as CLARE, CLPU and IK (Dart, 1997).

1.4 Factors Influencing Microbial Adhesion to Contact Lenses

1.4.1 Bacterial Characteristics

While cell surface appendages termed pili and flagella can aid in the adhesion processes of *P. aeruginosa* (Tran *et al.*, 2011; Hahn, 1997; Sato *et al.*, 1987), non-piliated *Pseudomonas* can also adhere to contact lenses (Fletcher *et al.*, 1993). The cell surface hydrophobicity of *P. aeruginosa* also contributes to its adhesion to contact lenses (Klotz *et al.*, 1989). This phenomenon could explain the greater adhesive nature of *P. aeruginosa* compared to *Staphylococcus*. *P. aeruginosa* GSU#3 is reported to be

highly hydrophobic with a surface water contact angle of 132° compared to that of various *S. aureus* strains which ranged from 20° to 36° (Bruinsma *et al.*, 2001). Adhesion of *P. aeruginosa* varies considerably between strains (Borazjani *et al.*, 2004; Vermeltfoort *et al.*, 2004; Williams *et al.*, 2003; Ahanotu *et al.*, 2001; Williams *et al.*, 1998; Klotz *et al.*, 1989; Miller *et al.*, 1988). *P. aeruginosa* strains can be classified as invasive or cytotoxic based on the presence of several genes. One report showed no direct correlation between the cytotoxic or invasive properties and degree of their adhesion to hydrogel and silicone hydrogel lenses (Borazjani *et al.*, 2004). However, cytotoxic strains (those carrying the *exoU* gene) are known to be strong biofilm producers on polystyrene surfaces (Choy *et al.*, 2008).

Overall, *P. aeruginosa* usually shows significantly greater adhesion to unworn silicone hydrogel or hydrogel lenses compared to *Staphylococcus* spp. (Vijay *et al.*, 2012; Kodjikian *et al.*, 2008; Henriques *et al.*, 2005; Bandara *et al.*, 2004; Borazjani *et al.*, 2004; George *et al.*, 2003; Ahanotu *et al.*, 2001; Bruinsma *et al.*, 2001). These findings are summarised in Table 1.5. *P. aeruginosa* also shows adhesion in higher numbers than *S. pneumoniae* (Bandara *et al.*, 2004), *H. influenzae* (Bandara *et al.*, 2004), *M. luteus* (Zhang *et al.*, 2005) or *S. marcescens* (Borazjani *et al.*, 2004). The greater ability to adhere to the contact lens surface is likely to contribute to its predominance as a causative microorganism in MK associated with contact lens wear.

Table 1.5: Comparison of bacterial adhesion levels to various lens materials for *P. aeruginosa* and *S. epidermidis.*

Ratio of *P. aeruginosa* adhesion compared to *S. epidermidis*	Lens material	Study
16 – 45	etafilcon A	Kodjikian *et al.* (2008)
533 – 717	etafilcon A	Bandara *et al.* (2004)
5–11	etafilcon A	Henriques *et al.* (2005)
2–3	polymacon	Slusher *et al.* (1987)
72 – 78	balafilcon A	Kodjikian *et al.* (2008)
1–5	balafilcon A	Henriques *et al.* (2005)
1–3	galyfilcon A	Henriques *et al.* (2005)
34 – 125	galyfilcon A	Kodjikian *et al.* (2008)
22 – 32	lotrafilcon B	Kodjikian *et al.* (2008)
1–5	lotrafilcon A	Henriques *et al.* (2005)

Table 1.6: Comparison of bacterial adhesion levels to various lens materials for *P. aeruginosa* and *S. aureus.*

Ratio of *P. aeruginosa* adhesion compared *to S. aureus*	Lens material	References
34–80	asmofilcon A	Vijay *et al*. (2012)
19–81	comfilcon A	Vijay *et al*. (2012)
21–155	enfilcon A	Vijay *et al*. (2012)
0.7	etafilcon A	Zhang *et al*. (2005)
26 – 85	etafilcon A	Subbaraman (2009)
0.9	etafilcon A	Willcox *et al*. (2010b)
185	etafilcon A	Borazjani *et al*. (2004)
9–43	filcon II 3	Vijay *et al*. (2012)
11–25	galyfilcon A	Vijay *et al*. (2012)
28	balafilcon A	Vermeltfoort *et al.* (2006)
28	balafilcon A	Borazjani *et al*. (2004)
41 – 47	balafilcon A	Subbaraman (2009)
10–18	balafilcon A	Vijay *et al*. (2012)
0.9	lotrafilcon A	Vermeltfoort *et al.* (2006)
8–11	lotrafilcon A	Vijay *et al*. (2012)
44 – 51	lotrafilcon B	Subbaraman (2009)
13–14	lotrafilcon B	Vijay *et al*. (2012)
37 – 65	senofilcon A	Subbaraman (2009)
8–25	senofilcon A	Vijay *et al*. (2012)
12–15	narafilcon A	Vijay *et al*. (2012)
7 – 17	undisclosed	Bruinsma *et al*. (2001)

Results from Bandara *et al.* (2004) (Table 1.5) show an order of magnitude higher adhesion of *P. aeruginosa* compared to *S. epidermidis* than most other studies. This is most likely a result of the strains tested; in this case *P. aeruginosa* 6294, which shows a greater level of adhesion than other *Pseudomonas* strains and *S. epidermidis* 5 which is less adhesive compared to other strains (unpublished data). Interestingly, results reported by Kodjikian *et al.* (2008) consistently showed higher adhesion of *P. aeruginosa* than *S. epidermidis* when compared to results from Henriques *et al.* (2005) to various lens materials. This is possibly due to the nutritionally limiting phosphate buffered saline (PBS) used by Kodjikian *et al.* (2008) compared to nutritionally rich artificial tears used by Henriques *et al.* (2005). In nutritionally poor media, *S. epidermidis* can show reduced cell viability especially if incubated for longer periods of time. Similarly, Borazjani *et al.* (2004) (Table 1.6), using PBS as media for both bacteria, showed a very high comparative adhesion ratio between *P. aeruginosa* and *S. aureus*. In contrast Vijay *et al*. (2012), Zang *et al.* (2005), Willcox *et al.* (2010b) and Vermeltfoort *et al.* (2006) (Table 1.6) reported a much lower adhesion ratio under similar assay conditions which appears to be mainly strain driven.

In most of the studies, PBS has been used as the bacterial suspension media (Kodjikian *et al.*, 2008; Borazjani *et al.*, 2004; George *et al.*, 2003; Williams *et al.*, 2003; Williams *et al.*, 1998; Boles *et al.*, 1992; Duran *et al.*, 1987; Slusher *et al.*, 1987). Some studies have used sterile saline instead (Giraldez *et al.*, 2010; Zhang *et al.*, 2005) but as both PBS and saline are nutritionally inert, longer incubation times when using PBS and saline may underestimate total cell numbers (especially for the more fastidious microbes such as staphylococci which may die upon prolonged exposure). Some studies have used a complex and nutritionally rich media such as tryptone soya broth (TSB) as bacterial suspension media, while other studies have used dilutions of these media in

PBS (Vijay *et al.*, 2012; Bandara *et al.*, 2004; Stapleton *et al.*, 1993a). In an attempt to more closely replicate adhesion that may occur during lens wear, several other studies have used artificial tears as the bacterial suspension fluid (Randler *et al.*, 2010; Henriques *et al.*, 2005). Any differences in adhesion levels observed between studies using these different media might be related to effects of electrolyte concentration and ionic charge of suspending media, as well as the nutritional fastidiousness of the bacteria. Furthermore, nitrogen or carbon limitation can significantly increase the ability of *P. aeruginosa* to adhere to etafilcon A lenses (Cowell *et al.*, 1999).

Interestingly, George *et al.* (2003) observed that the presence of *S. epidermidis* on hydrogel lens surfaces significantly reduces the adhesion of *P. aeruginosa*, but the presence of *P. aeruginosa* does not greatly alter the adhesion of *S. epidermidis* (George *et al.*, 2003). This phenomenon is yet to be understood though it further supports the general premise that the normal ocular microbiota (of which coagulase-negative staphylococci such as *S. epidermidis* are predominant members) may be protective to the eye. The strength of adhesion to contact lenses is also bacterial species dependent. A rinsing step after bacteria adhesion can remove significantly more *S. epidermidis* and *S. aureus* compared to *P. aeruginosa* from a lens surface (Kodjikian *et al.*, 2008; George *et al.*, 2003). In contrast, a recent investigation of adhesion forces between bacteria and contact lenses, lens storage cases or the cornea (measured using atomic force microscopy) suggested that staphylococci and *S. liquefactions* adhere significantly more strongly than *P. aeruginosa*, and this might result in slightly higher transmission rates of *P. aeruginosa* to the cornea (Qu *et al.*, 2011).

Using five different *P. aeruginosa* strains, Williams *et al.* (2003) sought to determine whether an increasing bacterial concentration ($1x10^7$ to $1x10^9$) resulted in greater viable bacterial counts on contact lenses. Maximum adhesion was seen for all

the strains when $1x10^9$ colony forming units (CFU) ml^{-1} bacteria were added to lenses, except in the case of *P. aeruginosa* 6294, which reached maximum adhesion to worn lenses at $1x10^8$ CFU ml^{-1}. Interestingly, George *et al.* (2003) found that re-exposure of the same suspension of *P. aeruginosa* to fresh lenses after the initial suspension had been previously allowed to adhere to a contact lens, resulted in lower adhesion than to the initial lens. This phenomenon suggests a limited number of bacterial cells in a standard inoculum are responsible for adhesion, suggesting a certain phenotype of bacterial cells within a population (approximately 10% of the cells) is responsible for most of the adhesion observed. This could be due to the use of cells grown in static culture to stationary phase, where cells of a differing phenotype could be present. Indeed, Williams *et al.* (2003), found that *P. aeruginosa* strains, grown to stationary phase adhered in higher levels that those grown to exponential phase this may be related to formation of biofilms under adverse conditions.

The time required for irreversible bacterial attachment and biofilm formation on the lens surface is a crucial factor, which can differ between bacterial types. Reaching maximum adhesion of cells to hydrogel lenses for a given inoculum takes up to 2 hr, for *S. epidermidis* while *P. aeruginosa* adhesion can be as rapid as 5 min (George *et al.*, 2003). Miller *et al.* (1987) reported that adhesion of *P. aeruginosa* increased with time, peaking after 3 hr and then remained constant. Duran *et al.* (1987) reported a steady increase in adhesion of an MK isolate of *P. aeruginosa* to polymacon and lidofilcon A lenses from 2 min to one hour. Subsequent studies by Stapleton *et al.* (1993a) supported these data and showed rapid attachment of up to 10^7 cells $lens^{-1}$, with adhesion reaching maximum after 45 min. Glycocalyx formation (i.e. biofilm formation) occurred after 30 min incubation with a bacterial inoculum of 10^7 organisms ml^{-1} (Stapleton *et al.*, 1993a). Andrews *et al.* (2001), using an ATP based bioluminescent assay and image

analysis, reported that the adhesion of *P. aeruginosa*, *S. epidermidis* and *S. marcescens* was maximal at 4–6 hr but this was followed by a metabolic decline after 18 hr. The decrease in metabolic activity is characteristic of a biofilm mode of growth (Hoiby *et al.*, 2010). However, others have shown that viable cell numbers on several lens materials (hydrogel and silicone hydrogel) significantly increased up to 16 to 24 hr after incubation (Randler *et al.*, 2010; Vermeltfoort *et al.*, 2004). Randler *et al.* (2010) noticed a decrease in viable bacterial numbers on silicone hydrogel lenses exposed to an artificial tear fluid within a few hours. This observed decrease might be due to antimicrobial components such as lysozyme in the artificial tear fluid.

Overall, the main factors that influence bacterial adhesion are cell surface hydrophobicity, differing strain characteristics, and the suspension medium. Most studies show that the adhesion of *P. aeruginosa* is higher than other bacterial types to most lens types, perhaps being one reason for the predominance of this bacterium in MK. Adhesion of *P. aeruginosa* to lenses is rapid, usually occurring within 1 hour, and biofilm formation can occur within 24 hr of initial adhesion.

1.4.2 Fungal and *Acanthamoeba* Characteristics

Fusarium spp. can directly adhere to contact lenses and also might form penetrating pegs (Willcox, 2013). Adhesion of fungus to contact lenses takes 1–2 days. Formation and adhesion by penetrating pegs, that are essentially the hyphae of the fungi, can take 2 – 14 days and can traverse into the matrix of lenses (Ahearn *et al.*, 2009; Ahearn *et al.*, 2007; Zhang *et al.*, 2007). Penetrating pegs have been observed in the contact lens storage cases received from the patients with *Fusarium* keratitis (Ahearn *et al.*, 2007). Formation of penetrating pegs occurs more rapidly on first generation silicone hydrogels, and somewhat more slowly on second generation silicone

hydrogels and HEMA-based hydrogel contact lenses (Ahearn *et al.*, 2009; Ahearn *et al.*, 2007; Zhang *et al.*, 2007).

The presence of bacteria on hydrogel or second generation contact lens surfaces encourages *Acanthamoeba* trophozoite adhesion to contact lenses (Simmons *et al.*, 1998). This phenomenon has the practical implication that a bacterially contaminated contact lens has a higher chance of *Acanthamoeba* adhesion, and subsequently may cause a predisposition to infection by these protozoa.

1.4.3 Contact Lens Material Characteristics

1.4.3.1 Ionicity and Water Content

Greater levels of adhesion of various strains of *P. aeruginosa* to lenses composed of non-ionic polymers, compared to those with ionic polymers have been reported (Stapleton *et al.*, 1993a; Miller *et al.*, 1987). In contrast, initial adhesion of *S. aureus* has been reported to be higher to an ionic hydrogel compared to a non-ionic hydrogel (Arciola *et al.*, 1995). Correlation of bacterial adhesion to lens water content is mostly consistent between studies, showing that bacterial adhesion is inversely proportional to water content (Kodjikian *et al.*, 2008; Garcia-Saenz *et al.*, 2002; Ahanotu *et al.*, 2001; Cook *et al.*, 1993b; Miller *et al.*, 1988; Miller *et al.*, 1987). Kodjikian *et al.* (2008), Miller *et al.* (1988; 1987) and Cook *et al.* (1993a; 1993b) showed increased adhesion to low water content hydrogel lenses for strains of *P. aeruginosa,* although Miller *et al.* (1987) noted strain differences. However, other studies did not establish a relationship between water content and adhesion (Vijay *et al.*, 2012; Gopinathan *et al.*, 1997; Lawin-Brussel *et al.*, 1991) and there appears to be no relationship between the numbers of bacteria isolated from lenses after wear and water content (Gopinathan *et al.*, 1997).

1.4.3.2 Hydrophobicity

Hydrophobicity is a crucial contact lens surface property as most bacterial isolates adhere in greater numbers to hydrophobic surfaces than hydrophilic ones (Giraldez *et al.*, 2010; Santos *et al.*, 2007; Pringle *et al.*, 1986; Fletcher *et al.*, 1979; Marshall *et al.*, 1973). Further, the strength of attachment is greater to hydrophobic surfaces than hydrophilic surfaces (Fletcher *et al.*, 1979). Hydrophobicity of contact lenses can be measured by captive bubble or sessile drop techniques (Maldonado-Codina *et al.*, 2007).

The hydrophobicity of silicone hydrogels such as lotrafilcon A or balafilcon A is higher than hydrogel lenses such as etafilcon A (Henriques *et al.*, 2005). Consistent with this, *P. aeruginosa, S. aureus* or *S. epidermidis* adhere in greater numbers to silicone hydrogel lenses compared to the more hydrophilic hydrogel lenses *in vitro* (Santos *et al.*, 2007; Henriques *et al.*, 2005; George *et al.*, 2003; Bruinsma *et al.*, 2001). Also, lotrafilcon A and balafilcon A are often reported as the most hydrophobic silicone hydrogel lenses and more bacteria adhere to these compared with other silicone hydrogels (Henriques *et al.*, 2005). It is likely that hydrophobic bacteria adhere in greater numbers to hydrophobic lenses, whereas hydrophilic bacteria adhere well to hydrophilic lenses (Vermeltfoort *et al.*, 2006). Vijay *et al*. (2012) suggested that the relationship between bacterial adhesion and contact lens surface hydrophobicity is parabolic and highest adhesion is observed between 40º and 60º contact angles, with a reduction toward less than 20º or above 100º. Despite reducing the hydrophobicity of first generation silicone hydrogels by plasma coating, it has been reported that these lenses are associated with high adhesion of *Acanthamoeba* trophozoites (Beattie *et al.*, 2009).

1.4.3.3 Roughness

Detailed information regarding contact lens topography and roughness can be determined by using atomic force microscopy. Giraldez *et al.* (2010) reported that comfilcon A and omafilcon A lens materials have relatively smooth surfaces compared to senofilcon A, nelfilcon A and ocufilcon B. *S. epidermidis,* has been shown to adhere in greater numbers to surfaces with greater roughness when the hydrophobicity of the material remained relatively constant (Giraldez *et al.*, 2010; Tang *et al.*, 2009). However, Vijay *et al*. (2012) was not in agreement with the previous results. By using 10 types of silicone hydrogel contact lenses, they showed no strict relationship between *S. aureus* viable and total adhesion with lens roughness, but *P. aeruginosa* adhesion was inversely correlated to surface roughness.

1.4.3.4 Lens Wear

Contact lens wear can have significant effects on the surface properties of lenses due to the deposition of tear film components during wear. There is also the possibility of deposition of components of MPDS during storage on a daily wear schedule. After wear, silicone hydrogel lenses show reduced surface hydrophobicity (Santos *et al.*, 2008; Vermeltfoort *et al.*, 2006; Cheng *et al.*, 2004). Worn hydrogel and silicone hydrogel lenses usually exhibit higher degrees of roughness than their unworn counterparts, presumably as a result of deposited proteins (Lira M, 2008; Santos *et al.*, 2008; Bhatia *et al.*, 1997). Therefore, these changes in surface characteristics of lenses during/after wear may influence bacterial adhesion. Worn galyfilcon A and lotrafilcon A adhere more *S. epidermidis* than unworn lenses (Santos *et al.*, 2008). The presence of sorbed protein can increase adhesion of *S. epidermidis* by 45% (Cook *et al.*, 1993b). *P. aeruginosa* colonise the surface of worn extended wear contact lenses in

direct proportion to lens surface deposits, preferentially adhering on areas of lens deposits (Butrus *et al.*, 1990). *In vitro Pseudomonas* adhesion is highly correlated with the number of large (more than 150 µm) focal deposits on the lens after wear (Aswad *et al.*, 1990). In contrast, though enzymatic cleaning is recommended for protein deposition, it does not appear to significantly reduce the adhesion of *P. aeruginosa* to worn lens surfaces (Butrus *et al.*, 1990).

Vijay *et al*. (2012) showed that adhesion of *P. aeruginosa* 6294 to one day worn senofilcon A and balafilcon A lenses was comparable to unworn counterparts. However, the same study observed decreased *P. aeruginosa* and *S. aureus* adhesion to daily worn galyfilcon A, lotrafilcon A and lotrafilcon B lenses. Borazjani *et al.* (2004) and Boles *et al.* (1992) reported that 1 week extended wear of balafilcon A and etafilcon A had no major effect on the adhesion of *P. aeruginosa.* However, continuous wear of lotrafilcon A lenses reduced adhesion of the hydrophilic *S. aureus* strain 835, whereas continuous wear of balafilcon A lens significantly increased adhesion of this same strain (Vermeltfoort *et al.*, 2006). Adhesion of the hydrophobic *P. aeruginosa* to lenses after continuous wear was generally less than unworn lenses, regardless of the type of lens (Vermeltfoort *et al.*, 2006). Interestingly, *P. aeruginosa* 6294 adhered to a greater extent to unworn etafilcon A lenses than to 30 nights continuously worn lenses (Willcox *et al.*, 2002). *In vitro* adhesion of *P. aeruginosa* significantly varies when lenses are worn by different individuals (Miller *et al.*, 1988). Brutus *et al.* (1987) demonstrated that *P. aeruginosa* adhere in greater numbers to worn extended wear soft contact lenses compared to unworn lenses. In another study, worn balafilcon A lenses have been shown to increase bacterial adhesion (Willcox *et al.*, 2001a).

The differing effects of lens wear on bacterial adhesion highlights the complexity of the adhesion reaction. From the above findings it would appear that

differing lens materials deposit different proteins or other components from the tear film, which then interact with differing bacterial surfaces to either promote or prevent adhesion. However, as discussed above the methods used to measure bacterial adhesion (examining live bacteria (CFU $lens^{-1}$) or total bacterial cells) can also have a significant effect on the observed levels of adhesion, making comparison between studies problematic.

It might be that it is the presence of specific adsorbed tear products on worn contact lenses that affect bacterial adhesion. Stern *et al.* (1986) and Taylor *et al.* (1998) showed adsorbed IgA, bovine submaxillary gland mucin, bovine serum albumin, lysozyme and human serum albumin enhanced the adhesion of *P. aeruginosa* to contact lenses. In contrast, Williams *et al.* (2003; 1998) and Subbaraman *et al.* (2009) showed that the presence of lactoferrin increased the total numbers of *P. aeruginosa* adhering to lenses but reduced their viability, killing the attached bacteria. *In vitro,* the presence of lysozyme on a lens surface has a variable impact on adhesion of *P. aeruginosa* (Williams *et al.*, 2003; Thakur *et al.*, 1999) and *S. aureus* (Zhang *et al.*, 2005), but markedly reduces the viability of *Micrococcus luteus* (Zhang *et al.*, 2005). The number of viable cells of *S. aureus* that adhere to contact lenses is reduced if those lenses are coated with secretory phospholipase A2 (Hume *et al.*, 2004b). Contact lenses can also absorb tear film lipids such as cholesterol or phospholipids during wear but they do not appear to modulate bacterial adhesion (Babaei Omali *et al.*, 2012; Babaei Omali *et al.*, 2011).

Overall, which lens surface properties are likely to favour bacterial adhesion are difficult to predict empirically as there is a complex interplay between factors which govern the promotion of adhesion to the lens. These include hydrophobicity and

roughness, polymer characteristics including water content and ionicity and factors related to the individuals wearing the lens and their tear film characteristics.

1.5 Antimicrobial Strategies with Contact Lenses

Key antimicrobial strategies in the past have focused on changing multipurpose disinfection solutions to increase their activity (Szczotka-Flynn *et al.*, 2013a), but recent epidemiological studies have confirmed that the incidence of contact lens driven microbial adverse events has remained unchanged for the last 20 years (Stapleton *et al.*, 2013; Stapleton *et al.*, 2008; Dart *et al.*, 2008; Keay *et al.*, 2007). Thus antimicrobial contact lenses have attracted increasing attention in the past decade, and some of these have been tested in animal and human trials (Zhu *et al.*, 2008; Mathews *et al.*, 2006). Table 1.7 details the antimicrobial strategies used to develop contact lenses that may have the capacity to reduce microbial contamination and thus reduce lens related microbial adverse events.

1.5.1 Passive, Non-Cidal Surfaces:

Grafting of hydrophilic polymers such as poly(ethylene glycol) (PEG) (Kingshott *et al.*, 2003; Kingshott *et al.*, 2002) or phosphorylcholine (Selan *et al.*, 2009) can suppress the adhesion of microorganisms. Selan *et al.* (2009) showed phosphorylcholine-bound to hydrogel lenses inhibited biofilm formation by *S. epidermidis* and *P. aeruginosa.* Furthermore, the biofilms that were formed on these lenses had significantly increased susceptibility to the antibitoics tazocin, gentamicin and imipenem antibiotics.(Selan *et al.*, 2009) This suggests that any biofilms that were formed were either incomplete or defective. Another study examined the effect of phosphylcholine-bound lenses to reduce adhesion of a different strain of *P. aeruginosa* (6294) and strains of *S. pneumoniae* and *H. influenzae* (Willcox et al., 2001a). In that

study, phosphylcholine appeared to reduce the adhesion of *H. influenzae* to lenses but did not affect adhesion of the strain of *P. aeruginosa* and increased adhesion for one strain (out of 2 tested) of *S. pneumoniae* (a bacterial type isolated from cases of keratitis during lens wear) (Sankaridurg *et al.*, 1999). Similar to quorum sensing inhibitors, incorporation of molecules such as PEG or phosphorylcholine may reduce the build up of dead or living microbes. However, *in vivo* or *in vitro* efficacy of these passive non-cidal substances against virulent microorganisms such as *Pseudomonas* or *Acanthamoeba* is yet to be established, and there have been no reports of reduced bacterially-driven adverse events (such as MK, CLARE, CLPU) in people wearing the phosphorylcholine containing lenses. A recent review has highlighted the potential for combinations of passive non-cidal surfaces (e.g. PEG) with active bactericides such as cationic peptides to be used together to reduce microbial contamination and reduce deposition of proteins or other substances on the surface that may mask the antimicrobial activity (Salwiczek *et al.*, 2013). This seems to be a potentially exciting new area of research for contact lenses.

Table 1.7: Antimicrobial strategies investigated with contact lenses.

Antimicrobial agents		Study	Substrate	Method	Microorganisms assessed	*In vivo / In vitro*	Result
Silver		Willcox *et al.* (2010b)	etafilcon A	impregnation	*P. aeruginosa, S. aureus*	*in vitro*	reduce bacterial colonisation (> 99%)
		Nissen *et al*. (2000)	weflex 55 hydrogel	adsorption	*P. aeruginosa, S. aureus*	*in vitro*	reduce bacterial adhesion (> 99%)
NSAIDs	Sodium salicylate	Beattie *et al.* (2011)	etafilcon A	adsorption	*P. aeruginosa, A. castellanii*	*in vitro*	inhibit biofilm formation and attachment of amoebal trophozoites (dose dependant inhibition; up to > 99%)
	Bendazac lysine	Arciola *et al.* (1998)	etafilcon A	adsorption	*P. aeruginosa, S. aureus, S. epidermidis*	*in vitro*	reduce bacterial adhesion (12% – 98%)
	Salicylic acid, Sodium diclofenac, Ketorolac	Bandara *et al.* (2004)	etafilcon A	adsorption	*P. aeruginosa, S. epidermidis, S. pneumoniae, H. Influenzae*	*in vitro*	more than 99% inhibition; within the same concentration, salicylic acid shows higher bacterial inhibition than ketorolac and sodium diclofenac
	Sodium salicylate	Tomlinson *et al.* (2000)	etafilcon A	adsorption	*P. aeruginosa, A. castellanii*	*in vitro*	reduce bacterial adhesion and attachment of amoebal trophozoite (up to 90% reduction in adhesion of trophozoites to *P. aeruginosa* biofilm-coated lenses)
Selenium		Mathews *et al.* (2006)	balafilcon A	covalently bound	*P. aeruginosa*	*in vivo / in vitro*	inhibit bacterial colonisation (not quantified); safe in rabbit animal model

Antimicrobial agents	Study	Substrate	Method	Microorganisms assessed	*In vivo / In vitro*	Result
Phosphorylcholine	Selan *et al.* (2009)	hydrogel	incorporated with polymer matrix	*P. aeruginosa, S. epidermidis*	*in vitro*	reduce minimal inhibitory concentration of the bacteria growing on the coated lenses
Fimbrolide (Furanone)	Zhu *et al.* (2008)	lotrafilcon A	covalently bound	*P. aeruginosa, S. aureus, S. marcescens, A. castellanii*	*in vivo / in vitro*	reduce bacterial & amoebal adhesion (67% – 92%); safe in guinea pig model, and one day human trial
	George *et al.* (2005)	tefilcon A, lotrafilcon A, alphafilconA, bufilcon A, vifilcon A, etafilcon A	adsorption	*P. aeruginosa, S. aureus, S. epidermidis*	*in vitro*	inconsistent inhibition depending on bacterial strain (up to 97%)
Melimine	Cole *et al.* (2010)	undisclosed silicone hydrogel	covalently bound	*P. aeruginosa, S. aureus*	*in vivo / in vitro*	reduce bacterial adhesion (80% – 96%); reduce/prevent contact lens related adverse response in rabbit, guinea pig model
	Willcox *et al.* (2008a)	etafilcon A	adsorption and covalently bound	*P. aeruginosa, S. aureus, S. pneumoniae*	*in vitro*	reduce bacterial adhesion (60% – 92%)

1.5.2 Silver

The antimicrobial properties of silver are well established. Silver has been used as an agent to produce functionalised antimicrobial polymers (Dallas *et al.*, 2011). Cationic silver is thought to disrupt protein function in the cell and in membranes by binding to thiol moieties in proteins and also catalysing the formation of di-sulphide bridges (Caiazzo *et al.*, 2009; Davies *et al.*, 1997). Silver can act on cell surfaces and can enter the cell, disrupting internal metabolic processes. For example, silver ions on the surface of bio-medical devices have been shown to interrupt bacterial respiration and synthesis of adenosine triphosphate (ATP) by binding to cell surface components (Kasuga *et al.*, 2004; Batarseh, 2004; Klueh *et al.*, 2000). In addition, silver ions are thought to intercalate between purine and pyrimidine bases, denaturing DNA molecules (Klueh, 2000) and disrupting cell replication and protein synthesis. Silver has the additional advantage that resistance does not easily develop as it acts on multiple cellular targets (Weisbarth *et al.*, 2007).

Currently, the only commercially available antimicrobial contact lens cases are silver impregnated. Silver lens cases have been shown to be active against a range of microorganisms *in vitro* including Gram positive bacteria such as *S. aureus,* and Gram negative bacteria such as *P. aeruginosa, S. marcescens, Delftia acidovorans, Citrobacter amalonaticus, K. pneumoniae, E. cloacae, A. calcoacetus* and *E. coli* (Dantam *et al.*, 2012; Dantam *et al.*, 2011; Weisbarth *et al.*, 2007; Amos *et al.*, 2006). Clinical trials reported by Amos *et al*. (2006) and Dantam *et al*. (2012) have shown reduced contamination of silver lens cases (26% to 71% of cases) compared to regular cases (67% to 82%). In addition, significantly lower numbers of microorganisms were isolated from silver lens cases (1.7 log CFU per well) compared to regular cases (4.1 log CFU per well) with lower recovery of Gram negative bacteria, Gram positive bacilli,

and fungi (Dantam *et al.*, 2012). Similar to storage cases, *in vitro* investigations by Nissen *et al*. (2000) and Willcox *et al*. (2010b) showed silver impregnated contact lenses reduce adhesion of *P. aeruginosa* and *S. aureus* by more than 5 log CFU. Silver released from nano-particle impregnated lenses inhibited growth of *Acanthamoeba* by up to 1 log (Willcox *et al.*, 2010b).

However, the reported efficacy of silver-containing or silver-releasing dressings designed to prevent burn infections is contradictory (Aziz *et al.*, 2012; Lo *et al.*, 2009). Meta-analysis of the literature on the efficacy of silver oxide and silver alloy impregnated catheters has shown that while the use of silver alloy can reduce the risk of developing bacteriuria by approximately one-third, the use of silver oxide-coated catheters might not decrease the risk of infection (Rai *et al.*, 2009; Schumm *et al.*, 2008). The reason behind this might be the inactivation of metallic silver particles when in contact with mammalian fluids such as plasma (Rai *et al.*, 2009). Similar problems may occur when silver impregnated contact lenses are in contact with tear film. In addition, the release of silver ions at the ocular surface has the possibility of inducing argyrosis. This is a toxic-blackening of the eye and mucus membrane, reported to arise from overuse of silver containing eye drops (Drake *et al.*, 2005) or occlusive contact lenses (Hau *et al.*, 2009).

1.5.3 Non-Steroidal Anti-Inflammatory Drugs

Capsular polysaccharides of bacteria play a key role in the production of biofilm formation. NSAIDs are known to reduce production of these polysaccharides. Muller *et al.* (1998) confirmed that salicylic acid inhibits up to 95% of the production of teichoic acid, slime-associated proteins, and Type I antigens of *S. epidermidis*. Teichoic acid is an essential component of slime produced by Gram-positive bacteria and a major

constituent of the bacterial cell surface (Hussain *et al.*, 1993; Muller *et al.*, 1993). Inhibition of these molecules is believed to reduce bacterial adhesion (Muller *et al.*, 1998). When contact lenses were soaked in NSAIDs such as salicylic acid, sodium salicylate or benzadak lysine, up to 100% inhibition of adhesion (viability) of *P. aeruginosa, S. aureus, S. epidermidis, S. pneumoniae* and *H. influenzae* adhesion was reported (Bandara *et al.*, 2004; Arciola *et al.*, 1998). Increasing concentrations of NSAID showed a dose-dependent inhibition in bacterial adhesion and biofilm formation on contact lenses (Beattie *et al.*, 2011; Tomlinson *et al.*, 2000; Farber *et al.*, 1995). Similar trends were seen against *Acanthamoeba* adhesion (Beattie *et al.*, 2011). Bacterial biofilm formation on contact lenses has been shown to enhance *Acanthamoeba* adhesion (Beattie *et al.*, 2006; Beattie *et al.*, 2003; Simmons *et al.*, 1998). Thus, reduction of bacterial biofilm by NSAIDs might contribute to the observed reduction in *Acanthamoeba* attachment via a secondary mechanism (Beattie *et al.*, 2011; Tomlinson *et al.*, 2000). These features suggest NSAIDs are potential candidates for antimicrobial strategies. However, these compounds were only investigated in a 'leach and release' form in contact lenses. These components may quickly disappear when used on a daily or extended wear modality and release high concentration of NSAID might expose to the wearer toxicity (Venter, 1982).

1.5.4 Selenium

Selenium compounds bound to organic molecules such as peptides, proteins or steroids, can work as catalysts and generate toxic superoxide (O_2^-) which in the presence of reducing agents such as glutathione (GSH) is active against viruses and bacteria (Palace *et al.*, 2004; Spallholz *et al.*, 2001). An organo-selenium compound, covalently coated onto hydrogel (etafilcon A) and a silicone hydrogel (balafilcon A) contact lenses effects up to 8 log reduction in adhesion of strains of *P. aeruginosa* and *S. aureus* and 7

log inhibition against *S. marcescens* adhesion and inhibited biofilm formation (Tran. P *et al.*, 2012; Reid *et al.*, 2012). These lenses were safe for up to 2 months extended wear in a rabbit model (Mathews *et al.*, 2006). Selenium can also inhibit biofilm formation of *S. aureus* when covalently incorporated into the polymer of contact lens case material (Reid *et al.*, 2012). Selenium coated contact lenses have not been reported yet in a human clinical trial. Additionally, bacterial resistance to selenium (e.g. selenium dioxide, selenium trioxide) can occur (Burton *et al.*, 1987), but its further implication in contact lens and care products needs further research.

1.5.5 Quorum Sensing Inhibitors

Materials, that interfere with bacterial signalling to prevent microbial proliferation and biofilm formation, offer an effective approach against microbial contamination of contact lenses (Hume *et al.*, 2004a). Many bacteria, such as *P. aeruginosa* use quorum sensing to regulate gene expression (Gambello *et al.*, 1991). Fimbrolides (also known as furanones) are a well-known class of quorum sensing inhibitors that, when covalently attached to contact lens surfaces, can confer 67 to 92% inhibition in *P. aeruginosa*, *S. aureus*, *S. marcescens* and *Acanthamoeba* adhesion *in vitro* (Zhu *et al.*, 2008). Fimbrolide-coated lenses have been shown to be safe for 28 days in guinea pig eyes and in humans during a 24 hour lens wear trial (Zhu *et al.*, 2008). However, the activity of quorum sensing inhibitors against various strains of Gram negative bacteria, especially *P. aeruginosa*, is reported to be inconsistent, at least when tested with lenses equilibrated with a fimbrolide solution (George *et al.*, 2005).

1.5.6 Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are a part of the innate immune system of all multicellular organisms and are able to inhibit microbial growth (McDermott, 2009;

McDermott, 2004; Ganz, 2003; Hancock, 2001). AMPs have been shown be active against a wide spectrum of microorganisms such as bacteria, viruses, fungi, protozoa, and parasites, including antibiotic resistant bacteria (McDermott, 2009; Zasloff, 2002; Hamamoto *et al.*, 2002; Hancock, 2001; Scott *et al.*, 2000; Hoffmann *et al.*, 1999; Masuda *et al.*, 1992; Murakami *et al.*, 1991). Additionally, these peptides modulate wound healing responses (Kolar *et al.*, 2011; Costa *et al.*, 2011) and may have additional immunomodulatory activities (Kolar *et al.*, 2011; Hancock *et al.*, 2006; Hancock, 2001). Although variable in length, they are mostly short chain of peptides, generally less than 50 amino acids (Nguyen *et al.*, 2011; McDermott, 2009; Hancock *et al.*, 1998). AMPs are mostly positively charged (+2 or higher) due to a higher number of positively charged amino acids, such as arginine and lysine (McDermott, 2009). There are also some anionic AMPs such as dermcidin (Nguyen *et al.*, 2011). AMPs can fold into three dimensional amphiphilic structures, in which hydrophobic areas are separated from hydrophilic zones, enabling interactions with bacterial cell membranes which have hydrophobic cores and hydrophilic head groups (Hancock, 2001).

1.5.6.1 Structure and Classification of AMPs

Over 900 (Yount *et al.*, 2006) to 1200 (Wang *et al.*, 2009) AMPs have been identified, with the majority of them sharing common biophysical features that are likely to be responsible for their activity. These features include small size, cationicity and amphipathicity. AMPs can have α helical, β sheet, or extended structures (Nguyen *et al.*, 2011; Hancock, 2001). The antimicrobial activity of the AMPs depends on net charge, hydrophobicity, size and amino acid sequence (Bulet *et al.*, 2004).

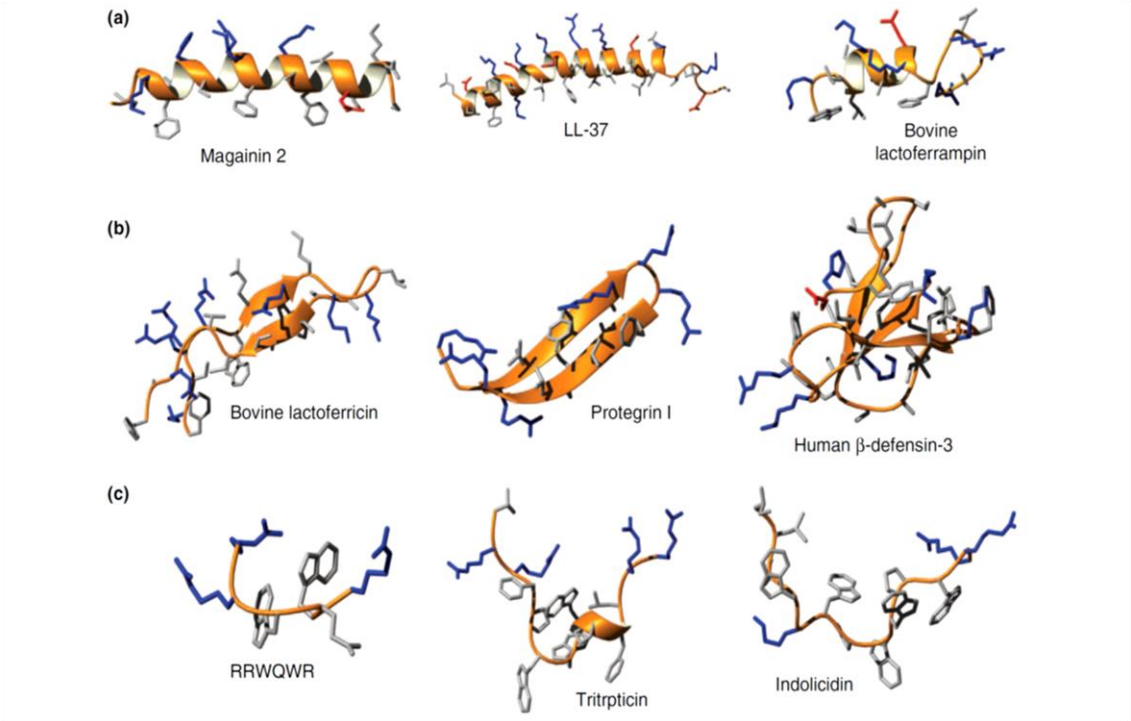

Figure 1-2: Major structural types of antimicrobial peptides (AMPs).

(a) α-helical peptides, (b) β-sheet peptides and (c) extended peptides. The positively charged side chains are coloured in blue, negatively charged side chains in red and remaining side chains in grey. From Nguyen et al. (2011) with permission.

1.5.6.1.1 α-Helical AMPs

Alpha-helical AMPs are the most commonly found structural group and have been reported in plants, invertebrates and vertebrates including humans (Figure 1-2) (Bulet *et al.*, 2004). These AMPs have activity against Gram negative and Gram positive bacteria, fungi and protozoa (Nguyen *et al.*, 2011; Wang *et al.*, 2009). In aqueous solution, these AMPs adopt a random conformation but during interaction with lipid surfaces, such as bacterial membranes they form an amphipathic α-helical structure (Bechinger *et al.*, 1993) on which their antimicrobial activity depends (Yeaman *et al.*, 2003; Marion *et al.*, 1988).

Melittin, cecropins, bovine lactoferrampin and magainin are examples of AMPs belonging to the α-helical group (Nguyen *et al.*, 2011; Haney *et al.*, 2009; Zasloff, 2002; Steiner *et al.*, 1981). Melittin often forms toroidal pores (Brogden, 2005) formed by interlocked phospholipid head groups of loosely associated peptides, which result to local changes in membrane thickness (Nguyen *et al.*, 2011; Ramadurai *et al.*, 2010; Shalet *et al.*, 1978). Melittin, composed of 26 amino acid residues, is derived from the venom of the European honey bee *Apis mellifera* (Habermann, 1972). Melittin has strong antimicrobial activity, especially against Gram positive bacteria (Willcox *et al.*, 2008a; Raghuraman *et al.*, 2007; Aliwarga *et al.*, 2001). However, even at sub-micromolar concentrations it is highly hemolytic and causes rapid lysis of erythrocytes (Raghuraman *et al.*, 2007). The sequence of melittin is presented in Table 1.8. The amino terminal region is mostly hydrophobic and the carboxy-terminal region is hydrophilic due to the presence of cationic amino acids (Raghuraman *et al.*, 2007).

Protamine is an arginine rich polycationic peptide which has high antimicrobial activity, especially against Gram negative bacteria (Willcox *et al.*, 2008a; Aspedon *et al.*, 1996; Johansen *et al.*, 1995). Although it forms a random coil conformation in aqueous solutions (Warrant *et al.*, 1978), during binding to transfer ribonucleic acid (tRNA) it assumes a secondary structure that includes α–helices with a shallow groove (Raukas *et al.*, 1999). It does not have the ability to assume the classic amphipathic structure required for channel formation in bacterial cells (Aspedon *et al.*, 1996). Its bactericidal mechanism includes inhibition of several metabolic processes that depend on a functional cytoplasmic membrane without triggering cell lysis (Aspedon *et al.*, 1996). The amino acid sequence of protamine is shown in Table 1.8.

Table 1.8: Amino acid sequences of melittin and protamine

Peptide	Sequence
Melittin	NH_2-G-I-G-A-V-L-K-V-L-T-T-G-L-P-A-L-I-S-W-I-K-R-K-R-Q-Q
Protamine	P-R-R-R-R-S-S-S-R-P-V-R-R-R-R-R-P-R-V-S-R-R-R-R-R-R-G-G-R-R-R-R

T, threonine; L, leucine; I, isoleucine; S, serine; W, tryptophan; K, lysine; N, asparagine; R, arginine; Q, glutamine; P, proline; V, valine; G, glycine; A, alanine; D, aspartic acid; F, phenylalanine; E, glutamic acid (Willcox et al., 2008a).

1.5.6.1.2 β-Sheet AMPs

β–sheet peptides (Naginton *et al.*, 1974) are mainly composed of 29–34 amino acids with frequent arginines providing much of the cationic charge (Figure 1-2) (Boman, 1995). Some β-sheet AMPs, such as bovine lactoferricin and human β–defensins (hBD) can act synergistically with other AMPs resulting in highly potent activity against microorganisms at very low concentrations (Gifford *et al.*, 2005; McDermott, 2004; Hancock, 2001). These AMPs are reported to be more active against Gram negative bacteria than Gram positive bacteria (Boman, 1995; Lehrer *et al.*, 1993).

1.5.6.1.3 Extended AMPs

The AMPs that do not fit into the regular secondary structure elements are termed extended AMPs and can be composed of high numbers of arginine, tryptophan and proline amino acids (Figure 1-2) (Nguyen *et al.*, 2011; Tsai *et al.*, 1998; Lehrer *et al.*, 1993). Unlike the β-sheet peptides, these peptides tend to be very flexible in solution and can rapidly form amphipathic structures upon contact with membranes or membrane mimicking environments (Hancock *et al.*, 2006). However, many of the extended AMPs are not membrane active, although, they can form defined amphipathic

structures in the presence of membranes, and can accumulate and may be active in the cytoplasm (Naginton *et al.*, 1974).

1.5.6.2 Mode of action of AMPs

A significant amount of work has been done to understand the diverse activities of the AMPs. Many AMPs form random structures in aqueous solution but form a stable amphipathic conformation when in contact with membranes (Huang *et al.*, 2010). The key mode of action of AMPs involves disruption of the integrity of the anionic bacterial cytoplasmic membrane using various electrostatic mechanisms (Figure 1-3) leading to cell lysis and ultimately cell death (Nguyen *et al.*, 2011; Hancock, 2001). Presently, there are three major proposed models that can explain the mode of action of AMPs.

i. The carpet model: The peptides orient parallel to the surface of the lipid bilayer of the microbial cell membrane and form an extensive layer of carpet like cluster of peptides, resulting in widespread wormhole formation (Costa *et al.*, 2011). Further, the lipid layer bends back on itself with lateral expansions in the polar head-group region, which provide gaps that, are occupied by AMPs. Ultimately, this membrane rearrangement causes inhibition of enzymatic activity, catastrophic breakdown of cytoplasmic membrane integrity and ultimately cell death (Costa *et al.*, 2011; Hancock, 2001). The carpet model is proposed to describe the mode of action of dermaseptin natural analogues, cecropins and LL-37 (Shai *et al.*, 2001).

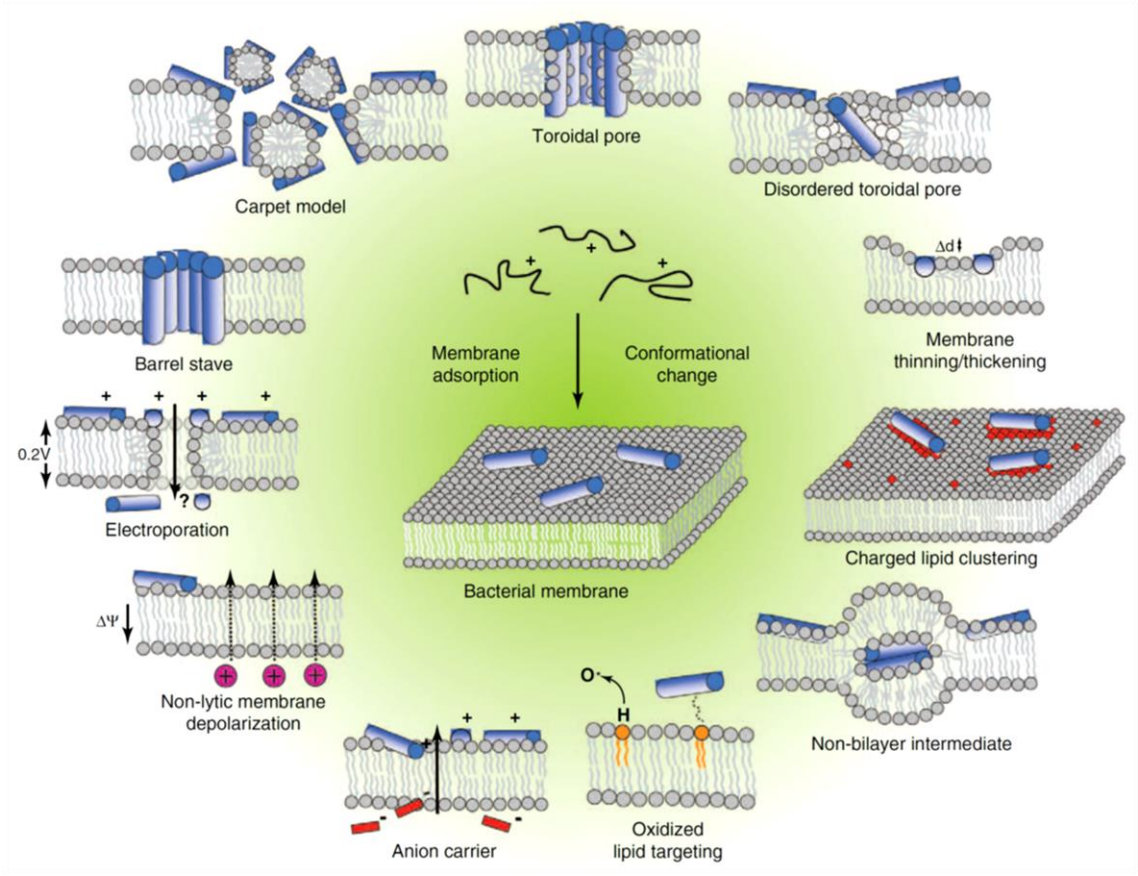

Figure 1-3: Interaction of antimicrobial peptides (AMPs) with bacterial cytoplasmic membranes.

All the models of peptide adsorption lead to cytoplasmic membrane disruption. From Nguyen et al. (2011) with permission.

ii. The barrel-stave model: Following initial electrostatic interaction with the outer layer of bacterial membrane, α-helical amphipathic peptides aggregate and insert into the membrane bilayer and form a barrel-like bundle. The hydrophobic peptide regions align with the lipid core regions and the hydrophilic peptide parts are oriented inward into the water-filled pore (Brogden, 2005). The antimicrobial activity of pardaxin (Rapaport *et al.*, 1991) and alamethicin (Nguyen *et al.*, 2011) is based on this mode of antimicrobial action. The pores formed in the membrane result in disruption to membrane potential and leakage of intracellular components (Nguyen *et al.*, 2011).

iii. The toroidal pore model: Following binding to phospholipid head groups, the AMPs insert into the microbial membrane and form full thickness unstructured bundles (Zhang *et al.*, 2000a). These channels are believed to be short-lived and responsible for leakage of ions and larger molecules from the inside of the bacteria. These channels allow more AMPs to cross the microbial membrane without causing further membrane depolarising, which attack internal targets (Brunengraber *et al.*, 2003). Magainin 2, melittin and protegrin-1 are few of the AMPs that are found to use this mode of antimicrobial action (Nguyen *et al.*, 2011).

Additionally, a few other modes of antimicrobial action have been described which include interruption in DNA and protein synthesis, disruption of protein folding, inhibition of enzymatic activity and cell wall synthesis (Nicolas, 2009; Brogden, 2005; Papo *et al.*, 2003). AMPs such as magainin, melittin and indolicidin have multiple modes of action resulting in highly efficient microbial killing and potentially reducing the chances of development of microbial resistance (Nguyen *et al.*, 2011; Peschel *et al.*, 2006). Considering the mode of actions for soluble AMPs, it is expected that antimicrobial mechanisms of immobilised AMPs are different. Surface tethered magainin attached through very short linkers displayed antimicrobial activity (Haynie *et al.*, 1995). This indicates that outer membrane interaction by attached magainin was sufficient for antimicrobial killing. However, direct immobilisation of AMPs through long linkers, in an attempt to allow the peptides to penetrate cell membrane, did not show activity (Bagheri *et al.*, 2009). It is believed that high local concentration of immobilised peptides may displace positively charged counter-ions associated with the outer surface of the microbial membrane, which could shift the ionic balance rapidly (Hilpert *et al.*, 2009). This may trigger clustering of ionic lipids, leakage of intracellular contents and activation of autolytic enzymes (Epand *et al.*, 2011). Hilpert *et al.* (2009)

have suggested that hydrophobic residues at the end of tethered AMP are responsible for the interaction with bacterial cell membrane.

MICs of all AMPs typically between 1–125 μg ml^{-1}, depending upon the kind of AMP and microorganism used (McDermott, 2004; Hancock, 2001; Schroder, 1999; Turner *et al.*, 1998; Ganz *et al.*, 1995). Generally, the MIC and minimal bactericidal concentration (MBC) of AMPs coincide or differ by no more than two-fold, emphasising the rapid bacterial killing by the AMPs compared to conventional antibiotics (Zhang *et al.*, 2000b; Steinberg *et al.*, 1997). In addition to the antibacterial mechanisms, a limited number of studies have explored antifungal modes of action, which include morphological distortions, rapid ion fluxes (De Samblanx *et al.*, 1997) and inhibition of energised mitochondria in fungal cells (Helmerhorst *et al.*, 1999). The mode of action against eukaryotic parasites and cancer cells is poorly understood (Hancock, 2001). A few AMPs, such as defensins, indolicidin and melittin exhibit antiviral activity against HIV, influenza A virus and herpes simplex virus by blocking viral cell fusion or the activity of retroviral long terminal repeats which control pro-viral gene transcription (Chia *et al.*, 2010; Zhang *et al.*, 2000a).

1.5.6.3 AMPs at the ocular surface

Defensins and LL-37 are the major AMPs present at the ocular surface (McDermott, 2004). Defensins have been classified as α-, β- and θ- defensins. Both α– and β–defensins are found at the ocular surface, and they are produced from infiltrating neutrophils and ocular surface epithelial cells respectively (McDermott, 2009). α-defensins, such as human neutrophil peptide (HNP) 1 to 3, can be present at the normal ocular surface in the range of 0.2 – 1.0 μg ml^{-1} (Zhou *et al.*, 2004). Elevated levels of α-defensins have been detected following corneal epithelial lesions (Hida *et al.*, 2005),

conjunctival inflammation (Haynes *et al.*, 1999) and removal of an ocular surface neoplasm (Hida *et al.*, 2005). hBD-1 is constitutively expressed (Haynes *et al.*, 1999), but expression of hBD-2 is up-regulated in response to corneal injury (McDermott *et al.*, 2001). hBD-3 is expressed by cornea and conjunctival cells, but hBDs 4 to 6 have not been found at the ocular surface (Huang *et al.*, 2007). hBDs can kill a variety of ocular pathogens including *Acanthamoeba, Candida albicans*, HSV-1, adenovirus and bacteria such as *P. aeruginosa, S. aureus* and *S. marcescens* (McDermott, 2004; Brunengraber *et al.*, 2003). McDermott (2004) have estimated that human corneal epithelium can produce microgram quantities of hBD-1 and hBD-2, which may accumulate in intracellular spaces at very high concentration and produce high antimicrobial activity. Additionally, these AMPs can work synergistically with other AMPs and antimicrobial substances, producing high activity at much lower concentrations (Yan *et al.*, 2001). However, sodium chloride present at the human tears can significantly reduce activity of hBD-2 *in vitro* (McDermott, 2004). This is possibly because the salt can interfere with the electrostatic interactions between hBD-2 and microbial membranes (McDermott, 2004). Due to six conserved cysteins in the primary structure of defensins, the exact configuration and mode of action is determined by three disulfide bonds (Brunengraber *et al.*, 2003). The antimicrobial mechanisms of hBDs have been discussed in section 1.5.6.2. The primary mechanism includes an electrostatic interaction with microbial cells membrane, followed by pore formation, DNA damage, and cell death.

LL-37 is the only member of the cathelicidin family of AMPs found in humans and is expressed by ocular tissues (Huang *et al.*, 2006). It is expressed by various tissues of the human eye including the cornea and conjunctiva (Kolar *et al.*, 2011). LL-37 is 37 amino acids long (Table 1.9) and has broad spectrum activity including activity, against

antibiotic resistant strains of bacteria (Durr *et al.*, 2006; Huang *et al.*, 2006; Gordon *et al.*, 2005; Turner *et al.*, 1998). Unlike other AMPs such as human β–defensin 1 or 2, it is less affected by the salt concentration in human tears (Huang *et al.*, 2006). This AMP is derived from its larger precursor hCAP-18 and its expression is upregulated in response to microbial challenge (Li *et al.*, 2008; Kumar *et al.*, 2007; Huang *et al.*, 2006). LL-37 interacts electrostatically with bacterial cytoplasmic membranes and then assumes an α–helical structure in the membrane (Nguyen *et al.*, 2011; Hancock *et al.*, 2006). Neutrophils, which produce LL-37, are recruited to the ocular surface in response to infection where LL-37 in addition to its antimicrobial activities, plays a role in the modulation of inflammation at the ocular surface including influencing the recruitment of additional polymorphonuclear granulocytes, monocytes and T-cells (Kolar *et al.*, 2011; Bowdish *et al.*, 2006; De *et al.*, 2000). Mice deficient in CRAMP, the murine homologue of LL-37, showed increased severity and delayed recovery from *P. aeruginosa* (Huang *et al.*, 2007) and *Candida* (Gao *et al.*, 2011) keratitis. However, the activity of LL-37 against *Acanthamoeba* is not yet clear, although Otri *et al*. (2010) have shown that *A. castellanii* stimulated *in vitro* expression of LL-37.

The other AMPs present at the ocular surface are MIP-3α, thymosin β-4, dermcidin, histains, statherin, CCL28, CXCL-1, LEAP-1 and LEAP-2 (McDermott, 2009; McIntosh *et al.*, 2005). However, among these AMPs only dermcidin has wide spectrum of antimicrobial activity (Schittek, 2012), MIP-3α and thymosin β-4 have weak bactericidal activity against *P. aeruginosa* and *S. aureus* (McDermott, 2009). As a group, the AMPs present at the ocular surface provide major defence against infection. Based on the AMP expression and antimicrobial activity, it is likely that hBD-1 and hBD-3 have major function to constituently protect against ocular infection, and during ocular inflammation and infection hBD-2 and LL-37 are additionally expressed to

provide greater protection. As each of these AMPs has different but sometimes overlapping spectra of antimicrobial activity, they provide protection against a wide range of ocular pathogens.

1.5.6.4 Melimine

Melimine is a synthetic peptide consisting of 29 amino acid residues (Table 1.9) derived from melittin and protamine (Willcox *et al.*, 2008a). Protamine has good activity against Gram negative bacteria but less effective against Gram positive bacteria. Melittin, on the other hand, has good activity against Gram positive bacteria but is less active against Gram negative bacteria (Willcox *et al.*, 2008a). The hybrid peptide melimine has a high activity *in vitro* which exceeds that of a mixture of the two parent peptides (Aliwarga *et al.*, 2001). Repeated exposure of bacteria to sub-minimal inhibitory concentrations of melimine did not readily induce resistance, suggesting that melimine may represent a good candidate for development as an antimicrobial strategy for preventing bacterially driven adverse events (Willcox *et al.*, 2008a).

Melimine adopts a random coil conformation in aqueous solution and addition of organic solvents allows the peptide to adopt a helical fold (Rasul *et al.*, 2010). In environments that mimic bacterial membranes, the alpha-helical content increases to approximately 40% (Rasul *et al.*, 2010). 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 major effect of melimine is on the integrity of the cytoplasmic membrane both for *P. aeruginosa* and *S. aureus*, however it is of particular interest that there are differences in the relationship between depolarisation of the cytoplasmic membrane and the kinetics of loss of viability of these bacteria (Rasul, 2010). For *P. aeruginosa*, the rapid loss of cytoplasmic membrane integrity correlates

directly with loss of cell viability whilst for *S. aureus*, membrane depolarisation occurred at concentrations where there was no significant loss of viability (Rasul *et al.*, 2010). 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 (Rasul *et al.*, 2010). Figure 1-4 shows transmission electron micrographs of the changes in the structure of both *P. aeruginosa* 6294 and *S. aureus* 31 cell membranes when exposed to melimine in solution. It would be anticipated that the mechanism of action of melimine may differ when constrained by attachment to the surface of a biomaterial.

Table 1.9: Amino acid sequences of melimine and LL-37

Peptide	Sequence
LL-37	L-L-G-D-F-F-R-K-S-K-E-K-I-G-K-E-F-K-R-I-V-Q-R-I-K-D-F-L-R-N-L-V-P-R-T-E-S
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

T, threonine; L, leucine; I, isoleucine; S, serine; W, tryptophan; K, lysine; N, asparagine; R, arginine; Q, glutamine; P, proline; V, valine; G, glycine; A, alanine; D, aspartic acid; F, phenylalanine; E, glutamic acid (Willcox et al., 2008a; McDermott, 2004).

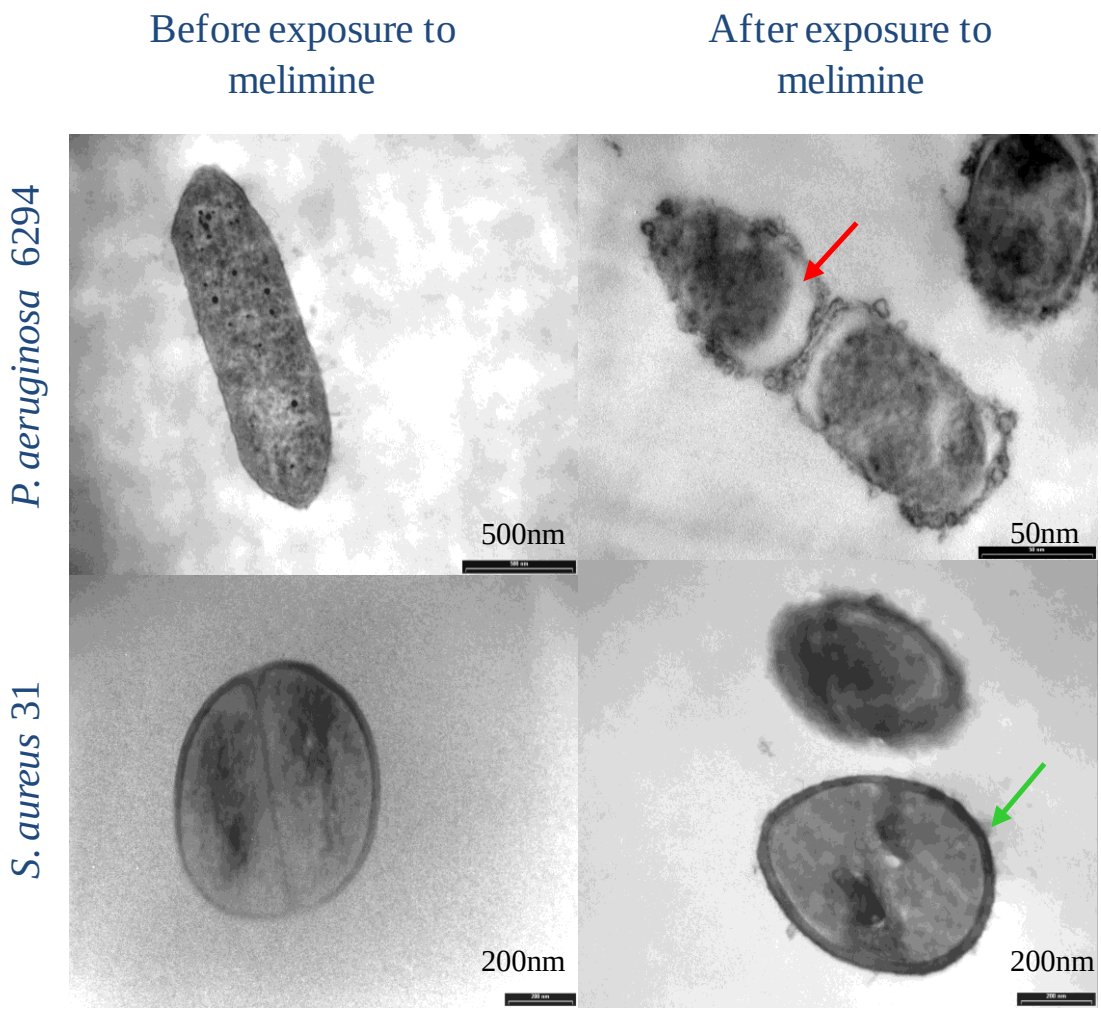

Figure 1-4: Transmission electron micrographs showing the exposure of both *P. aeruginosa* and *S. aureus* to melimine at the minimal inhibitory concentration produced changes in the bacterial cell structure.

After exposure to melimine, P. aeruginosa 6294 cells showed bleb formation, loss of membrane integrity, condensation of DNA and separation of membrane from the cell wall (red arrow). After exposure, S. aureus 31 showed fibres extending from cell surface and DNA condensation (green arrow). From Willcox et al.(2008a) with permission.

In addition to the activity in solution, melimine covalently attached to glass surfaces via an azide linker also has been shown to retain high bactericidal activity (Chen *et al.*, 2009). The activity was characterised by substantial membrane damage, flattening and a transparent appearance of *P. aeruginosa*. In addition immobilised melimine showed significant reduction in adhesion of *S. aureus* (Figure 1-5). However,

the efficacy of melimine is dependent upon the method of covalent attachment (Chen *et al.*, 2009).

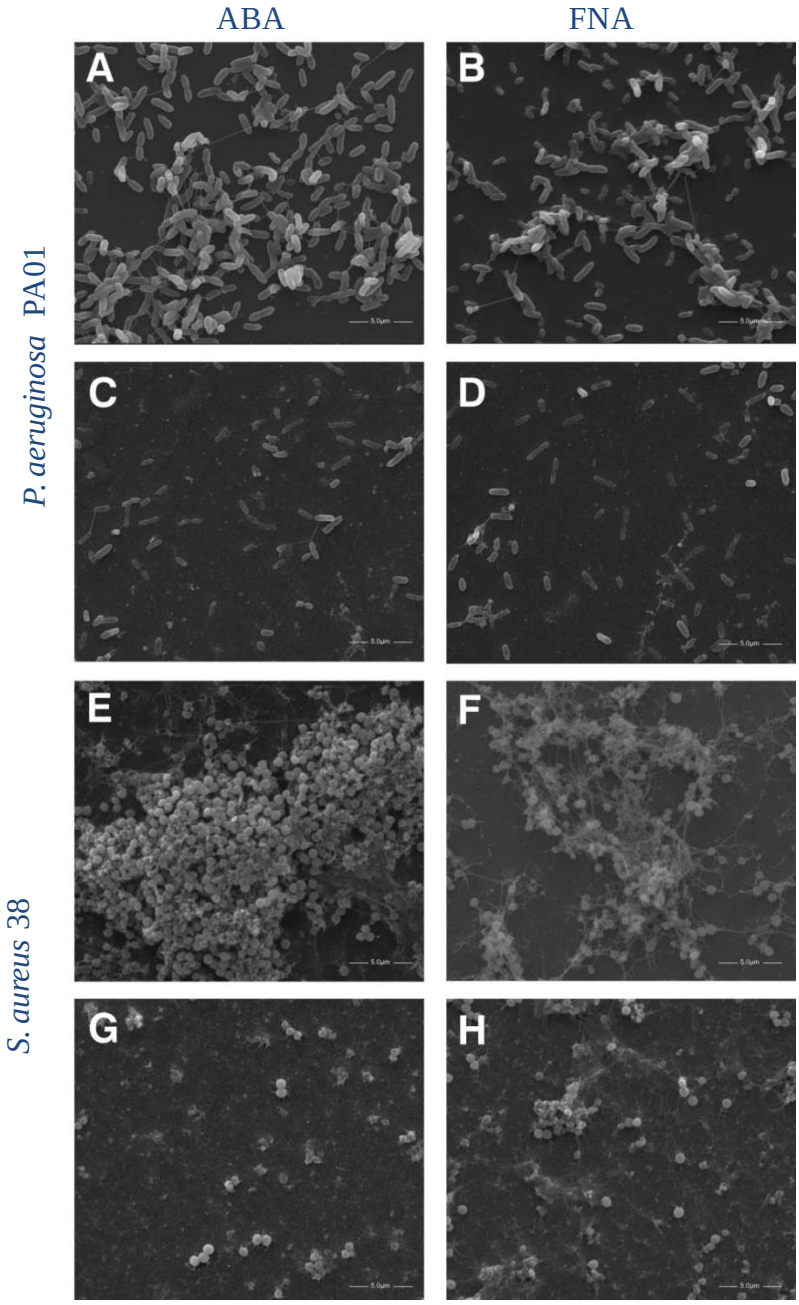

Figure 1-5: Scanning electron micrographs of *P. aeruginosa* and *S. aureus* adhesion to melimine-attached glass surfaces via two azide linkers, 4-azidobenzoic acid (ABA) and 4-fluoro-3-nitrophenyl azide (FNA).

(A) P. aeruginosa adherent to ABA process control. (B) P. aeruginosa adherent to FNA process control. (C) P. aeruginosa adherent to ABA–melimine surface. (D) P. aeruginosa adherent to FNA–melimine surface. (E) S. aureus adherent to ABA process control. (F) S. aureus adherent to FNA process control. (G) S. aureus adherent to ABA–melimine surface. (H) S. aureus adherent to FNA–melimine surface. From Chen et al. (2009) with permission.

When covalently attached to the surface of a contact lens, melimine showed activity against both Gram positive and Gram negative bacteria *in vitro* (Willcox *et al.*, 2008b). Melimine attached to contact lenses, successfully reduced levels of bacterial contamination *in vitro* and the incidence and severity of CLARE and CLPU in an animal model (Cole *et al.*, 2010)

1.6 *In Vitro* Antimicrobial Activity Required for Contact Lenses

An estimation of the number of bacteria on contact lenses required to produce microbially-driven adverse events has been reported in the eyes of guinea pigs (Vijay *et al.*, 2009) and rabbits (Wu *et al.*, 2005). Around 11,000 CFU of *S. aureus* on contact lenses gave a 100% CLPU-like response in rabbit eyes. Reducing this level to between 6500 and 4,000 CFU of *S. aureus* led to a reduced incidence and/or a less severe response; this was reflected in the reduction of the average diameter of the epithelial defect from 7 mm to 3 mm, for the rabbits that had defects respectively (Wu *et al.*, 2005). Similarly, 94,000, 23,000 and 10,000 CFU of *P. aeruginosa* on contact lenses were required to produce a CLARE-like response in 67%, 50% and 33% of guinea pig eyes, respectively (Vijay *et al.*, 2009). This finding indicates that 33% guinea pigs still developed a CLARE-like response after reduction of bacterial load from 94,000 to 10,000 (around one log). Extrapolation from these results indicate that the load below which CLARE may not occur is approximately <1,000 CFU. Considering that when contact lenses are removed from the eye aseptically they are usually only colonised by <1000 CFU bacteria (Szczotka-Flynn *et al.*, 2010b), a 1.5 log reduction for any antimicrobial lens would be required to lower the incidence of bacterially-driven adverse responses to below 1%. At the time of contact lens-induced keratitis contact

lenses may be colonised by <5000 CFU of bacteria (Szczotka-Flynn *et al.*, 2010a), and again an antimicrobial lens that reduced this level to <500 (i.e. a 1 log inhibition) may significantly reduce the incidence of keratitis. Another study has shown that lenses associated with CLARE were colonised by a median of $3x10^5$ CFU Gram-negative bacteria (Range $2x10^5$-$3x10^5$ CFU; counts from lenses plus the transport saline into which lenses were collected from eyes (aseptically) and transported to the laboratory for analysis), lenses associated with infiltrates (equivalent to IK) were colonised by a median of $2x10^4$ CFU Gram-negative bacteria (Range 4-$2.7x10^5$ CFU), whereas lenses of eyes that had no infiltration were colonised by a median of 0 CFU (Range 0-$3x10^5$ CFU) (Holden *et al.*, 1996). Antimicrobial lenses that reduce the median colonisation by ≥1.5 log CFU would be expected to reduce the severity of any infiltrate response.

1.7 Rationale for Research

Despite the advances in contact lens materials and solutions, infection and inflammation is still a major concern in contact lens wear for practitioner and wearers alike. There is a need to reduce the number of adverse events occurring among the large number of contact lens wearers. Limiting microbial adhesion and contamination to contact lenses may prevent contact lens related adverse events.

1.8 Thesis Aims

This thesis aims to investigate the broad spectrum antimicrobial activity of AMP-coated contact lenses. Additionally, this thesis will examine the biocompatibility of AMP-coated lenses in an animal model and human clinical trial.

There is currently no commercially available antimicrobial contact lens. A contact lens based on AMP-derived inhibition of adhesion of various pathogenic

microorganisms that is biocompatible with the ocular environment may be efficacious in reducing the incidence and/or severity of contact lens related microbial adverse events.

1.9 Thesis Hypotheses

Covalent immobilisation of AMPs onto the surface of a contact lens will result in broad spectrum antimicrobial activity. The AMP coating will be safe and biocompatible in both the animal models and in humans, and retain activity after human contact lens wear.

1.10 Thesis Overview

Chapter 2 determines a set of assays best suited for evaluating *in vitro* bacterial inhibition for contact lenses. The set of assays established for Gram positive and Gram negative bacteria will be used throughout the thesis.

Chapter 3 evaluates the antimicrobial activity of melimine and cathelicidin bound to contact lenses.

Chapter 4 evaluates the antimicrobial activity of melimine-coated contact lenses against multi-drug resistant Gram positive and Gram negative bacteria, *Acanthamoeba* and ISO panel microorganisms. In addition, endotoxin association and total bacterial adhesion is also determined.

Chapter 5 validates the physical dimensions, wettability and heat stability of the melimine-coated coated contact lenses. Further, this chapter determines the compatibility of melimine-coated coated lenses with lens care solutions.

Chapter 6 evaluates *in vivo* safety and ocular irritation of melimine-coated coated contact lenses in rabbit eyes.

Chapter 7 investigates the clinical performance and retention of antimicrobial activity of melimine-coated coated contact lenses in a prospective, randomised, double-masked, contralateral human clinical trial.

Chapter 8 summarises and discusses the findings and limitations of this work and recommends future studies to extend the findings.

Chapter 2: Defining Assay Conditions for *In Vitro* Evaluation of Bacterial Adhesion to Contact Lenses

This chapter has been published as:

- Dutta D, Willcox MD. A laboratory assessment of factors that affect bacterial adhesion to contact lenses. *Biology*. 2013;2(4):1268-1281.

2.1 INTRODUCTION

Bacterial adhesion to contact lenses is a complex and multifactorial process and previous *in vitro* and *ex vivo* adhesion data differ widely between various studies (Willcox, 2013). This is mainly due to the variety of methodologies used to evaluate the numbers of adherent bacteria and the range of assay conditions under which bacterial adhesion to contact lenses has been evaluated. These conditions have included different strains or types of bacteria, contact lens materials, inoculum size, the nutritional content of media and incubation times for adhesion to occur. Methods used to quantify microbial adhesion to contact lenses include viable plate counts (Giraldez *et al.*, 2010; Willcox *et al.*, 2008a; Garcia-Saenz *et al.*, 2002), number of cells adherent in parallel plate flow chambers (Vermeltfoort *et al.*, 2006), scanning electron microscopy (Mathews *et al.*, 2006), bioluminescent ATP assays (Kodjikian *et al.*, 2008), light microscopy (Williams *et al.*, 2003), and assessment of the number of cells after radio-labelling (Babaei Omali *et al.*, 2012; Babaei Omali *et al.*, 2011; George *et al.*, 2003). The solutions used during adhesion experiments vary from phosphate buffered saline (PBS) (Borazjani *et al.*, 2004) which is nutritionally inert, to growth media such as tryptone soya broth (TSB) (Bandara *et al.*, 2004) or Mueller Hinton broth, which are nutritionally rich. The reported inoculum sizes in bacterial adhesion assays vary from $1x10^3$ colony forming units (CFU) ml^{-1} to $1x10^9$ CFU ml^{-1} (Willcox *et al.*, 2010b; Giraldez *et al.*, 2010) and the incubation period for adhesion has ranged from 10 min (Williams *et al.*, 2003) to 72 hr (Randler *et al.*, 2010).

The wide variety of bacterial assays used in previous antibacterial studies and consequent differences in bacterial numbers adhering to lenses signify a need to develop a set of standardised *in vitro* assays that will allow comparisons within and between

studies of adhesion of different bacterial strains to different contact lens surfaces to facilitate antimicrobial research. It would be of benefit to design conditions that closely mimic the scenario that is likely to occur during lens wear. This study aimed to determine an assay condition that is best suited to laboratory evaluation of bacterial adhesion to antibacterial contact lenses. The hypotheses were that the conditions under which bacteria are allowed to adhere to contact lenses and the method of evaluation will affect the numbers of bacteria detected.

2.2 METHODS

Two of the most widely used contact lens materials (Efron *et al.*, 2011) were used, the hydrogel etafilcon A (ACUVUE® 2; Johnson & Johnson Vision Care Inc., Jacksonville, FL; Base curve: 8.7 mm, Diameter: 14.0 mm, Power: -3.00 Ds) and the silicone hydrogel contact lens material senofilcon A (Acuvue® OASYS™; Johnson & Johnson Vision Care, Jacksonville, FL ; Base curve: 8.4 mm, Diameter: 14 mm, Power: -3.00 Ds). The properties of these materials are described in Table 2.1.

Table 2.1: Properties of contact lens materials used in the study

Proprietary name	Acuvue® 2	Acuvue® OASYS™
United States Adopted Name (USAN)	etafilcon A	senofilcon A
Manufacturer	Johnson & Johnson	Johnson & Johnson
Water content (%)	58	38
Oxygen permeability (Dk)	21	103
Centre thickness (mm) - 3.00 Ds	0.08	0.07
Oxygen transmissibility (Dk/t) at 35°C	25	147
Surface area (mm^2)	387.2	384.0
FDA group	IV	I
Surface treatment	None	No surface treatment. Internal wetting agent (PVP) that also coats the surface
Principal monomers	HEMA + MA	mPDMS + DMA + HEMA + siloxane macromer + PVP + TEGDMA

mPDMS, (monofunctional polydimethylsiloxane); DMA, (N,N-dimethylacrylamide); HEMA, (2-hydroxyethyl methacrylate); PVP, (polyvinyl pyrrolidone); TEGDMA (tetraethyleneglycol dimethacrylate); MA, (methacrylic acid)

2.2.1 Bacterial Strains

As most contact lens related microbial adverse events are caused by either Gram negative *Pseudomonas aeruginosa* or Gram positive *Staphylococcus aureus* (Otri *et al.*, 2012; Green *et al.*, 2008b), selected strains of these species were used.

Table **2.2** details the bacterial strains used in this study. None of the selected strains was known to have resistance against any antibiotic (Schubert *et al.*, 2008; Borazjani *et al.*, 2004; Lakkis *et al.*, 2001; Fleiszig *et al.*, 1997; Parment *et al.*, 1993).

Table 2.2: Details of bacteria used in the study

***S. aureus* strains**	**Isolation site**
S. aureus 31[a]	CLPU – contact lens
S. aureus 38[a]	MK

***P. aeruginosa* strains**	**Isolation site**	**Genotype**		
		ExoS	**ExoT**	**ExoU**
P. aeruginosa 6294[b]	MK	Positive	Positive	Negative
P. aeruginosa ATCC 9027[c]	Otic infection	Negative	Negative	unclear
P. aeruginosa GSU3[d,e]	Human Corneal Ulcer	Positive	Positive	Negative

a = Schubert *et al.* (2008); b = Fleiszig *et al.* (1997); c = Lakkis *et al.* (2001); d = Borazjani *et al.* (2004); e = Parment *et al.* (1993)

2.2.2 Assay Media

The four different media used were PBS pH 7.4 (NaCl 8 g l^{-1}, KCl 0.2 g l^{-1}, Na_2HPO_4 1.15 g l^{-1}, KH_2PO_4 0.2 g l^{-1}), TSB (Oxoid, Basingstoke, UK), TSB diluted 10-times in sterile PBS (1/10 TSB), or 1/10 TSB containing glucose (0.25% w/v) (TSBG). PBS acted as a nutritionally inert media and TSB as a highly nutritious media.

2.2.3 Incubation Period

A literature review revealed that initial bacterial adhesion to contact lenses increased with time, peaking at 3 to 18 hr of incubation and then remaining steady, suggesting the end point of primary adhesion (Randler *et al.*, 2010; Andrews *et al.*, 2001; Miller *et al.*, 1987). To evaluate bacterial adhesion during two phases of the process, 2 hr and 18 hr exposure of contact lenses to bacterial suspension were chosen.

2.2.4 Inoculum Size

It is difficult to quantify the numbers of pathogenic bacteria adherent to contact lenses during lens wear that result in a microbial adverse event in human eyes. Therefore, three different inoculum sizes were selected for investigation; 10^3 CFU ml^{-1}, 10^6 CFU ml^{-1} and 10^{10} CFU ml^{-1}, representing low, medium and very high inocula respectively.

2.2.5 Adhesion Conditions

Stock cultures were stored in 30% glycerol at -80°C. Bacteria were grown overnight in TSB at 37°C with aeration. The bacterial cells were centrifuged for 10 min at 3,000 rpm and washed three times with PBS. Bacteria were then resuspended in one of the four media to an OD_{660nm} of 0.1 ($1x10^8$ CFU ml^{-1}). The bacterial cell suspensions were then diluted to $1x10^3$ and $1x10^6$ CFU ml^{-1}. The bacterial suspension of $1x10^{10}$ CFU ml^{-1} was made by centrifuging 10 ml of $1x10^9$ CFU ml^{-1} (OD_{660nm} of 1.0) suspension and resuspending the in 1 ml in the respective media. Contact lenses were washed three times in PBS and placed concave side up in the wells of 24-well tissue culture plates (CELESTAR®, Greiner bio-one, Frickenhausen, Germany). One ml of bacteria suspension was added to the wells. To allow adhesion of bacterial cells, lenses were incubated for two hr or 18 hr at 37ºC with shaking (120 rpm). Lenses were aseptically

removed from the suspension and gently washed three times with 1 ml PBS in a 24-well plate by shaking at 120 rpm for 1 min to remove non-adherent cells. Following washing, contact lenses were stirred rapidly in a vial containing 2 ml of PBS and a small magnetic stirring bar. Following ten-fold serial dilutions in PBS, 3 x 50 µl of each dilution were plated on nutrient agar plates (NA; Oxoid, Basingstoke, UK). After 24 hr incubation at 37ºC, viable bacteria were enumerated as CFU mm^{-2} lens. The inoculum size was determined retrospectively by a standard dilution and plate count method. Results are expressed as the numbers of adherent viable bacteria from three independent experiments performed with triplicate samples.

2.2.6 Data Analysis

The adhesion data were $\log_{10}(x+1)$ transformed prior to data analysis where x is the number of adherent bacterial colonies mm^{-2}. All data were analysed using Statistical Package for Social Science for Windows version 21.0 (SPSS, Inc, Chicago, IL). Interactions between different factors influencing bacterial adhesion to contact lenses such as bacterial strain type, assay media, incubation time and inoculum size were investigated in a nested model of all the variables. Based on this estimation, the estimated mean was calculated which was adjusted for the other variables in the model. To evaluate and compare the influence of tested assay conditions on bacterial adhesion, the partial eta squared was estimated. Bacterial adhesion was analysed using an independent two sample *t* test. Differences between the groups were analysed using a linear mixed model ANOVA, which adjusts for the correlation due to repeated observations. Post hoc multiple comparisons were done using a Bonferroni correction. Statistical significance was set at 5%.

2.3 RESULTS

Figure 2-1 and Figure 2-2 represent the adhesion of *P. aeruginosa* and *S. aureus* respectively when incubated in the four different media and at three different bacterial concentrations over time. Analysis of strain differences within a genera/species found that only *P. aeruginosa* ATCC 9027 showed higher adhesion to etafilcon A than senofilcon A ($p < 0.05$). *P. aeruginosa* adhered at higher numbers compared to *S. aureus* ($p < 0.05$).

For each bacterial type and strain, there was a significant increase in adhesion from 2 to 18 hr ($p < 0.05$) when incubated with $1x10^3$ CFU ml^{-1} or $1x10^6$ CFU ml^{-1} bacterial suspension. For *P. aeruginosa* strains, adhesion to the contact lenses increased as the initial inoculum increased ($p < 0.05$). However, for strains of *S. aureus* adhesion reached a maximum when $1x10^6$ CFU ml^{-1} bacterial cells were incubated with lenses for 18 hr; addition of bacteria at $1x10^{10}$ CFU ml^{-1} did not increase adhesion. The differences between the number of bacterial cells recovered from the washed solutions of the contact lenses incubated with different concentrations of bacteria was less than 0.3 log.

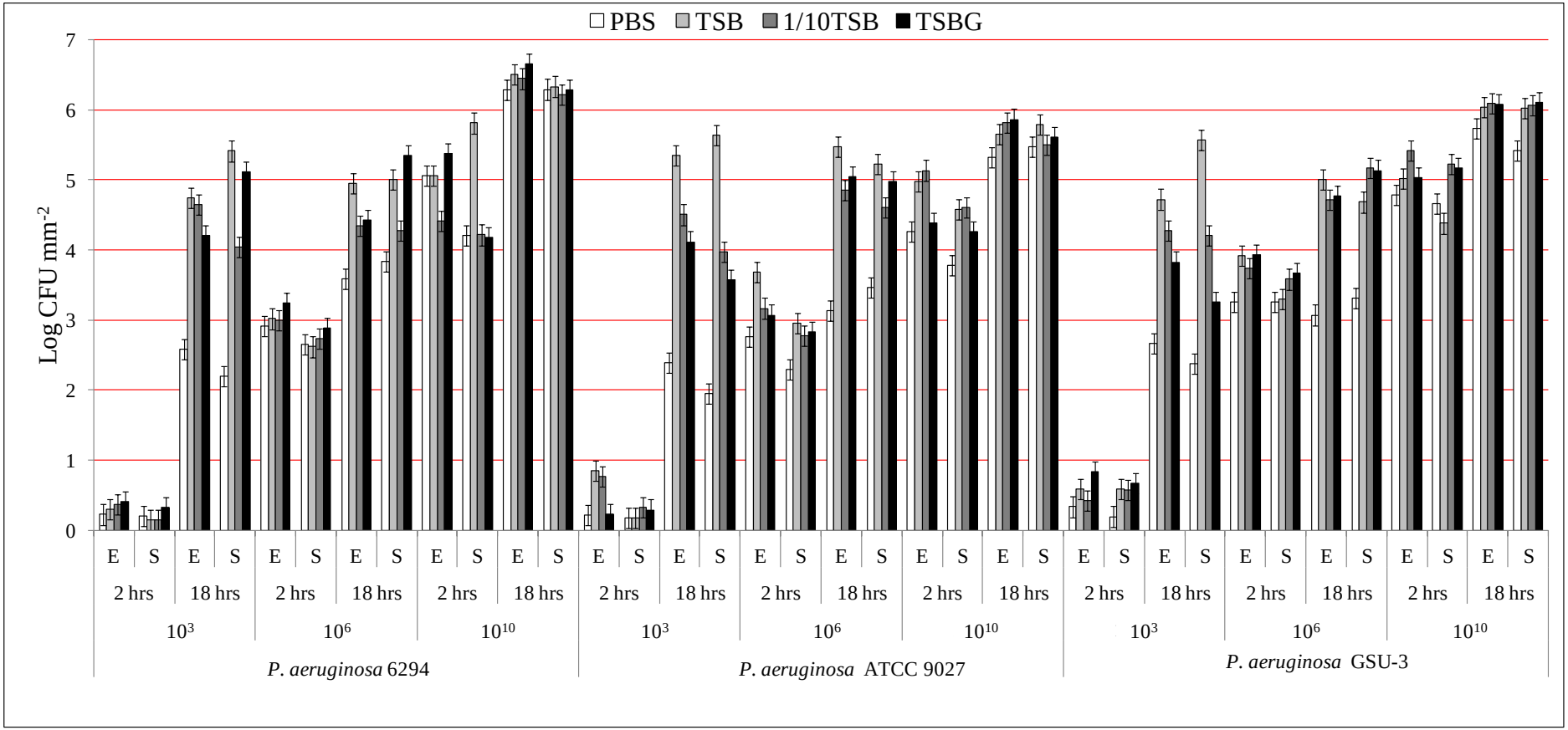

Figure 2-1: Adhesion of *P. aeruginosa* to contact lenses under different conditions.

(E = etafilcon A lenses; S = senofilcon A lenses)

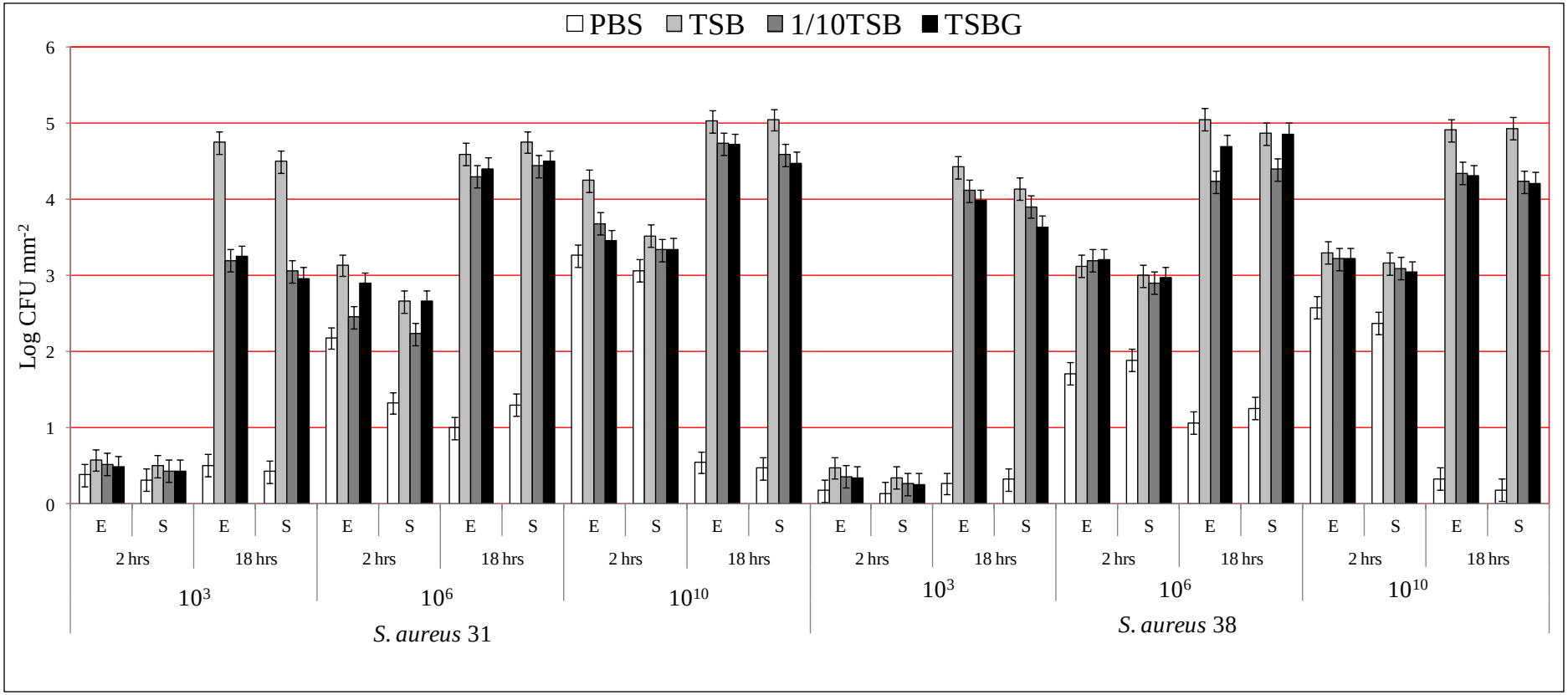

Figure 2-2: Adhesion of *S. aureus* to contact lenses under different conditions.

(E = etafilcon A lenses; S = senofilcon A lenses)

When comparing the effect of media on adhesion, there were differences between the bacterial genera/species. For *P. aeruginosa*, adhesion was significantly lower ($p < 0.05$) when incubated in PBS compared to the other media after 18 hr for concentrations up to and including $1x10^6$ CFU ml^{-1}, but not at $1x10^{10}$ CFU ml^{-1}. At low bacterial numbers ($1x10^3$ CFU ml^{-1}), adhesion of *P. aeruginosa* to the surface of contact lenses was significantly higher (up to $1.8x10^3$ fold) when incubated with high nutrition (TSB; $p < 0.05$) compared to media where nutrition was limited. However, this effect became less significant as bacterial numbers increased. For *S. aureus*, adhesion was significantly lower in PBS (up to $5.6x10^4$-fold; $p < 0.05$) than all other media at all bacterial concentrations, at all time points and on both contact lens types. When lens surfaces were exposed to higher bacterial innocula ($1x10^6$ or $1x10^{10}$ CFU ml^{-1}) of *S. aureus* in PBS, there was up to 518-fold ($p < 0.05$) reduction in adherent bacteria after 18 hr adhesion compared to 2 hr adhesion. This was not observed with the higher nutrition media, where longer incubations were always associated with higher adhesion.

After adjusting for the effects of incubation time, inoculum size and lens material, incubation with PBS showed significantly ($p < 0.05$) less adhesion for all bacteria studied. There were no significant differences in bacterial adhesion ($p > 0.05$) when incubated with 1/10 TSB or TSBG. Incubation in the nutritionally rich TSB was associated with higher adhesion, when incubated with $1x10^3$ CFU ml^{-1} and $1x10^6$ CFU ml^{-1} cells (Figure 2-1 and Figure 2-2), compared to other media, especially after 18 hr.

Table 2.3 shows the estimated degree of association between bacterial adhesion and assay conditions that influenced the adhesion. A higher partial eta squared value implies greater influence over bacterial adhesion. Differences in the two *S. aureus* strains did not influence bacterial adhesion (partial eta squared = 0.00; $p = 0.41$), while

the strains of *P. aeruginosa* did influence adhesion ($p < 0.05$). Assay media had a greater influence on *S. aureus* than *P. aeruginosa* adhesion. The remaining factors examined – incubation period and inoculum size – had significant influence ($p < 0.05$) on adhesion. The ranking of higher to lower influencing factors for *P. aeruginosa* adhesion were inoculum size > incubation period > assay media > bacterial strains > type of lens and for *S. aureus* were assay media > inoculum size > incubation period > type of lens > bacterial strains.

Table 2.3: Effect of factors that influence bacterial adhesion

Influencing factor for bacterial adhesion	Partial eta squared	
	P. aeruginosa	*S. aureus*
Inoculum size	0.75	0.43
Incubation period	0.64	0.37
Assay media	0.19	0.54
Type of lens	0.01	0.01
Bacterial strains	0.02	0.00

A higher partial eta squared value implies higher influence over bacterial adhesion.

2.4 DISCUSSION

In this study, adhesion of a number *P. aeruginosa* and *S. aureus* strains to contact lenses was assessed under a variety of assay conditions. In most cases there was no significant difference in levels of bacterial adhesion for each strain examined to either the hydrogel etafilcon A or silicone hydrogel senofilcon A lenses. This is consistent with an earlier study (Borazjani *et al.*, 2004). However, the results reported here differ from those of others where adhesion of both *P. aeruginosa* and *S. aureus* to silicone hydrogel contact lenses was found to be higher than to hydrogel lenses (Giraldez *et al.*, 2010; Kodjikian *et al.*, 2008; Henriques *et al.*, 2005). These differences may result from the use of balafilcon and lotrafilcon lenses in these previous studies, as Subbaraman *et al*. (2011) have demonstrated that senofilcon A lenses which were used in the current study show lower levels of bacterial adhesion than either balafilcon A or lotrafilcon B.

There was significant difference in adhesion between the strains of *P. aeruginosa*, but *S. aureus* strains examined in this study did not differ significantly in their adhesion to contact lenses. Previous studies have shown considerable variation in adhesion between different strains ranging up to $2.00x10^5$ CFU mm^{-2} for *P. aeruginosa* and $1.23x10^5$ CFU mm^{-2} for *S. aureus* (Vijay *et al.*, 2012; Borazjani *et al.*, 2004; Klotz *et al.*, 1989; Miller *et al.*, 1987). Thus, it is important to use the same strains across studies for meaningful comparisons to be made. However, other strains may need to be incorporated for comprehensive evaluation of surface effects on adhesion. *P. aeruginosa* adhered at higher levels than *S. aureus* and this is consistent with previous reports (Vijay *et al.*, 2012; Zhang *et al.*, 2005; Borazjani *et al.*, 2004). The reasons for these strain differences are not known in any great detail. It is known that

cell surface appendages such as flagella and pili aid in the adhesion of *P. aeruginosa* (Tran *et al.*, 2011) as does the relatively hydrophobic nature of some strains of *P. aeruginosa* compared to *S. aureus* (Bruinsma *et al.*, 2001). This greater ability to bind to both hydrogel and silicone hydrogel lens surfaces has been hypothesised (Borazjani *et al.*, 2004) to contribute to the finding that *P. aeruginosa* predominates as a causative agent in contact lens induced-MK. Although the difference was not significant, *P. aeruginosa* strain 6294 and *S. aureus* strain 31 showed slightly higher adhesion to both hydrogel and silicone hydrogel contact lenses compared to other strains within the respective genera/species. Given that both the bacterial strains have been widely used for antimicrobial contact lens studies (Cole *et al.*, 2010; Willcox *et al.*, 2010b; Willcox *et al.*, 2008a; Bandara *et al.*, 2004; Zhu *et al.*, 2002), both of these ocular isolates were selected as *P. aeruginosa* and *S. aureus* representative strains to be used in all subsequent studies in this thesis.

In the current study, numbers of viable adherent bacteria after 18 hr were higher than after 2 hr of adhesion, an observation that agrees with previous studies (Randler *et al.*, 2010; Szczotka-Flynn *et al.*, 2009c). With the use of laminar-flow optical chamber and phase contrast microscopy, Tran *et al*. (2011) showed linear kinetics of bacterial adhesion up to 70 min and Randler *et al*. (2010) using a three-phase *in vitro* system investigated adhesion up to 72 hr but determined incremental adhesion to lenses only until 24 hr. Combining results from the current study with those from Tran *et al*. (2011) and Randler *et al*. (2010), it is clear that adhesion to contact lenses increases in a time-dependent manner up to 18–24 hr of incubation. The observed reduction in recovery of viable bacteria after these times may be due to the bacteria entering a biofilm mode of growth. Bacteria in biofilms are known to show reduced viable recovery (Donlan *et al.*, 2002; Evans *et al.*, 1990) or biofilm dispersal may occur,

particularly when there is nutrient limitation. In contrast, Stapleton *et al.* (1993a) and Andrews *et al.* (2001) reported a plateau in adhesion after 45 min and 4 hr incubation respectively, with the adhesion remaining constant thereafter for more than 18 hr. These results may be associated with slightly higher inoculum ($1x10^7$ CFU ml^{-1} and $2x10^8$ CFU ml^{-1}) and varying bacterial count methods (viable plate count and ATP based bioluminescence assay) being used, respectively. This finding illustrates that there is a need to select the incubation period of a bacterial adhesion carefully, depending upon study methodology and the hypothesis being tested.

Bacterial incubation in the nutritionally rich TSB resulted in the highest levels of adhesion for both bacterial types. PBS, being nutritionally inert, may have resulted in death of the more fastidious *S. aureus* strains used in the current study. Consequently PBS is not recommended as an assay medium for *S. aureus*. A reduction in the number of viable *S. aureus* cells over an 18 hr period in PBS was found for both low and high innocula of $1x10^3$ CFU ml^{-1}, $1x10^6$ CFU ml^{-1} and $1x10^{10}$ CFU ml^{-1} with approximately 1, 3 and 5-log reductions observed respectively, lending support to the unsuitability of PBS for assessment of adhesion in future studies. This study identified diluted TSB as the preferred medium for comparison of *S. aureus* adhesion to contact lens surfaces. However, PBS is well-suited to the assessment of adhesion of *P. aeruginosa* to contact lens surfaces. The use of TSBG conferred no advantage over 1/10 TSB and the addition of glucose is not recommended.

In this study three different inoculum sizes of $1x10^3$, $1x10^6$ and $1x10^{10}$ CFU ml^{-1} were investigated. $1x10^{10}$ CFU ml^{-1} was associated with highest adhesion when incubated for 2 hr, however this not always the case for longer incubation. Previous studies have also used higher inoculum sizes when incubation times were short (Williams *et al.*, 1998; Stapleton *et al.*, 1993a; Miller *et al.*, 1988) and a lower inoculum

size when incubated for longer (Willcox *et al.*, 2010b; Bandara *et al.*, 2004). Contact lenses are rarely exposed to bacterial numbers as high as those used in this study ($1x10^{10}$ CFU ml^{-1}) either during contact lens wear or in lens cases. The range of bacterial numbers isolated from contact lens storage cases has been reported to be between $1.2x10^4$ CFU/case to $6.3x10^4$ CFU/case (Dantam *et al.*, 2012; Willcox *et al.*, 2010a; Pens *et al.*, 2008; Fleiszig *et al.*, 1992; Wilson *et al.*, 1990; Donzis *et al.*, 1987). Therefore, exposing contact lenses to $1x10^{10}$ CFU of bacteria may not reflect in use situations.

Inoculum size was the greatest factor influencing *P. aeruginosa* adhesion, followed by incubation period and assay medium. Interestingly, assay medium was the greatest influencing factor determining *S. aureus* adhesion, confirming that *S. aureus* is sensitive to the nutritional potential of the environment.

It is important to carefully select assay conditions appropriate for the purpose of the study. Adhesion of *P. aeruginosa* to contact lenses ranged from approximately $1x10^{-1}$ to $1x10^6$ CFU mm^{-2} and similarly, *S. aureus* adhesion ranged from approximately $1x10^{-1}$ to $1x10^5$ CFU mm^{-2}. For experiments designed to investigate whether antimicrobial lenses can reduce adhesion, it is important that the assay conditions be chosen that allow a medium-to-high adhesion to control lenses so that the inhibition of adhesion to test lenses can be measured.

Table 2.4 shows assay conditions that provided moderate to high adhesion of between $1x10^3$ CFU mm^{-2} to $1x10^5$ CFU mm^{-2} for both *P. aeruginosa* and *S. aureus*. These conditions will allow a sensitive assessment of the surfaces to be tested in these studies.

Table 2.4: List of assay conditions with estimated mean (95% CI) adhesion of $1x10^3$ CFU mm^{-2} to $1x0^5$ CFU mm^{-2} for *P. aeruginosa* and *S. aureus* for both etafilcon A and senofilcon A contact lenses.

Bacteria	Inoculum size (CFU ml^{-1})	Media	Incubation time (hr)	etafilcon A			senofilcon A		
				Marginal mean (CFU mm^{-2})	95% CI (CFU mm^{-2})		Marginal mean (CFU mm^{-2})	95% CI (CFU mm^{-2})	
					Lower bound	Upper bound		Lower bound	Upper bound
P. aeruginosa 6294	10^{10}	1/10 TSB	2	4.4	4.3	4.6	4.2	4.1	4.4
	10^6	1/10 TSB	18	4.3	4.2	4.5	4.3	4.1	4.4
	10^6	PBS	18	3.6	3.4	3.7	3.8	3.7	4.0
	10^3	1/10 TSB	18	4.7	4.5	4.8	4.0	3.9	4.2
P. aeruginosa ATCC 9027	10^{10}	TSBG	2	4.4	4.2	4.5	4.3	4.1	4.4
	10^{10}	PBS	2	4.3	4.1	4.4	3.8	3.6	3.9
	10^6	1/10 TSB	18	4.9	4.7	5.0	4.6	4.5	4.8
	10^6	PBS	18	3.1	3.0	3.3	3.5	3.3	3.6
	10^3	1/10 TSB	18	4.5	4.4	4.7	4.0	3.8	4.1
	10^3	TSBG	18	4.1	4.0	4.3	3.6	3.4	3.7
P. aeruginosa GSU-3	10^{10}	PBS	2	4.8	4.6	4.9	4.7	4.5	4.8
	10^6	1/10 TSB	2	3.7	3.6	3.9	3.6	3.4	3.7
	10^6	TSBG	2	3.9	3.8	4.1	3.7	3.5	3.8
	10^6	PBS	2	3.3	3.1	3.4	3.3	3.1	3.4
	10^6	PBS	18	3.1	2.9	3.2	3.3	3.2	3.5
	10^6	TSB	2	3.9	3.8	4.1	3.3	3.2	3.4
	10^6	1/10TSB	18	4.3	4.1	4.4	4.2	4.1	4.4
	10^3	TSBG	18	3.8	3.7	4.0	3.3	3.1	3.4

Bacteria	Inoculum size (CFU ml^{-1})	Media	Incubation time (hr)	etafilcon A			senofilcon A		
				Marginal mean (CFU mm^{-2})	95% CI (CFU mm^{-2})		Marginal mean (CFU mm^{-2})	95% CI (CFU mm^{-2})	
					Lower bound	Upper bound		Lower bound	Upper bound
S. aureus 31	10^{10}	TSBG	18	4.7	4.6	4.9	4.5	4.3	4.6
	10^{10}	1/10TSB	2	3.7	3.5	3.8	3.3	3.2	3.5
	10^{10}	TSBG	2	3.5	3.3	3.6	3.3	3.2	3.5
	10^{10}	TSB	2	4.2	4.1	4.4	3.5	3.4	3.7
	10^{10}	TSB	2	4.2	4.1	4.4	3.5	3.4	3.7
	10^{6}	1/10TSB	18	4.5	4.4	4.7	4.4	4.3	4.6
	10^{6}	TSB	18	4.6	4.4	4.7	4.8	4.6	4.9
	10^{3}	TSB	18	4.7	4.6	4.9	4.5	4.3	4.7
S. aureus 38	10^{10}	1/10TSB	18	4.3	4.2	4.5	4.2	4.1	4.4
	10^{10}	TSBG	18	4.3	4.2	4.5	4.2	4.1	4.4
	10^{10}	TSB	2	3.3	3.2	3.4	3.2	3.0	3.3
	10^{3}	1/10TSB	18	4.1	4.0	4.3	3.9	3.8	4.0
	10^{3}	TSB	18	4.4	4.3	4.6	4.1	4.0	4.3
	10^{6}	1/10TSB	18	4.2	4.1	4.4	4.4	4.3	4.5
	10^{3}	TSBG	18	4.0	3.8	4.1	3.6	3.5	3.8

For experimental purposes it is essential to have a common assay that could be used to test various hypotheses, and no single assay condition could provide $1x10^3$ CFU mm^{-2} to $1x10^5$ CFU mm^{-2} adhesion for all the five bacterial strains. Hence, Table 2.5 details a short-listed set of adhesion conditions from

Table 2.4 which provided $1x10^3$ CFU mm^{-2} to $1x10^5$ CFU mm^{-2} adhesion for either *P. aeruginosa* or *S. aureus.* Two sets of assay conditions provided the desired levels of adhesion for *P. aeruginosa* in Table 2.5 and the inoculum sizes that resulted in the desired adhesion levels are $1x10^3$ and $1x10^6$ CFU ml^{-1}. As discussed earlier, more than $1x10^4$ CFU bacteria were regularly recovered from contact lens cases (Dantam *et al.*, 2012; Willcox *et al.*, 2010a; Pens *et al.*, 2008), thus an experimental model with an inoculum size of 10^6 CFU ml^{-1} was preferred over 10^3 CFU ml^{-1}. In addition, it is hypothesised that antimicrobial contact lenses that provide a reduction in bacterial adhesion when 10^6 CFU ml^{-1} are used will also be effective against 10^3 CFU ml^{-1}. Among the five common sets of assay conditions for *S. aureus* in Table 2.5, three had an inoculum of $1x10^{10}$ CFU ml^{-1} which is extremely high. The assay conditions with a lower inoculum size of 10^3 CFU ml^{-1} was in TSB which is very high in nutrition and consequently unlikely to reflect conditions which occur in practise. For these reasons, 1/10 TSB with 18 hr incubation was chosen as an assay condition for *S. aureus*. The final bacterial strains and adhesion conditions which will be used for further evaluation of efficacy of antimicrobial contact lenses are detailed in Table 2.6.

Table 2.5: Shortlisted common assay conditions that provided estimated mean (95% CI) to $1x10^3$ CFU mm^{-2} to $1x10^5$ CFU mm^{-2} adhesion for either *P. aeruginosa* or *S. aureus* strains.

Bacteria		Inoculum size (CFU ml^{-1})	Media	Incubation time (hr)	etafilcon A			senofilcon A		
					Estimated mean (CFU mm^{-2})	95% Confidence interval (CFU mm^{-2})		Estimated mean (CFU mm^{-2})	95% Confidence interval (CFU mm^{-2})	
						Lower bound	Upper bound		Lower bound	Upper bound
P. aeruginosa strains	6294	10^3	1/10 TSB	18	4.7	4.5	4.8	4.0	3.9	4.2
	9027				4.5	4.4	4.7	4.0	3.8	4.1
	GSU3				4.3	4.1	4.4	4.2	4.1	4.4
	6294	10^6	PBS	18	3.6	3.4	3.7	3.8	3.7	4.0
	9027				3.1	3.0	3.3	3.5	3.3	3.6
	GSU3				3.1	2.9	3.2	3.3	3.2	3.5
S. aureus strains	31	10^3	TSB	18	4.7	4.6	4.9	4.5	4.3	4.7
	38				4.4	4.3	4.6	4.1	4.0	4.3
	31	10^6	1/10 TSB	18	4.5	4.4	4.7	4.4	4.3	4.6
	38				4.2	4.1	4.4	4.4	4.3	4.5
	31	10^{10}	1/10 TSB	18	4.7	4.6	4.9	4.6	4.4	4.7
	38				4.3	4.2	4.5	4.2	4.1	4.4
	31	10^{10}	G sol	18	4.7	4.6	4.9	4.5	4.3	4.6
	38				4.3	4.2	4.5	4.2	4.1	4.4
	31	10^{10}	TSB	2	4.2	4.1	4.4	3.5	3.4	3.7
	38				3.3	3.2	3.4	3.2	3.0	3.3

Table 2.6: Selected *P. aeruginosa* and *S. aureus* strains and assay conditions for further antimicrobial contact lens research.

Bacteria	Media	Inoculum size (CFU ml^{-1})	Incubation time (hr)
P. aeruginosa 6294	PBS	10^6	18
S. aureus 31	1/10 TSB	10^6	18

In summary, the results of this study indicate that bacterial adhesion to contact lenses is multifactorial and sensitive to changes in assay components. *P. aeruginosa* adheres to contact lenses in higher numbers than *S. aureus* and this may contribute to its predominance as the causative organism in contact lens-associated MK. *S. aureus* is a fastidious species and requires greater nutrition than *P. aeruginosa* in an experimental assay, especially when incubated for longer periods. An inoculum of 10^6 CFU ml^{-1} resulted in levels of adhesion that may enable greatest discrimination between test materials. A longer incubation time such as 18 hr provides higher bacterial adhesion. Selection of this time frame may be relevant to overnight contact lens wear or when lenses are in contact lens case, and provides information as to efficacy over clinically relevant time frames which may not be inferred from shorter incubation times. Careful selection of bacterial assay components is important. The results of this study indicate a 10^6 CFU ml^{-1} inoculum size with an 18 hr incubation period is most appropriate for *P. aeruginosa* and *S. aureus* with PBS and 1/10 TSB as media respectively for antimicrobial contact lens investigation. The conditions described above will be used further to evaluate the antimicrobial activity of peptide coated contact lenses.

Chapter 3: Antimicrobial Activity of Melimine and Cathelicidin Bound to Contact Lenses

This chapter has been presented as:

- Dutta D, Willcox MD (2013). Antimicrobial activity of melimine or cathelicidin bound to contact lenses. *Invest Ophthalmol Vis Sci, ARVO*. Seattle, USA. 54: E-Abstract 507.

3.1 INTRODUCTION

There is a need for development of antimicrobial contact lenses as contamination of contact lenses has been associated with contact lens related microbial adverse events. *Pseudomonas aeruginosa* (Lam *et al.*, 2002; Houang *et al.*, 2001; Cheng *et al.*, 1999) and *Staphylococcus aureus* (Green *et al.*, 2008b; Houang *et al.*, 2001; Alexandrakis *et al.*, 2000) are the two dominant microorganisms responsible for majority of the contact lens related adverse events. Antimicrobial peptides (AMPs) are part of the innate immune system of all multicellular organisms (Kolar *et al.*, 2011; McDermott, 2009; McDermott, 2004; Ganz, 2003), have a wide spectrum of antimicrobial activity (Hancock, 2001) and can act at very low concentrations compared to other antimicrobial agents (McDermott, 2009). These advantages make AMPs an attractive option for development of an antimicrobial contact lens. LL-37 is a cryptic peptide obtained by enzymatic cleavage of its precursor and is a human member of the family of AMPs called cathelicidins (Hancock *et al.*, 1998). LL-37 is expressed by corneal and conjunctival epithelial cells (Nizet *et al.*, 2003; Zanetti *et al.*, 1995). LL-37 has a wide spectrum of activity which is not influenced by the presence of tears (McDermott, 2009; Huang *et al.*, 2007). Melimine is a synthetic cationic peptide produced from naturally occurring AMPs which has also demonstrated promising antimicrobial activity against *P. aeruginosa*, *S. aureus* and *Streptococcus pneumoniae* (Rasul *et al.*, 2010; Willcox *et al.*, 2008b).

Antimicrobial efficacy can be retained when covalently attaching AMPs to different surfaces such as polymide resins, cellulose or glass coverslips (Costa *et al.*, 2011). Covalent immobilisation of cathelicidin LL-37 on titanium surfaces shows bactericidal activity against *E. coli* (Gabriel *et al.*, 2006). However, retention of antimicrobial

activity of these naturally occurring AMPs once bound onto surfaces such as contact lenses has not yet been investigated. Retention of antimicrobial activity against Gram negative and Gram positive bacteria by covalent immobilising of the synthetic peptide, melimine onto the contact lens surface has been demonstrated (Willcox *et al.*, 2008b). The aim of the study described here was to evaluate the threshold concentration of peptide attached to contact lens that provides more than 1.5 to 2 log inhibition of bacterial adhesion to contact lenses. It is hypothesised that to be effective, an antimicrobial lens should reduce bacteria adhesion to the lens surface by ≥ 1.5 log as it has been shown that current commercially available contact lenses such as lotrafilcon A and balafilcon A, can differ in adhesion of bacteria to their surface by up to 1 log (Vijay *et al.*, 2012; Borazjani *et al.*, 2004) and yet epidemiological studies have not demonstrated a difference in the rates of microbial keratitis between these lens types (Stapleton *et al.*, 2012; Dart *et al.*, 2008).

3.2 METHODS

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) and cathelicidin LL-37 (L L G D F F R K S K E K I G K E F K R I V Q R I K D F L R N L V P R T E S) were synthesised by conventional solid-phase peptide synthesis by the American Peptide Company (CA, USA). Peptides with >80% purity were used in all experiments.

3.2.1 Determination of Minimum Inhibitory Concentration (MIC)

MICs for both LL-37 and melimine were investigated following the modified protocol suggested by R.E. Hancock (Hancock, 1999), based on the classical microtitre broth dilution recommended by the National Committee of Laboratory Safety and Standards (NCLSS) (Amsterdam, 1996). The microorganisms used in this study are detailed in Table 3.1.

Table 3.1: Details of microorganisms used for evaluation of MIC

Bacteria	Source
P. aeruginosa ATCC 19660	Human septicaemia
P. aeruginosa 6294	Microbial keratitis
S. aureus ATCC 29213	Wound
S. aureus 31	Contact lens peripheral ulcer

In brief, bacteria were grown overnight in Mueller Hinton broth (MHB; Oxoid, Basingstoke, UK) and then washed three times in phosphate buffered saline (PBS). Serial dilutions of the peptides were made in 0.4% bovine serum albumin (Sigma-Aldrich Co., St Louis, USA) and 0.2% acetic acid (Ajax Finechem Pty Ltd, NSW,

Australia) in 96-well polypropylene microtitre plates (CELLSTAR®, Greiner bio-one, Frickenhausen, Germany) with MHB. The molecular weights of melimine and LL-37 are 3786.6 amu and 4493.3 amu respectively. Each well was inoculated with 2 – 7 x 10^5 colony forming units (CFU) ml^{-1} of the test organism to a final volume of 250 µl in MHB containing the test peptide at final concentrations ranging from 528.10 nmol ml^{-1} to 0.79 pmol ml^{-1} and 445.10 nmol ml^{-1} to 0.66 pmol ml^{-1} for melimine and LL-37 respectively. A well with microorganisms but without peptide acted as positive control and a well without both peptide and microorganisms acted as negative control. The 96 well plate was wrapped with Parafilm® (Sigma chemical co, S. Louis, USA) to prevent evaporation and incubated for 18 – 24 hr at 37ºC with shaking at 120 rpm. After incubation, cultures in the 96 well plates were log serial diluted, plated on nutrient agar (Oxoid, Basingstoke, UK) and incubated overnight. CFU for each dilution were enumerated to determine viable counts. MIC was taken as the lowest concentration of peptide that reduced growth by more than 50% of the positive control (MIC 50).

3.2.2 Production of AMP Coated Contact Lenses

Melimine and cathelicidin LL-37 were diluted in sterile PBS. One of the most widely used contact lens materials, etafilcon A (Efron *et al.*, 2011) (Johnson & Johnson Vision Care Inc., Jacksonville, USA; Base curve: 8.7 mm, Diameter: 14.0 mm, Power: -3.00 Ds), was used for this study. Contact lenses were removed from the manufacturer's vials, and washed three times in 1 ml PBS. Both the AMPs were covalently attached to lenses using a modification of a previously described method (Willcox *et al.*, 2008b). A schematic of the reaction steps is illustrated in Figure 3-1.

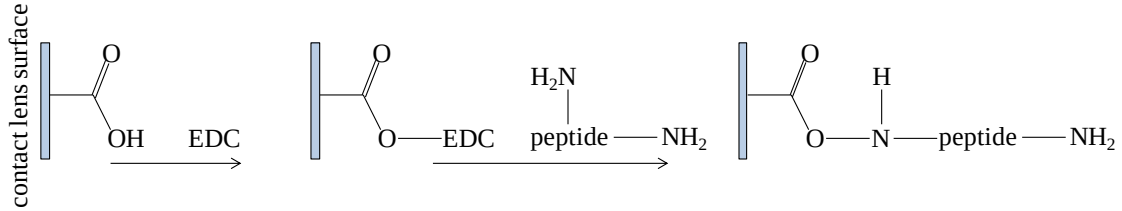

Figure 3-1: Attachment of peptide to contact lens surface via EDC coupling

Briefly, lenses were washed twice in 0.1 mol l^{-1} sodium acetate buffer pH 5.0 and soaked in 2 ml 0.1 mol l^{-1} sodium acetate buffer pH 5.0 containing 2 mg ml^{-1} 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for 15 min at 25ºC. Subsequently, lenses were washed three times in 2 ml PBS and then resuspended in 1 ml of 100 µg ml^{-1}, 500 µg ml^{-1}, 1 mg ml^{-1}, 3 mg ml^{-1} and 5 mg ml^{-1} of melimine or LL-37 in PBS and incubated for 2 hr at 37ºC with gentle shaking. The incubation range of 100 µg ml^{-1} to 5 mg ml^{-1} of peptide essentially indicate lenses were incubated with 1320.45 nmol ml^{-1} to 26.40 nmol ml^{-1} melimine or 1112.75 nmol ml^{-1} to 22.25 nmol ml^{-1} LL-37. This means the lenses were incubated with a common range of 1112.75 nmol ml^{-1} to 26.40 nmol ml^{-1} of both the peptides. Lenses were washed three times in 2 ml sterile PBS, and then resuspended in 2 ml of 10% w/v NaCl overnight followed by soaking in PBS for 2 hr to extract any dissolved peptide remaining within the lens matrix. All the lenses were sterilised by autoclaving at 121ºC for 10 min and stored in glass vials at 5ºC in sterile PBS until use. Unprocessed contact lenses acted as controls.

3.2.3 Quantification of Peptide Attachment

The amount of peptide present on the lens was quantified using amino acid analysis (AAA) which is detailed elsewhere (Kaspar *et al.*, 2009). Briefly, lenses were washed in Milli-Q® (Millipore Corp. Billerica, USA) water, and then underwent 24 hr gas phase hydrolysis in 6M HCl (Ajax Finechem Pty Ltd, NSW, Australia) at 110ºC. Lenses were then dried and amino acids extracted in 20% acetonitrile in trifluoroacetic

acid (Themo Fisher Scientific, Rockford, USA). Amino acids in the hydrolysates were analysed using the AccQ-Tag Ultra chemistry kit (Waters Corp., Milford, USA). As the amino acids asparagine and glutamine are hydrolysed to the amino acids aspartic acid and glutamic acid respectively, the amount of these acids was regarded as the sum of the respective original type of amino acid. The sum of all the amino acids derived from each contact lens was regarded as total amount of peptide attachment to a contact lens. This test was done in triplicate.

3.2.4 Strains and Adhesion Conditions

The bacterial adhesion conditions have been described previously (Table 2.6). Briefly, PBS and 10x diluted tryptone soya broth (1/10 TSB; Oxoid, Basingstoke, UK) were used as assay media for evaluation of antimicrobial efficacy of peptide coated contact lenses against *P. aeruginosa* 6294 and *S. aureus* 31 respectively. Peptide coated and uncoated contact lenses were incubated with 1 ml bacterial suspension of 10^6 CFU ml^{-1} at 37ºC for 18 hr.

Contact lenses were washed 3 times with PBS to remove non-adherent cells and then stirred rapidly in 2 ml of PBS containing a small magnetic stirring bar. Following log serial dilutions in Dey Engley neutralising broth (DE; Becton, Dickson and Company, USA), 3 x 50 µl of each dilution were plated on a tryptone soya agar (TSA; Oxoid, Basingstoke, UK) containing Tween 80 (0.5%; Sigma-Aldrich, St Louis, USA) and lecithin (0.07%; Sigma-Aldrich, St Louis, USA) for recovery of cells. After 24 hr incubation at 37ºC, the viable microorganisms were enumerated as colony forming units (CFU) mm^{-2} of the lens surface. Results are expressed as the reduction in adherent viable bacteria compared to the uncoated control lens of triplicate measurements performed on a minimum of three separate occasions.

3.2.5 Data Analysis

The adhesion data were $\log_{10}$ (x+1) transformed prior to data analysis where x is the adherent bacteria in CFU mm^{-2}. All data were analysed using Statistical Package for Social Science for Windows version 21.0 (SPSS, Inc, Chicago, IL). Differences in bacterial adhesion were analysed using independent 2-sample *t*-test. Statistical significance was set at 5%.

3.3 RESULTS

3.3.1 MIC of Peptides

MIC of LL-37 and melimine against *P. aeruginosa* and *S. aureus* strains is detailed in Table 3.2. LL-37 showed more than 4.7 fold and 73.3 fold lower MIC ($p < 0.05$) than melimine against *P. aeruginosa* and *S. aureus* respectively. This suggests greater relative antibacterial activity compared to melimine for all strains except *S. aureus* 31.

Table 3.2: Minimal Inhibitory Concentration (MIC) of LL-37 and melimine

Bacteria	Minimal inhibitory concentration	
	LL-37	Melimine
	nmol ml^{-1} (μg ml^{-1})	nmol ml^{-1} (μg ml^{-1})
P. aeruginosa ATCC 19660	0.9 (3.9)	132.0 (500)
P. aeruginosa 6294	13.8 (62)	66.0 (250)
S. aureus ATCC 29213	0.9 (3.9)	66.0 (250)
S. aureus 31	55.6 (250)	33.01 (125)

3.3.2 Quantification of Covalently Attached Peptide

Figure 3-2 shows that higher amounts of melimine were able to bind to contact lenses compared to LL-37. The amount of melimine bound to the contact lenses from the initial starting concentrations of 1 mg ml^{-1}, 3 mg ml^{-1} or 5 mg ml^{-1} melimine were 109 ± 4 μg, 152 ± 43 μg and 168 ± 1 μg respectively. The final amount of melimine bound to a lens was not significantly different ($p > 0.05$) between the concentrations of 3 mg ml^{-1} and 5 mg ml^{-1}. EDC covalent coupling did not bind more than 19 μg or 4.2 nmol of LL-37 to contact lenses even when reacted with as high as 5 mg ml^{-1} LL-37. In

comparison, using a starting concentration of only 0.1 mg melimine resulted 9.8 nmol melimine attachment (Figure 3-2).

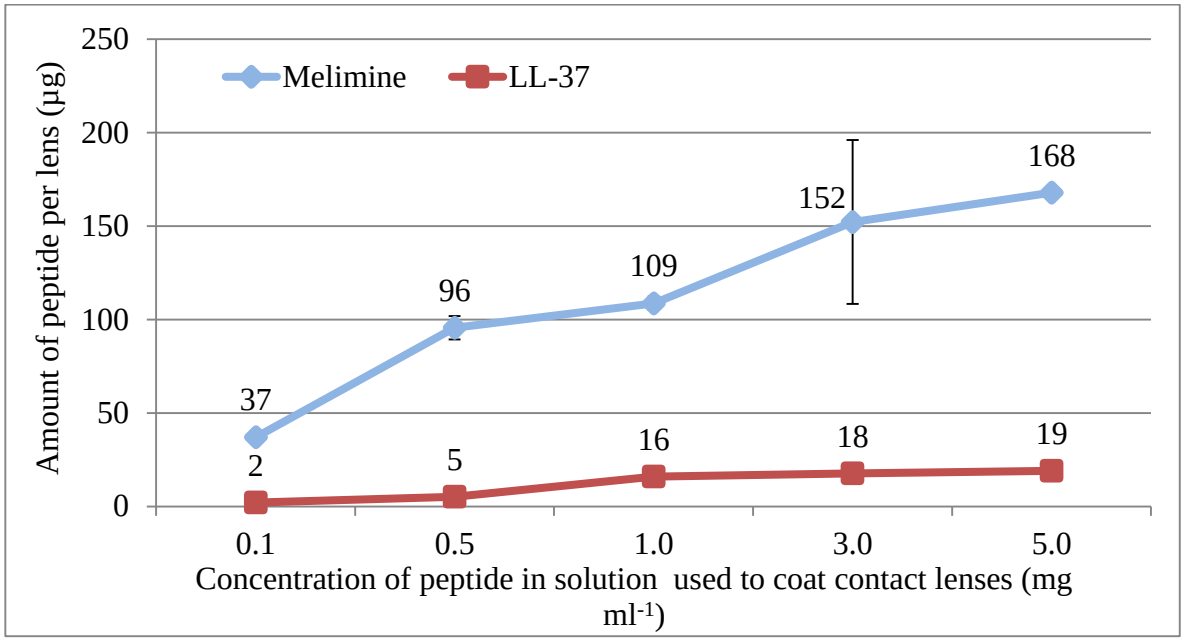

Figure 3-2: Quantification of melimine and LL-37 attached to contact lenses.

3.3.3 Antibacterial Efficacy of Peptide Attached Lenses

Figure 3-3 shows the level of bacterial inhibition by either LL-37 or melimine-coated contact lenses compared with control lenses. Bound LL-37 showed no antimicrobial activity against *S. aureus* 31, but did show 3.2 – 3.3 log reduction with *P. aeruginosa* 6294 when 18 – 19 µg (3.9 – 4.2 nmol) lens^{-1} were attached to lenses. Increasing concentrations of melimine bound to contact lenses resulted in higher log inhibition of both *P. aeruginosa* 6294 and *S. aureus* 31. LL-37 was more active against *P. aeruginosa* on a molecule/molecule basis than melimine (i.e. 3.9 nmol of LL-37 gave approximately the same level of antimicrobial activity as 40.1 nmol of melimine), but was not active against *S. aureus*.

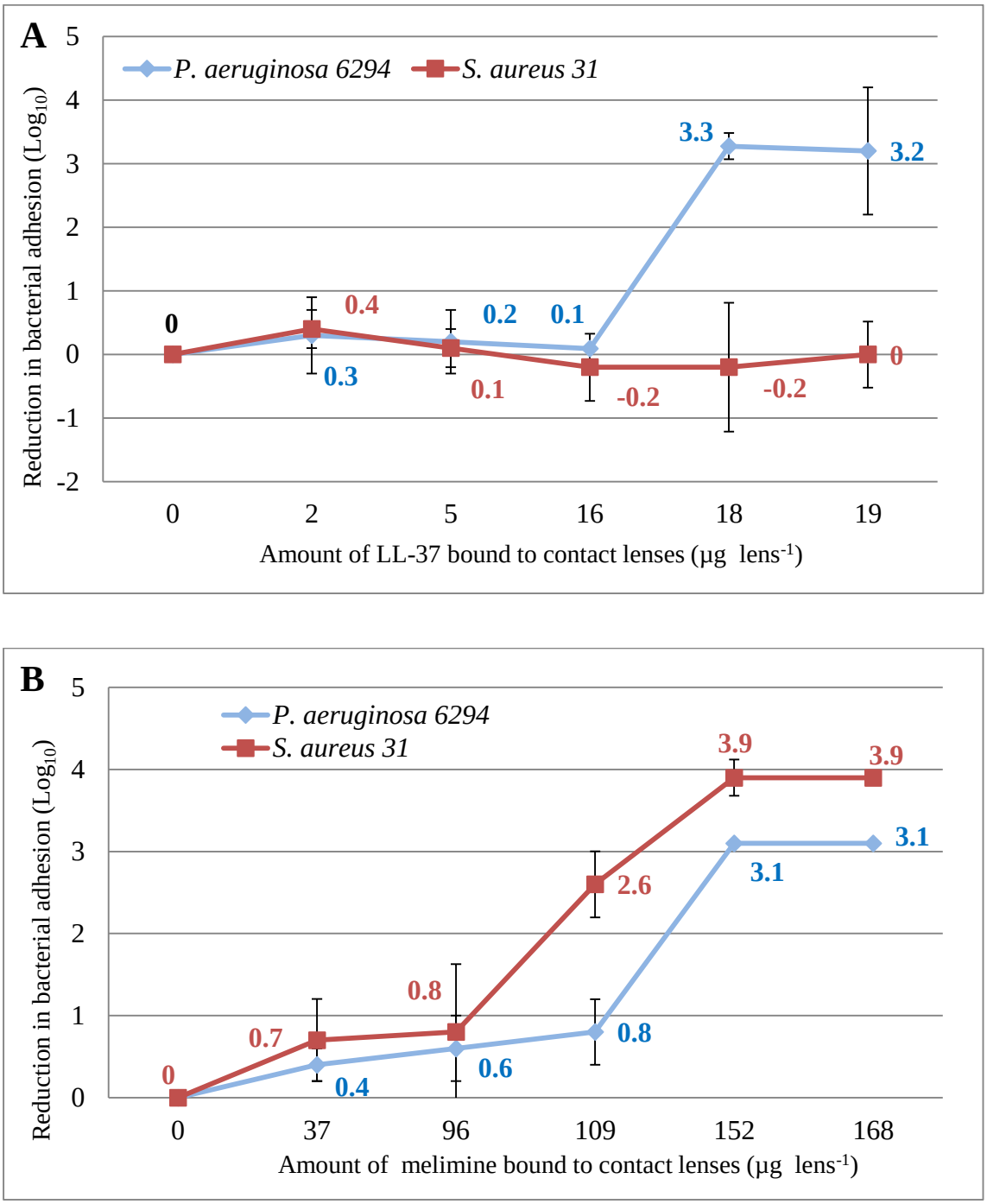

Figure 3-3: Antimicrobial activity of covalently bound LL-37 (A) or melimine (B) contact lenses against *P. aeruginosa* 6294 and *S. aureus* 31

(n ≥ 9 lenses per peptide concentration).

3.4 DISCUSSION

Results of this study indicate that both LL-37 and melimine have antimicrobial activity in free form against *P. aeruginosa* and *S. aureus*, however the efficacy varies against different strains of bacteria. EDC covalent coupling resulted in a high concentration of melimine bound to contact lenses, but this was not the case with LL-37. Increasing concentrations of melimine bound to contact lenses resulted in high inhibition of adhesion of both bacterial strains.

Results from this study are consistent with previously published data on the antimicrobial activity of LL-37 in solution against *P. aeruginosa* (<10 to 16 μg ml^{-1}) (Huang *et al.*, 2007; Turner *et al.*, 1998) and *S. aureus* (<10 to 50 μg ml^{-1}) (Durr *et al.*, 2006; Turner *et al.*, 1998). However, MIC of LL-37 in the current study was much higher against *S. aureus* 31 compared to *S. aureus* ATCC 29213. The strain differences which have lead to this difference in efficacy remain to be investigated, but are of interest as *S. aureus* 31 was isolated from a case of contact lens-induced peripheral ulcer and an understanding of the factors which make it more resistant may be of particular pertinence to the development of contact lens coatings if these characteristics are more generally found in ocular isolates. Overall, melimine showed lower antimicrobial activity (higher MIC; typically between 66 to 132 nmol ml^{-1}) in solution compared to LL-37 (0.9 – 55.6 nmol ml^{-1}). Melimine is rich in arginine and its efficacy probably relies only on membrane permeabilisation (Rasul *et al.*, 2010). Whereas, the antibacterial activity of LL-37 has been attributed to multiple modes of action in its free state including 'toroidal-pore' (McDermott, 2009; Durr *et al.*, 2006; Henzler Wildman *et al.*, 2003), 'carpet-model' (Durr *et al.*, 2006; Oren *et al.*, 1999), 'membrane thinning

and thickening' (Nguyen *et al.*, 2011) and 'charged lipid clustering' (Nguyen *et al.*, 2011).

There was major disparity between the amount of LL-37 and melimine that could be attached to contact lenses. Incubation with 792.2 nmol (3 mg) ml^{-1} melimine resulted in 40.1 nmol (152 µg) $lens^{-1}$ melimine being attached, whereas incubation with 1112.7 nmol (5 mg) ml^{-1} LL-37 resulted in only 4.2 nmol (19 µg) $lens^{-1}$ attached to lenses. This study used covalent attachment via EDC coupling which allows binding of any cationic amine group of a peptide with carboxyl groups of methacrylic acid present in the contact lens. These amine groups are present only in arginine and lysine amino acids in the sequence of melimine and LL-37. The lower concentration of LL-37 bound to lenses maybe due to the lower numbers of arginine and lysine present in LL-37 (11 Arginine and Lysine) compared to melimine (16 Arginine and Lysine). LL-37 also has 3 glutamic acid and 2 aspartic acid residues that bear 5 negative charges, resulting a net charge at physiological pH of +6, whereas melimine does not have any negatively charged amino acids producing net charge of +16. Unlike the random-EDC attachment method used in this study, Gabriel *et al.* (2006) used selective N-terminal coupling of Cys-LL-37 which resulted in reduced adhesion of *Escherichia coli* at the titanium surface. In addition, Kolar *et al.* (2010) and McDermott *et al.* (2010) used Cu-catalysed alkyl-azide cycloaddition ("click" reaction) to bind LL-25 (a truncated version of LL-37) and found surface-attached LL-25 significantly reduced *P. aeruginosa* adhesion. These results imply that refinement of the attachment technique and redesigning the peptide are required to increase concentration and efficiency of surface bound LL-37.

In an organic aqueous environment that potentially mimics the bacterial membrane environment (Hancock *et al.*, 1998), melimine alters its chain conformation

towards adopting a classical right-handed helical fold (Rasul *et al.*, 2010). It can form up to 40% helical conformation and the rest remains in a random coil structure (Rasul *et al.*, 2010). On the other hand, LL-37 forms a near to perfect helix with a 70 – 80% α-helical secondary structure (Durr *et al.*, 2006) under similar conditions. This appears to be the reason for the higher antibacterial efficacy showed by LL-37 in solution (lower MIC). However this may not be the case following EDC covalent attachment of the peptides. The lack of activity of LL-37 bound to contact lenses against *S. aureus* 31 may be due to the reduction in its α-helical structure, and also perhaps due to the lower level of peptide attachment by EDC coupling. LL-37 binds well to lipopolysaccharides (LPS) (Ciornei *et al.*, 2005), a component of the Gram negative bacteria cells wall. It is possible that activity against *P. aeruginosa* was mediated via LPS binding at low levels while surface attached LL-37 did not reach the bactericidal concentration needed for activity against *S. aureus*. Higher antimicrobial activity was associated with melimine-coated lenses, probably due to melimine's higher concentration after attachment.

The antibacterial mode of action of the immobilised peptides is believed to be largely dependent on 'charged lipid clustering' (Nguyen *et al.*, 2011; Banerjee *et al.*, 2011). The activity demonstrated by attached melimine satisfied the criteria discussed in Chapter 1, i.e. bound melimine showed more than 1.5 to 2 log inhibition in bacterial adhesion *in vitro*. Thus, melimine-coated contact lenses may have the potential to reduce contact lens related microbial adverse events. This study demonstrated the minimal starting concentration (3 mg ml^{-1}) in solution of which there was no further significant increase in antimicrobial efficacy over the range of concentrations tested against *P. aeruginosa* 6294 and *S. aureus* 31 (3.1 ± 0.1 and 3.9 ± 0.2 log inhibition respectively). Therefore, for all subsequent experiments, melimine-coated lenses were produced by incubating in 3 mg ml^{-1}.

In summary, biocidal mechanisms of various AMPs vary with their innate structures and therefore AMP-specific surface attachment techniques may be necessary for optimal outcomes. This study was able to optimise and demonstrate very high antimicrobial activity against Gram negative and Gram positive bacteria by attaching melimine onto the contact lens surface by EDC covalent coupling offering excellent potential for development as an antimicrobial coating for contact lenses.

Chapter 4: Broad Spectrum Antimicrobial Activity of Melimine Covalently Bound to Contact Lenses

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- Dutta D, Cole N, Willcox MD (2012) Broad spectrum antimicrobial efficacy of the cationic peptide melimine. Molecular Microbiology Meeting, Sydney, Australia.

4.1 INTRODUCTION

A variety of microorganisms have been implicated in contact lens-related microbial keratitis (MK), such as *Pseudomonas aeruginosa, Staphylococcus aureus,* coagulase-negative staphylococci, *Serratia marcescens, Escherichia coli, Acanthamoeba castellanii, Fusarium solani* and *Candida albicans* (Haas *et al.*, 2011; Tu *et al.*, 2010; Green *et al.*, 2008b). Contact lens related acute red eye (CLARE), contact lens peripheral ulcer (CLPU) and infiltrative keratitis (IK) are also associated with microbial (mainly Gram-negative bacteria or *S. aureus*) colonisation of contact lenses (Wu *et al.*, 2003; Sankaridurg *et al.*, 2000; Holden *et al.*, 1996). Thus, there is a need to develop an antimicrobial contact lens that has broad spectrum activity.

Antibiotic resistance among ocular pathogens has been increasing in parallel with the increase observed in systemic bacterial infections (Sharma, 2011). A significant proportion of ocular infections caused by *S. aureus* and *P. aeruginosa* have been associated with antibiotic resistant strains (Haas *et al.*, 2011; Willcox, 2011). Rates of resistance to ciprofloxacin, a common first line monotherapy for MK (Willcox, 2012), in ocular isolates of *S. aureus* from cases of MK treated in Florida increased from 3–8% in the early 1990s to 27–40% in 2000–2001 (Marangon *et al.*, 2004). This was largely due to the more frequent isolation of methicillin-resistant *S. aureus* (MRSA), which had rates of resistance to ciprofloxacin of 30–97% in the same time period (Marangon *et al.*, 2004). Whilst resistance to ciprofloxacin in *P. aeruginosa* isolates has remained relatively low in Australasia and USA, rates of resistance of 19–23% have been reported in India (Bharathi *et al.*, 2010; Kunimoto *et al.*, 1999; Garg *et al.*, 1999) and China (Zhang *et al.*, 2008). MK associated with drug-resistant bacteria can increase morbidity, treatment cost and result in a poor prognosis (French, 2005).

Furthermore, biofilm formation by clinical bacterial isolates from contact lenses has been reported to increase resistance to several contact lens disinfecting solutions (Szczotka-Flynn *et al.*, 2009c) and decrease the ability of polymorphonuclear leukocytes to phagocytose the bacteria (Hume *et al.*, 2003).

Outbreaks of fungal and *Acanthamoeba* keratitis associated with specific multipurpose contact lens disinfecting solutions have highlighted these microorganisms as causative agents of disease during contact lens wear (Keay *et al.*, 2011; Verani *et al.*, 2009; Ahearn *et al.*, 2008; Patel *et al.*, 2008; Iyer *et al.*, 2006). Although the rate of contact lens related *Fusarium* keratitis slowly decreased after withdrawal of the implicated solutions (Tu *et al.*, 2010), the overall incidence of *Acanthamoeba* keratitis remained higher than prior to the epidemic even after the removal of the solution from world-wide sale (Yoder *et al.*, 2012; Tu *et al.*, 2010). The incidence of fungal or amoebal keratitis during lens wear still remains much lower than for bacterial keratitis (Green *et al.*, 2008b; Keay *et al.*, 2006b) but these non-bacterial infections continue to be difficult to diagnose and treat (Otri *et al.*, 2012; Thomas *et al.*, 2007).

The broad range of microorganisms responsible for contact lens related microbial adverse events led to the exploration of the spectrum of antimicrobial activity of melimine-coated contact lenses. In addition, melimine-coated lenses were screened for their safety to mammalian cells. The aims of the study were to evaluate melimine-coated lenses for activity against fungi, *Acanthamoeba* and multi-drug resistant *S. aureus* and *P. aeruginosa,* and to confirm that melimine-coated lenses were non-toxic to mammalian cells. The hypotheses that were tested were that melimine-coated contact lenses have high antimicrobial activity against fungi, *Acanthamoeba* and multi-drug

resistant *S. aureus* and *P. aeruginosa*, and that the melimine lens coating is non-toxic to mammalian cells.

4.2 METHODS

Melimine was synthesised by conventional solid-phase peptide synthesis protocols and was obtained from American Peptide Company (CA, USA; > 80% purity). The procedure used for covalent attachment of melimine to etafilcon A (Johnson & Johnson Vision Care Inc., Jacksonville, FL, USA; Base curve: 8.7mm, Diameter: 14.0 mm, Power: -3.00 Diopter) contact lenses has been described previously in section 3.2.2. However, in the current study contact lenses were only incubated with 3 mg ml^{-1} of melimine in phosphate buffered saline (PBS) which resulted in 152 ± 43 µg $lens^{-1}$ covalent attachment of melimine. Lenses that were reacted only with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (no melimine added) served as process controls. A separate batch of lenses prepared by soaking in melimine (also 3 mg ml^{-1} in PBS) solution for 2 hr without EDC-mediated covalent coupling was used to determine effectiveness of the attachment. All the lenses were stored in glass vials at 5ºC in sterile PBS prior to use.

4.2.1 Strains and Adhesion Conditions

All microorganisms used in this study and their sources are listed in Table 4.1. Antimicrobial activity was tested against the drug resistant strains of *P. aeruginosa* (Conibear, 2006), *S. aureus* (Schubert, 2008), ISO panel micro-organisms (ISO 14729, 2001) and *Acanthamoeba* as well as *P. aeruginosa* 6294 and *S. aureus* 31 as positive controls.

Table 4.1: Details of microorganisms

Bacterial strains	Isolation site	Resistant to*
P. aeruginosa 6294	MK	Not determined (ND)
S. aureus 31	CLPU – contact lens	ND
ISO panel organisms		
P. aeruginosa ATCC 9027	Otic infection	ND
S. aureus ATCC 6538	Human Isolate	ND
S. marcescens ATCC 13880	Pond water	ND
C. albicans ATCC 10231	Bronchomycosis	ND
F. solani ATCC 36031	MK	ND
Acanthamoeba		
A. castellanii ATCC 50370	Eye infection	ND
Drug resistant and strong biofilm producer bacterial strains[a, b]		
P. aeruginosa 31	MK	GEN, TOB, PRL, NOR, OFX, MXF and CIP
P. aeruginosa 34	MK	GEN, TOB, TIC, PRL, NET, OFX and MXF
P. aeruginosa 35	MK	GEN, TOB, NOR, OFX, MXF and CIP
P. aeruginosa 37	MK	PRL, GEN, TOB, NOR, OFX, MXF and CIP
P. aeruginosa 142	MK	Strong biofilm producer
S. aureus 60	Hospital strain	PCN, MET, TET, GEN, ERY and CIP
S. aureus 61	MK	PCN, MET, TET, GEN, ERY and CIP
S. aureus 62	MK	PCN, MET, TET, GEN and ERY
S. aureus 110	MK	MET, TOB, ERY and CIP
S. aureus 103	Conjunctivitis	MET, TOB, ERY and CIP

**CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; MET, methicillin; MXF, moxifloxacin; NET, netilmicin; NOR, norfloxacin; OFX, ofloxacin; PCN, penicillin; PRL, piperacillin; TET, tetracycline; TIC, ticarcillin; TOB, tobramycin*

a = Schubert et al. (2008); b = Willcox et al. (2004)

Bacteria were grown overnight in tryptone soya broth (TSB) and then washed three times in PBS. *S. aureus* strains were re-suspended in 1/10 (10%) TSB, and *S. marcescens* and *P. aeruginosa* strains were re-suspended in PBS to an OD_{660nm} of 0.1 (1.0×10^8 colony forming unit (CFU) ml^{-1}). The bacterial cell suspensions were then diluted (1/10) to 1.0×10^6 CFU ml^{-1} for adhesion assays. Fungal strains were grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) plates by incubating for 7–10 days at 25ºC for *F. solani* and for 24 hr at 37ºC for *C. albicans*. Both fungal strains were then suspended in sterile PBS and filtered through sterile 70 µm and 40 µm filters to remove hyphal fragments and finally re-suspended to an OD_{660nm} of 2.6 and 1.5 (1.0×10^8 CFU ml^{-1}) respectively. Fungal suspensions were then diluted in PBS to 1.0×10^6 CFU ml^{-1} for adhesion assays.

A. castellanii ATCC 50370 was used in this study. Cryo-preserved *Acanthamoeba* cysts were inoculated into 25 ml of peptone yeast extract glucose broth (PYG; 20 g l^{-1} proteose peptone, 2 g l^{-1} yeast extract, 0.48 g l^{-1} $MgSO_4$, 59 mg l^{-1} $CaCl_2$, 1 g l^{-1} sodium citrate.$2H_2O$, 20 mg l^{-1} $Fe(NH_4)_2$ $(SO_4).6H_2O$, 0.34 g l^{-1} KH_2PO_4, 188 mg l^{-1} Na_2HPO_4, 18 g l^{-1} glucose) and incubated at 32ºC for 7–10 days to obtain motile trophozoites. A sterile cell scraper was used to gently detach the trophozoites adhered to the base of the flask. Aliquots of this culture were added to flasks containing fresh PYG and incubated for a further 3–4 days to obtain more trophozoites which were collected by centrifuging for 12 min at 1,000 rpm and re-suspended in Page's saline (0.12 g l^{-1} NaCl, 4 mg l^{-1} $MgSO_4$. $7H_2O$, 4 mg l^{-1} $CaCl_2$. $2H_2O$, 142 mg l^{-1} Na_2HPO_4, 136 mg l^{-1} KH_2PO_4). The cells were counted using a Neubauer haemocytometer and the final inoculum adjusted using Page's saline to approximately $1.0 - 1.5 \times 10^5$ cells ml^{-1}.

Peptide-coated and un-coated control lenses were washed in PBS and transferred to 1 ml of bacterial, fungal or acanthamoebal suspensions in the wells of 24- well tissue

culture plates (CELLSTAR®, Greiner bio-one, Frickenhausen, Germany). To allow adhesion of microbial cells, lenses were incubated 18 hr at 37ºC for bacteria, 18 hr at 25ºC for fungi and 6 hr at 25°C for amoeba with shaking (120 rpm).

Contact lenses were washed three times with PBS to remove non-adherent cells and then stirred rapidly in 2 ml of PBS containing a small magnetic stirring bar. This PBS was serially diluted (1/10) in Dey Engley (DE) neutralising broth (Becton, Dickson and Company, USA). For bacteria, 3 x 50 µl of each dilution was transferred to tryptone soya agar (TSA; Oxoid, Basingstoke, UK) containing Tween 80 and lecithin for recovery of cells. For fungal strains, each dilution was transferred to 100 µl PDA for recovery of viable cells. For *Acanthamoeba,* 4 x 100 µl of each dilution was transferred to non-nutrient agar (NNA; Oxoid, Basingstoke, UK) plates pre-incubated with *Escherichia coli* and incubated at 32ºC for up to 2 weeks. The plates were inverted and examined under a microscope on day 7 for tracks or excystment indicating viability, and survivor numbers were determined using Reed and Muench computation (Buck *et al.*, 1996). After 24 hr incubation at 37ºC for bacteria or 2 days incubation at 37°C for *C. albicans* and 4 days incubation at 25°C for *F. solani*, the viable microorganisms were enumerated as CFU mm^{-2}. Results are expressed as the reduction in adherent viable bacteria, fungi or *Acanthamoeba* on melimine coated lenses compared to uncoated control lenses. Measurements were performed in triplicate on a minimum of three separate occasions.

4.2.2 Effect on Total Bacterial Adhesion

To determine the effect of melimine-coated lenses on the total number (viable + nonviable cells) of bacterial adhesion to lenses, *P. aeruginosa* 6294 and *S. aureus* 31 were used. Bacteria were grown overnight with 20 µl of 3H-uridine (PerkinElmer®,

Glen Waverley, Australia) in TSB. Lenses were incubated with bacteria for 18 h, washed with PBS to remove non-adherent cells, and then lenses were vortexed vigorously using a small magnetic stirrer. Subsequently, 0.5 ml of the vortexed bottle contents were added with 4.5 ml Opti-Fluor scintillation cocktail (Packard Instrument Co., Downers Grove, USA) in 6 ml Pony H-I vials (PerkinElmer®, Turku, Finland). The vials were vortexed and then placed in a β scintillation counter (Wallac 1400 DSA; PerkinElmer®, Turku, Finland) to estimate radioactivity associated with the lenses. The disintegrations per min (dpm) from each lens were converted to number of cells mm^{-2} of lens surface area based on a standard calibration curve. Standard calibration curve was plotted for each experiment from radioactivity measurement of known counts of radio-labelled bacteria. Triplicates of each lens types were included in each adhesion experiment and the experiment was repeated for minimum of three times for both the bacteria to collect the data from a minimum of nine lenses for each group.

In addition, contact lenses with adherent *P. aeruginosa* 6294 and *S. aureus* 31 were stained with LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Inc, Eugene, OR) following the manufacturer's guidelines and described in Chen *et al.* (2009). Microscopic observation and image acquisition was performed with a Leitz diaplan fluorescent microscope and an Olympus FV1000 confocal inverted microscope. Images obtained from 8 representative areas on each of triplicate samples for each surface were analysed using Image J software (Rasband 1997–2008). This experiment was repeated three times and the image analysis results were measured as the average area of live cells and the average area of dead cells per field of view and are reported as the average percentage coverage of the fields of view.

4.2.3 Detection of Endotoxin

Melimine coated and un-coated contact lenses were incubated overnight with *P. aeruginosa* 6294 following the method described in section 3.2.4. Following incubation, lenses were washed to remove loosely bound bacteria and stirred rapidly in 2 ml pyrogen-free cell culture grade water (PromoCell GMBH, Heidelberg, Germany). Hence, the solution was diluted 100 times in pyrogen-free container and endotoxin associated with both the melimine-coated and uncoated contact lenses were determined by Limulus amoebocyte lysate (LAL) assay (Lonza, Walkersville, MD, USA). In brief, 50 µl LAL reagent was added to 50 µl of 100x diluted samples in a 96-well endotoxin-free plate (CELLSTAR®, Greiner bio-one, Frickenhausen, Germany) followed by 100 µl pre-warmed (37ºC ± 1ºC) chromogenic substrate after 10 min and 100 µl stop reagent (Acetic acid, 25% v/v glacial acetic acid; Ajax Finechem Pty Ltd, NSW, Australia) after 16 min. During this time the side of 96-well plate was gently tapped to facilitate mixing. Endotoxin units (EU) $lens^{-1}$ were calculated after measuring the absorbance by SpectrafluorPlus microplate reader (Tecan Group Ltd, Männedorf, Switzerland) at 405 – 410 nm and converting the absorbance into EU by reference to a standard curve. Standard curves were plotted for each experiment from four standard endotoxin solutions that were prepared from standard *Escherichia coli* endotoxin of known potency and following manufacturer's guidelines. EU $lens^{-1}$ was calculated and measurements were performed in triplicate.

4.2.4 Cytotoxicity

In vitro cytotoxicity of the melimine-coated contact lenses were determined using a direct contact method as outlined in ISO 10993–5 (2009). Briefly, murine L929 cells were grown in plastic petri dishes to near confluence and melimine-coated lenses

or non-coated controls were placed directly on the cell monolayer and incubated for 24 hr with fresh medium. After this incubation, the cytotoxicity was assessed using bright field and phase contrast microscopy after staining with trypan blue. Cytotoxic responses, i.e. zone of extent of cell damage were graded on a scale of 0 to 4 (ISO 10993-5, 2009). Additional controls used were silastic medical grade tubing (Dow Corning Corporation, MI USA) as a negative control and samples of surgical latex gloves (Ansell Medical Victoria, Australia) as positive control. Grades of above 1 are suggestive of cytotoxic responses under the conditions specified. Three melimine-coated and three uncoated contact lenses were used for this test.

4.2.5 Data Analysis

Data were analysed using Excel® (Office; Microsoft®, Redmond, USA) and Statistical Package for the Social Sciences software (SPSS) for Windows software version 21.0 (SPSS, Inc., Chicago, IL, USA). The bacterial adhesion data were $\log_{10}$ (x+1) transformed prior to data analysis where x is the adherent bacteria or fungi in CFU mm^{-2} or amoeba in track forming units mm^{-2}. Microbial adhesion and lens-associated endotoxin were analysed using independent 2-sample *t*-test. Prior to comparing the fluorescence microscopy images, equality of variances was tested using Levene's test. Unequal variances were adjusted by transforming the data using square root transformation. Differences between the groups were analysed using linear mixed model ANOVA, which adjusts the correlation due to repeated observations. Post hoc multiple comparisons were done using Bonferroni correction. Statistical significance was set at 5%.

4.3 RESULTS

Figure 4-1 A and B show total and viable adhesion of *P. aeruginosa* 6294 and *S. aureus* 31 to melimine-coated and control lenses. Melimine-coated lenses gave 3.0 ± 0.4 log and 2.7 ± 0.2 log inhibition in viable adhesion against the two bacteria respectively. Compared to viable adhesion, there was substantially higher total adhesion by the two bacteria to melimine-coated lenses. This reflects in the larger differences between total and viable count for melimine-coated lenses than control lenses. Melimine-coated lenses demonstrated a small inhibition in total adhesion for *P. aeruginosa* 6294 and *S. aureus* 31 (0.4 log and 0.1 log inhibition respectively). Even so, the total adhesion to melimine-coated lenses was statistically significantly lower than control lenses ($p < 0.05$ for *P. aeruginosa* and $p = 0.01$ for *S. aureus* 31). The total adhesion by the two bacteria to control lenses was higher than viable adhesion.

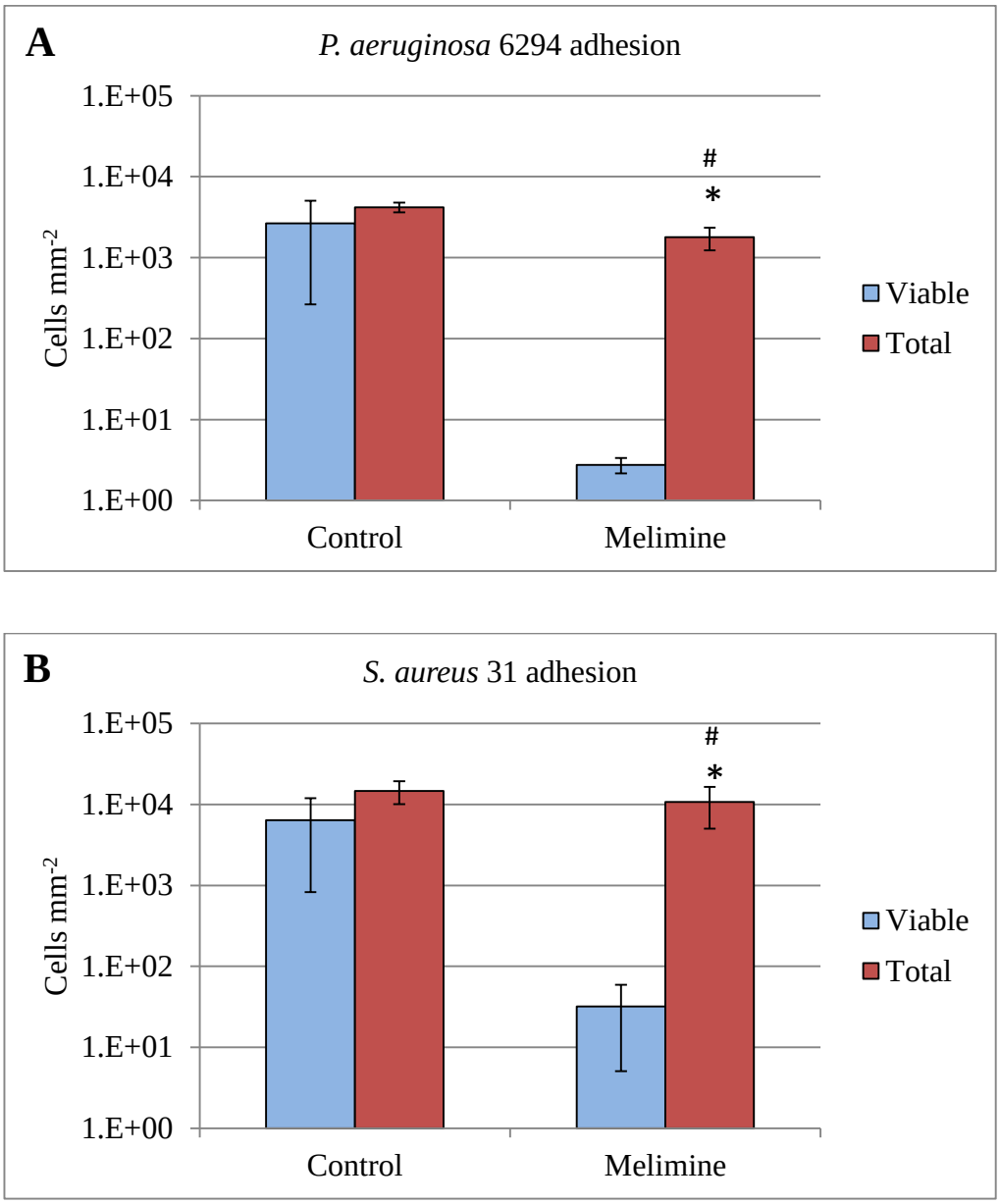

Figure 4-1: Total and viable *P. aeruginosa* 6294 (A) and *S. aureus* 31 (B) adhesion and to melimine-coated and control contact lenses.

Asterisk '' represent significantly higher total bacterial adhesion than viable bacterial adhesion to melimine-coated lenses and '#' represent significantly lower total bacterial adhesion to melimine-coated lenses than uncoated control lenses.*

The effect of melimine-coated contact lenses on the numbers of adherent bacteria was investigated also by fluorescence microscopy (Figure 4-2) and image analysis (Figure 4-3). For both *P. aeruginosa* 6294 ($p = 0.01$) and *S. aureus* 31 ($p < 0.05$) there was a significant decrease in the numbers of bacteria staining green (indicating intact cell membrane) on the melimine-coated contact lens surfaces compared to control contact lenses. There was no significant difference between areas of the surfaces covered by red stained (membrane damaged) *P. aeruginosa* 6294 ($p = 0.08$) on the melimine-coated contact lenses when compared to control contact lenses. In contrast, red stained *S. aureus* 31 covered a higher percentage area ($p < 0.05$) of melimine-coated lenses than control lenses. Overall, there was significantly ($p < 0.05$) decreased bacterial adhesion (dead and live combined) on melimine-coated contact lenses compared to control lenses.

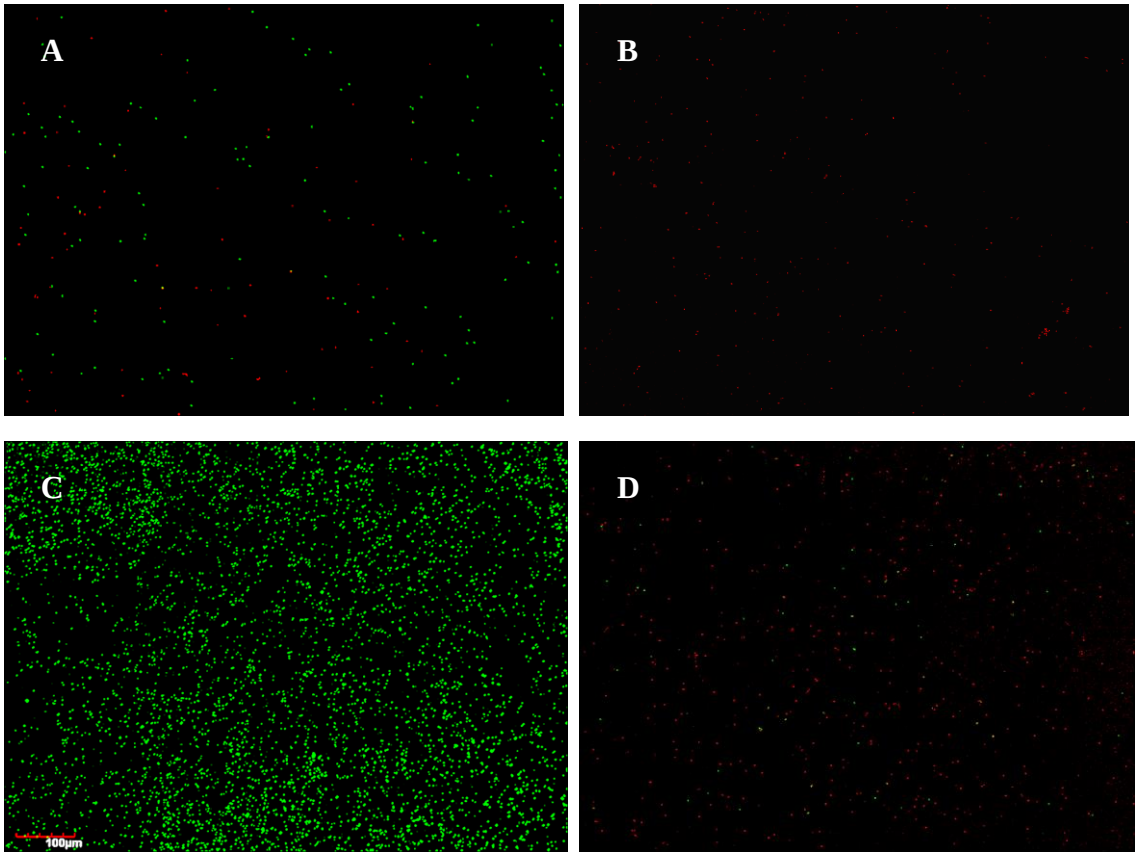

Figure 4-2: Representative confocal micrographs of *P. aeruginosa* 6294 and *S. aureus* 31 adhered to contact lenses in the presence and absence of covalently bound melimine.

Bacterial cells with intact membranes stain green, while those with permeabilised membranes were red. (Captured using a 20x objective, magnification = 200x. (A) P. aeruginosa 6294 adherent to control contact lens. (B) P. aeruginosa 6294 adherent to melimine-coated lens. (C) S. aureus 31 adherent to control lens. (D) S. aureus 31 adherent to melimine-coated lens).

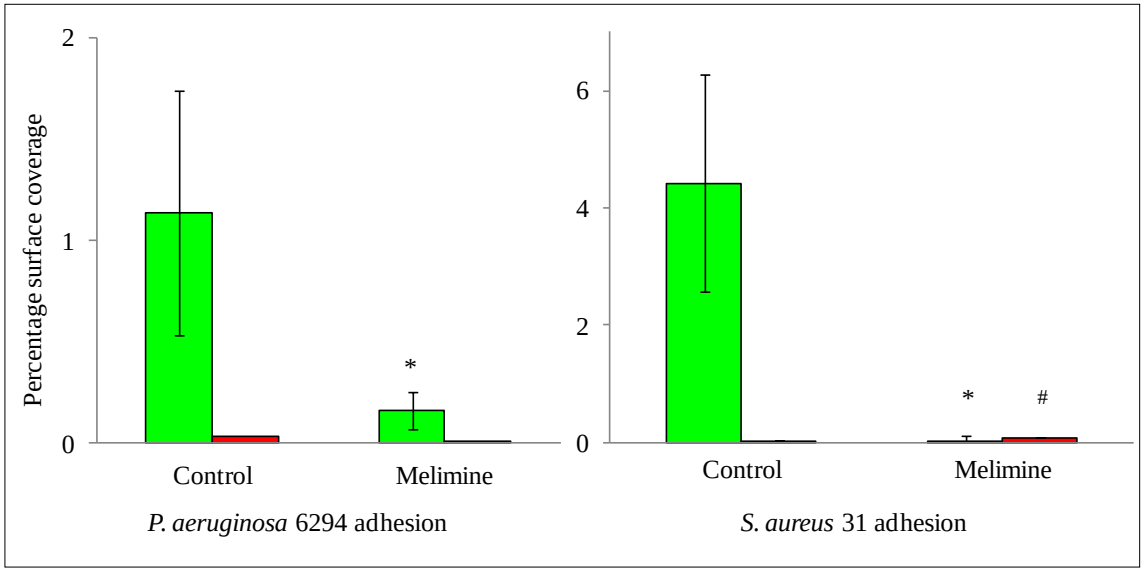

Figure 4-3: Percentage surface coverage of live bacteria (green) and dead bacteria (red) on contact lens (n ≥ 9) surfaces in the presence and absence of covalently bound melimine.

The image analysis results were measured as the average percentage area of live cells and the average percentage area of dead cells per field of view. Areas covered by green staining bacteria are represented by green bars and the area covered by red-staining bacteria by red bars. The asterisk '' represent significant (P < 0.05) reduction for green stained (live) bacteria and '#" represent significant increase of red stained (dead) S. aureus 31 on the melimine-coated contact lens.*

4.3.1 Detection of Endotoxin

Each melimine-coated contact lens on average was associated with 110 ± 11 EU. Control lenses after adhesion of *P. aeruginosa* had a higher endotoxin level of 143 ± 32 EU lens^{-1} (Figure 4-4). However, the difference was not statistically significant (p = 0.14).

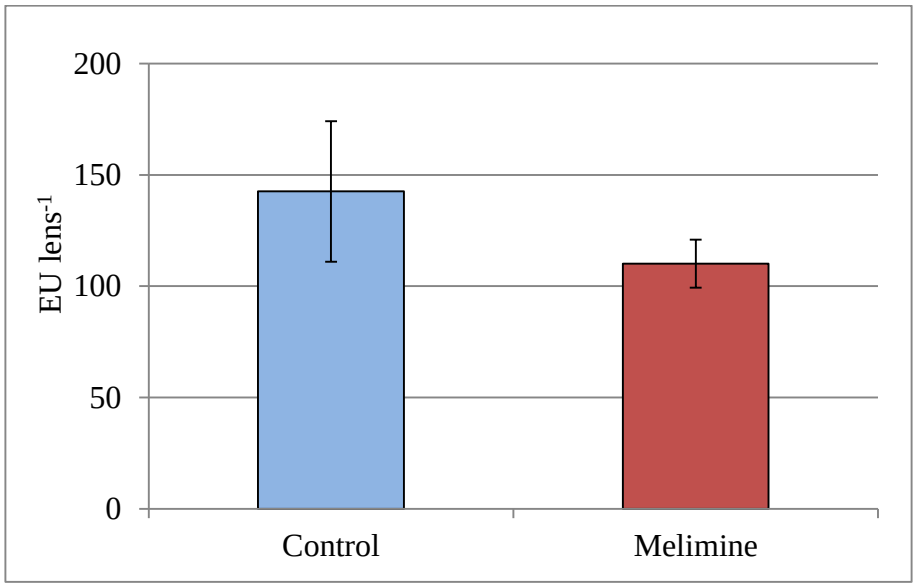

Figure 4-4: Endotoxin of Gram negative bacteria associated with control and melimine-coated lenses

4.3.2 Cytotoxicity of Melimine-Coated Lenses

Cytotoxicity responses were graded according to a standard key, which quantified the zonal extent of cell damage (0 to 4). A grade of 1 represented slight damage including physical damage to the cell monolayer due to movement of the sample. Positive and negative controls produced results as expected; the positive and negative controls gave a response of grade 4 and 1, respectively. All three melimine-coated lenses and commercially available etafilcon A lenses showed a response of grade

1, indicating no cytotoxicity, with only a small zone of dead cells under the contact area. Thus the melimine-coated lenses were determined to be nontoxic.

4.3.3 Efficacy of Melimine-Coated Lenses against Drug Resistant Bacteria

Melimine-coated lenses significantly ($p < 0.01$) reduced the viability of all the adherent drug resistant bacterial strains as well as the high biofilm producing strain of *P. aeruginosa* (Figure 4-5). Table 4.2 shows melimine-coated lenses inhibited viable adhesion of all the drug resistant bacteria by more than 2 log. The viable counts of bacteria associated with melimine-coated lenses ranged from 0 - 16 CFU mm^{-2} compared to controls which ranged from 3.6×10^2 to 2.0×10^4 CFU mm^{-2}.

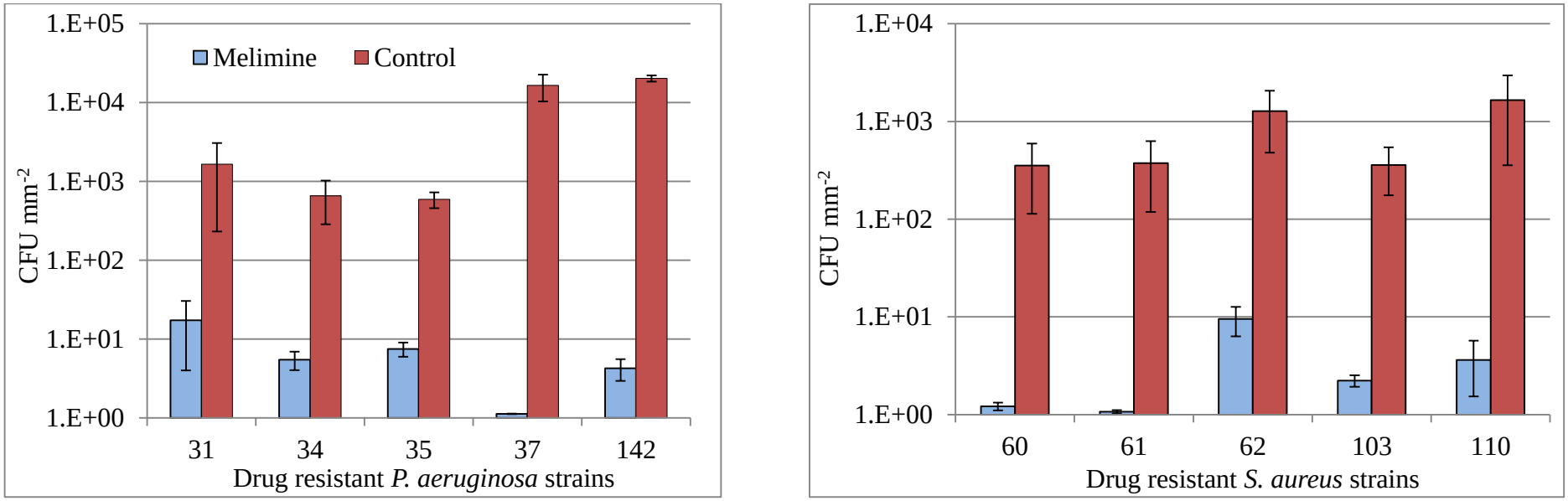

Figure 4-5: Adhesion of antibiotic resistant *P. aeruginosa* and *S. aureus* to melimine-coated and uncoated control contact lenses (n ≥ 9).

Table 4.2: Inhibition of adhesion of antibiotic resistant bacteria by melimine-coated lenses (SD = standard deviation)

Drug resistant strains	Mean log inhibition	± SD
P. aeruginosa 31	2.0	0.2
P. aeruginosa 34	2.2	0.1
P. aeruginosa 35	2.0	0.1
P. aeruginosa 37	4.2	0.2
P. aeruginosa 142	3.4	0.4
S. aureus 60	2.4	0.5
S. aureus 61	2.6	0.1
S. aureus 62	2.1	0.6
S. aureus 103	2.4	0.3
S. aureus 110	2.4	0.2

4.3.4 Activity against ISO Panel Strains

The ability of melimine-coated lenses to reduce viable adhesion of ISO panel organisms is shown in Figure 4-6. Melimine-coated lenses significantly ($p < 0.05$) inhibited the number of viable cells adherent to lens surfaces of all the organisms tested. The log inhibitions of each microorganism are detailed in Table 4.3.

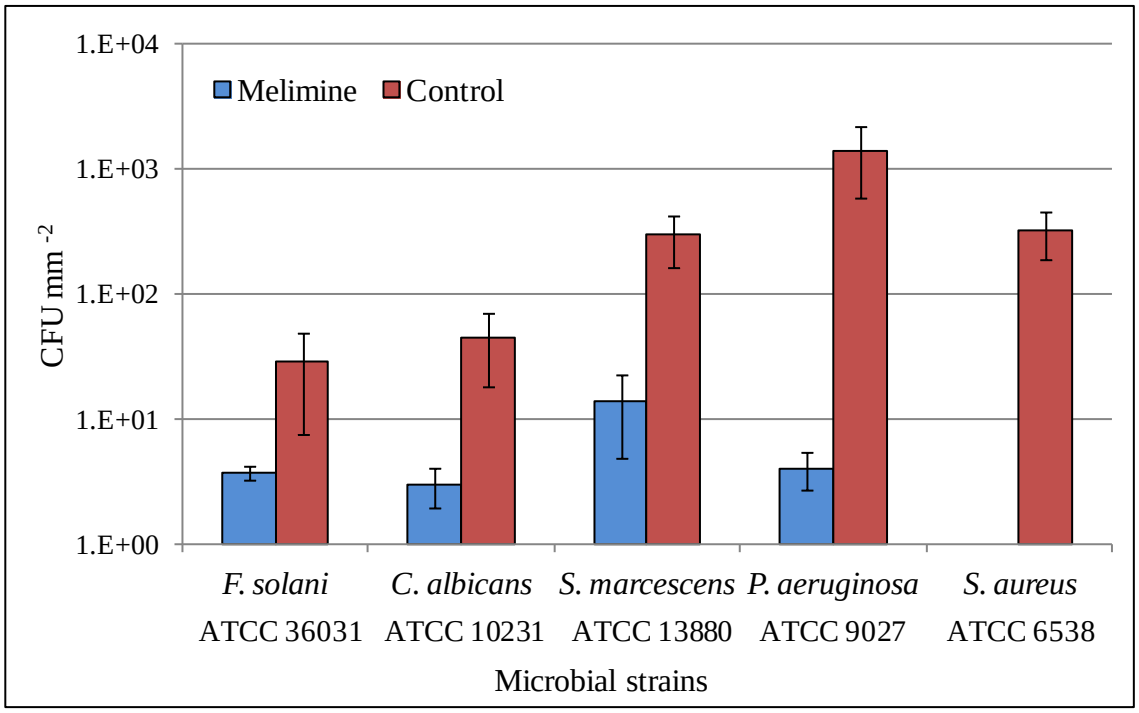

Figure 4-6: Adhesion of ISO panel bacterial and fungal microorganisms to melimine-coated and control contact lenses (n ≥ 9).

Table 4.3: Inhibition of viable adhesion of ISO panel microorganisms by melimine-coated lenses

Drug resistant strains	Mean log inhibition	± SD
F. solani ATCC 36031	1.0	0.2
C. albicans ATCC 10231	1.1	0.2
S. marcescens ATCC 13880	0.9	0.3
P. aeruginosa ATCC 9027	2.8	0.1
S. aureus ATCC 6538	2.4	0.3

4.3.5 Activity against *Acanthamoeba*

There were on average 1801 mm^{-2} viable *A. castellanii* ATCC 50370 cells adhered to control contact lens surfaces compared to 70 mm^{-2} cells on the melimine-coated contact lens surface, resulting in 1.4 ± 0.2 log inhibition ($p < 0.05$) of viable cells.

4.4 DISCUSSION

This study has demonstrated for the first time antimicrobial activity of melimine-coated contact lenses against *Acanthamoeba*, fungi and antibiotic resistant strains of *P. aeruginosa* and *S. aureus*. This extends previous data (section 3.3) and data from Willcox *et al.* (2008a) which demonstrated activity against one additional strain each of *S. aureus* (CK5) and *P. aeruginosa* (ATCC 15442) as well as a strain of *Streptococcus pneumoniae* (Spneu 10). Moreover, the current study shows that melimine bound to a lens surface was not cytotoxic to fibroblasts. This finding reinforces the previously published non-hemolytic activity of melimine in solution (Willcox *et al.*, 2008b).

There was a significant reduction in the numbers of viable bacteria adherent to melimine coated contact lenses. Confocal microscopy indicated significant reductions in the numbers of dead *P. aeruginosa* adherent to melimine-coated lenses. On the other hand, there was an increase in the level of dead (red stained) adherent *S. aureus*. This difference may be due to the nutritious disparity in the media used in the bacterial assays or to the known differences in activity of melimine in solution on these two types of bacteria (Rasul *et al.*, 2010). However, total bacterial adhesion results obtained using the radio-labelled procedure did not support confocal microscopy data. Radio-labelled total bacterial adhesion to melimine-coated lenses was substantially higher than live (green stained) and dead (red stained) bacteria together. In this study, total bacterial adhesion was determined by growing bacteria with radioactive 3H-uridine and then plotting the radiation emitted from lenses in a standard calibration curve resulting in CFU mm^{-2}. Ribonucleic acid (RNA) content is directly proportional to bacterial growth and uptake of 3H-uridine during bacterial growth and incorporation into bacterial RNA

is an established method of tracking adhesion (Yamauchi *et al.*, 1978). The lack of visible intact dead bacterial cells (or at least to levels comparable to the amount of radioactive RNA present) indicates that measured radiation from contact lenses were essentially the emission from lens of attached radioactive RNA rather than intact bacterial cells. Interaction of surface-attached melimine with both types of bacteria is predominantly electrostatic, which causes rapid disruption of bacterial cytoplasmic membranes resulting cell death (Rasul *et al.*, 2010). This may result in diffusion of bacterial cell material out of cells. Given that RNA is a negatively charged molecule (polyanion) (Lee *et al.*, 2004), bacterial radioactive RNA may adhere to the positively charged melimine surface. This suggests that radioactivity determined on the melimine-coated lenses may not necessarily be due to the presence of live or dead bacterial cells, which is in agreement with confocal microscopy results. Due to the presence of methacrylic acid, control etafilcon A lenses are negatively charged and so similar mechanisms are unlikely to occur. In addition, a previous study also has reported similar total adhesion results (with 44 µg melimine $lens^{-1}$) with melimine lenses (Cole *et al.*, 2010) and likely due to the same reasons.

Endotoxin is also known as lipopolysaccharide (LPS), which is a major constituent of the outer cell membrane Gram negative bacteria (Rietschel *et al.*, 1994). Expression of endotoxins is essential for bacterial growth and survival, but once released from bacterial cells, endotoxin can induce a variety of pathophysiological effects (Seydel *et al.*, 1994). Endotoxins are also released when Gram negative bacteria are killed (Elsbach, 2000). Endotoxins in human eyes, especially during contact lens wear due to build up of microbial bioburden, may result in contact lens related inflammation, such as CLARE (Schultz *et al.*, 1997; Holden *et al.*, 1996). Attached melimine may kill bacteria by cytoplasmic membrane destabilisation (Rasul *et al.*,

2010). This may have resulted in LPS being released from cells. LPS, bacterial RNA and DNA at the ocular surface can trigger a rapid host immune response followed by up-regulation of expression of pattern recognition receptors such as toll like receptors (TLR) (Micera *et al.,* 2005). Specifically, the presence of *P. aeruginosa* derived LPS and bacterial RNA and DNA on melimine-coated contact lenses could be recognised by TLR4 and TLR9 respectively, which are shown to be expressed by polarised corneal epithelial cells, bulbar conjunctiva and stromal fibroblasts (Chang *et al.*, 2006b). Activation of TLRs are critical in the pathogenesis of endotoxin induced keratitis. They are the key components of innate immune systems and if expressed adequately, have the capacity to trigger inflammation, resulting in responses such as CLARE at the ocular surface. However, this inflammatory response may not occur due to the presence of normal microbiota. It has been suggested that ocular surface epithelial cells may initiate an immunosilent condition for TLR mediated innate immunity to inhibit unwanted inflammatory responses to non-pathogenic microbiota (Ueta *et al.*, 2004). The ocular surface epithelial cells are regularly exposed to these bacteria and thus may create a downregulatory mechanism to avoid unnecessary TLR mediated stimulation cascades (Ueta *et al.*, 2004). However, previous studies have confirmed that melimine-coated lenses can reduce incidence and severity of inflammatory events when contaminated with Gram negative bacteria in animal trial (Cole *et al.*, 2010), and presumably even in the presence of relatively high concentration of LPS. Human clinical trials aiming to evaluate incidence of melimine-coated lens related inflammatory events will be able to assess the implications of any endotoxin production and bacterial RNA adhesion to contact lenses.

Contact lens related fungal keratitis is a rare but severe form of infectious keratitis generally associated with poor prognosis (Tuli *et al.*, 2007). The incidence has

progressively increased even after the recent *Fusarium* keratitis epidemic (Gorscak *et al.*, 2007). Fungi can be resistant to the activity of several contact lens multipurpose disinfecting solutions (Retuerto *et al.*, 2012). Furthermore, a recent study investigating *in vitro* antimicrobial activity of three commercially available silver impregnated contact lens cases revealed high activity against bacteria but all the lens cases were essentially ineffective against *C. albicans* after 6, 10 and 24 hr, and only one lens case showed limited activity (0.5 log) against *F. solani* (Dantam *et al.*, 2011). The current study demonstrated that the melimine-coated lenses produced more than 1 log inhibition against both *C. albicans* and *F. solani* strains, indicating the possibility of controlling colonisation of lens surfaces by fungi as well as bacteria.

Acanthamoeba keratitis associated with contact lens wear is a serious eye infection with poor prognosis and significant ocular morbidity (Otri *et al.*, 2012; Yoder *et al.*, 2012; Por *et al.*, 2009). Keratitis caused by *Acanthamoeba* often has limited treatment options, significantly higher duration of hospital admission and unpredictable outcome (Otri *et al.*, 2012). Many commonly used contact lens disinfecting solutions have only limited amoebicidal efficacy (Boost *et al.*, 2012). The recent outbreak of contact lens related *Acanthamoeba* keratitis associated with use of Complete® MoisturePlus™ contact lens disinfecting solution (Tu *et al.*, 2010) and persistent elevated numbers of events even after removal of this solution from sale (Yoder *et al.*, 2012; Tu *et al.*, 2010), clearly indicates a need for an effective strategy to help reduce the incidence of this disease. In this study, for the first time an antimicrobial peptide attached to contact lens surface was shown to have amoebicidal activity. This activity was much higher than that previously reported for fimbrolide-coated contact lenses (70% inhibition) against *Acanthamoeba* trophozoites (Zhu *et al.*, 2008).

Development of bacterial resistance against conventional antibiotics is a major problem. Resistance increases the risk of treatment failure with potentially serious consequences. In the last decade, various reports have confirmed antibiotic resistance of *P. aeruginosa* and *S. aureus* ocular isolates (Sharma, 2011; Green *et al.*, 2008b; Conibear, 2006; Mayo *et al.*, 1986). This study reported at least 2 log inhibition of adhesion by melimine-coated lenses for five *P. aeruginosa* and five *S. aureus* strains which were resistant against commonly used antibiotics such as ciprofloxacin, gentamicin, moxifloxacin and tobramycin. This combined with the previous finding of the inability of bacterial strains to become resistant after 30 days repeated exposure to sub-inhibitory concentrations of melimine (Willcox *et al.*, 2008b) is a promising finding towards controlling these resistant bacteria.

In summary, this study demonstrated that melimine-coated contact lenses have broad spectrum antimicrobial activity including activity against fungi, *Acanthamoeba* and multi-drug resistant *S. aureus* and *P. aeruginosa*. They are also non-toxic. This, coupled with previous demonstration of the ability of melimine-coated lenses to control adverse events in animal models (Cole *et al.*, 2010), makes melimine-coated lenses potentially ideal as an antimicrobial coating. However, these lenses were associated with endotoxin of Gram negative bacteria and possibly higher level of dead bacterial debris such as RNA, which can activate TLR at the ocular surface and trigger inflammation. Although, the dead bacteria associated with melimine-coated lenses did not induce as high a CLARE-like response in guinea pig eyes (Cole *et al.*, 2010) comapred to uncoated eyes. Any possible consequence of this needs to be explored with human clinical trials. Further investigations are required to evaluate whether melimine-coated contact lenses maintain acceptable physical parameters for *in vivo* studies and retain antimicrobial activity when treated with commercially available multipurpose

disinfection solutions. Also, *in vivo* exploration in animal and human clinical trial is necessary to evaluate the safety and biocompatibility.

Chapter 5: Validation of Melimine-Coated Contact Lenses

Part of this chapter has been presented as:

- Dutta D, Zhu H, Willcox MD (2013). Antimicrobial activity of multipurpose disinfection solution soaked contact lenses. *7th International conference on the Tear Film and Ocular Surface: Basic Science and Ocular Relevance*, Taormina, Italy. Page 24, E-Abstract 54.

5.1 INTRODUCTION

The previous chapter demonstrated that melimine-coated contact lenses have high broad spectrum antimicrobial activity including activity against fungi, *Acanthamoeba* and multi-drug resistant bacteria. For clinical applications, various validations of the melimine-coated contact lenses are necessary. Since, melimine is attached to contact lenses via covalent coupling, it is important that the lenses maintain acceptable physical parameters and surface wettability to be appropriate for *in vivo* use. In addition, it would be useful if the lenses maintain antimicrobial activity following heat sterilisation, a process commonly used during lens manufacture. More than 90% of the soft lens wearers use multipurpose disinfection solutions (MPDS) for disinfection, cleaning and overnight contact lens storage (Efron *et al.*, 2013; Efron *et al.*, 2010). For practical purposes, it is important that melimine-coated contact lenses are compatible with MPDS. Thus, it would be worthwhile to investigate the compatibility of melimine-coated lenses with marketed MPDS.

The aims of this chapter were to:

1. Determine retention of antimicrobial activity of melimine-coated contact lenses after heat sterilisation
2. Investigate any changes in contact lens parameters after covalent coupling with melimine
3. Determine surface hydrophilicity of contact lenses after melimine attachment
4. Evaluate compatibility of melimine-coated contact lenses by investigating the retention of antimicrobial activity after soaking in MPDS and subsequent contact lens surface characterisation

5.2 METHODS

5.2.1 Contact Lenses

Hydrogel contact lenses of etafilcon A material (ACUVUE® 2; Johnson & Johnson Vision Care Inc., Jacksonville, FL; Base curve: 8.3 mm, Diameter: 14.0 mm, Power: -3.00 Diopter) were used. These lenses were covalently coated with melimine (>80% purity; American Peptide Company, Sunnyvale, CA, USA) following the procedures described in section 3.2.2. This procedure was shown to result in 152 ± 43 µg $lens^{-1}$ covalent attachment of melimine onto contact lenses. Lenses processed only with 1-ethyl-3-(3-dimethylaminopropyl (EDC; without melimine) acted as process controls. Uncoated etafilcon A lenses were used as controls. A separate batch of lenses prepared by soaking in melimine with same concentration used for EDC coupling (3 mg ml^{-1} melimine) solution for 2 hr without EDC covalent coupling was used to determine effectiveness of the covalent attachment.

5.2.2 Effect of Autoclaving on Activity of Melimine-Coated Lenses

Melimine-coated, melimine soaked, EDC process control, and untreated control contact lenses were autoclaved (121ºC) in phosphate buffered saline (PBS) for 15 min, after which the lenses were cooled to ambient temperature (~20ºC). *Pseudomonas aeruginosa* 6294 and *Staphylococcus aureus* 31 adhesion to these lenses were evaluated using the same procedure described in section 3.2.4. Retention of antimicrobial activity of the melimine-coated autoclaved lenses compared to melimine-coated non-autoclaved lenses was determined using the same bacteria. Three lenses were used for each experiment and experiments were repeated on a minimum of three separate occasions.

5.2.3 Lens Parameter Measurements

To test whether reacting lenses with melimine resulted in any changes to lens parameters, five uncoated contact lenses were selected for metrological evaluation before and after being coated with melimine. Lenses were immersed in PBS at ambient temperature (20°C ± 2°C) for 24 hr prior to testing. Centre thicknesses were measured using a Heidenhain soft contact lens thickness gauge following the ISO: 18369-3 (2006), 9339-2 (1998) and American National Standard ANSI Z80.20-1998 (1998) protocols. The diameter of lenses were measured following the ISO: 18369-3 (2006) and 9338 (1996) protocols in a wet cell using a Nikon profile projector with horizontal x-y table and digital position readout. Sagittal depth was measured by profile projector following ISO:18369-3 (2006) and ANSI Z80.20-1998 (1998) protocols. Base curve equivalents were calculated using measured lens diameters, centre thickness and sagittal depth measurements. All procedures were repeated to obtain five independent measurements for each lens and these were then averaged.

5.2.4 Effect of Covalent Attachment of Melimine on Lens Surface Hydrophobicity

Contact lens hydrophobicity was evaluated through dynamic water contact angle measurement using a captive bubble technique (Read *et al.*, 2010) and a contact angle goniometer (Model no. 200-F1; Rame-Hart, Inc NRL USA). Melimine treated and control contact lenses were soaked in PBS for 2-3 hr at ambient temperature (20°C ± 2°C), then lenses were carefully rested on a custom made holder so that the convex lens surface faced downward directly into a PBS-filled optically clear chamber. An air bubble was dispensed from a 1.25 mm diameter blunt-ended steel needle positioned 2 mm directly below the lens apex. The size of the bubble was slowly

increased to 3 µl using a micro-syringe. Assessment of the receding and advancing contact angles was achieved by first enlarging the air bubble and then shrinking until the bubble detached from the surface. The angle between bubble and lens surface was measured with 50 mm Cosmicar Television Lens (Tokyo, Japan). Image J software (version 1.47r, National Institutes of Health, USA) was used to calculate advancing and receding contact angle. A minimum of eight measurements was made on five samples of each contact lens and were averaged.

5.2.5 Compatibility of Melimine-Contact Lenses with Contact Lens Care Solutions

Compatibility of melimine-coated contact lenses was evaluated by determining the retention of antimicrobial activity after soaking for 1 day, 10 days and 30 days in MPDS and subsequent surface characterisation by X-ray photoelectron spectroscopy (XPS) to determine changes to the atomic structures of the lens surface.

5.2.5.1 Retention of Antimicrobial Activity after 1 Day, 10 Days and 30 Days Soaking

PureMoist®, Biotrue™ and RevitaLens OcuTec™ were used as MPDS in this study (Table 5.1). Retention of antimicrobial activity of melimine-coated contact lenses with *P aeruginosa* 6294 and *S. aureus* 31 was investigated after soaking for 1 day, 10 days and 30 days in these MPDS. Uncoated etafilcon A lenses were also treated similarly and acted as controls. Both melimine-coated and control lenses were soaked in 2 ml of MPDS in lens storage cases. In addition, melimine-coated lenses were soaked in sterile PBS in lens cases. The MPDS or PBS was replaced each day with 2 ml aliquot of fresh solutions. The antimicrobial assay used in this study followed the same procedures described in section 3.2.4. Triplicates of each lens type were included in each adhesion

experiment and the experiment was repeated for minimum of three times, using both bacterial strains.

Table 5.1: Constituents of multipurpose disinfection solutions (MPDS) used in this study

Contact lens care solutions	PureMoist®	Biotrue™	Revitalens OcuTec™
Manufacturers	Alcon laboratories Inc., Fort Worth, USA	Bausch + Lomb, Rochester, USA	Abbott Medical Optics, Illionis, USA
Commercial brand	OPTI-FREE® PureMoist®	Biotrue™	RevitaLens OcuTec®
Constituents	0.001% Polyquad® (Polyquaternium-1), 0.0006% Aldox® (myristamidopropyl dimethylamine), sodium citrate, sodium chloride, boric acid, sorbitol, aminomethylpropanol, disodium EDTA, Tetronic® 1304 and HydraGlyde®Moisture Matrix [EOBO-41™-Polyoxyethylene-Polyoxybutylene]	0.00013% polyaminopropyl biguanide, 0.0001% polyquaternium, hyaluronan, sulfobetaine, poloxamine, boric acid, sodium borate, edetate disodium and sodium chloride	0.00016% alexidine dihydrochloride, 0.0003% polyquaternium-1, boric acid, sodium borate, tetronic 904, edetate disodium, sodium citrate and sodium chloride

5.2.5.2 XPS Characterisation

After soaking for 1 day, 10 days or 30 days in MPDS or PBS, one set each of melimine-coated and control contact lenses was processed for high resolution XPS (ESCALAB220-iXL, VG Scientific, West Sussex, England) to determine the elemental composition (atomic percentages) of the surface-bound species. All the contact lenses were air-dried in a closed container at room temperature before XPS analysis. The X-ray source was monochromated Al Kα and the photo-energy was 1486.6 eV with a source power of 120 W. Vacuum pressure was ≤ 10-8 mbar. XPS uses x-ray beams to release electrons from a surface which penetrates the surface to a depth up to 5 nm (Ray *et al.,* 2011). The electrons emited from the surface have a characteristic kinetic energy, which represents how tightly electrons are bound to the nucleus. Thus, the elemental composition of a surface can be revealed by measuring the kinetic energy at which electrons escape the surface. In high-resolution nitrogen spectra (N1s), the N1 component represents C-N, N2 corresponds to nitrogen bound to carbon that is doubly bonded to oxygen such as amides (N-C=O), N3 corresponds to protonated N bound to carbon ($^{+}$HN-C), and N4 to quaternarised nitrogen ($^{+}$N-C) groups. Increased surface concentration of amide nitrogen indicates presence of melimine on the lens surface. A minimum of four samples was used for each group and the test was repeated twice with each lens being analysed at three points on its surface.

5.2.6 Data Analysis

Data were analysed using Excel® (Office; Microsoft®, Redmond, USA) and Statistical Package for the Social Sciences software (SPSS) for Windows software version 21.0 (SPSS, Inc., Chicago, IL, USA). All data were summarised using descriptive statistics. The bacterial adhesion data were $\log_{10}$ (x+1) transformed prior to

data analysis where x is the adherent bacteria in colony forming units (CFU) mm^{-2}. Differences in bacterial adhesion and contact angle were analysed using independent 2-sample *t*-test. For the surface analysis by XPS, differences in the lens surface compositions were assessed using analysis of variance. Changes in lens parameters were analysed using paired-*t* test. The significance of comparisons between the control and test conditions was assessed using post hoc (Bonferroni) analysis. Statistical significance was set at 5%.

5.3 RESULTS

5.3.1 Effect of Autoclaving on Activity of Melimine-Coated Lenses

For both *P. aeruginosa* 6294 and *S. aureus* 31, autoclaved melimine-coated contact lenses showed no significant ($p > 0.05$) reduction in antimicrobial activity compared to non-autoclaved melimine-coated lenses. Both the untreated control and EDC process control lenses showed 3.5 log *P. aeruginosa* 6294 and 4.3 log *S. aureus* 31 adhesion, respectively. There was no significant difference ($p > 0.05$; 0.3 to 0.7 log inhibition) between bacterial adhesion to the melimine-soaked and control lenses (Figure 5-1), indicating that the autoclaving had removed most of the adsorbed and non-covalently bound melimine.

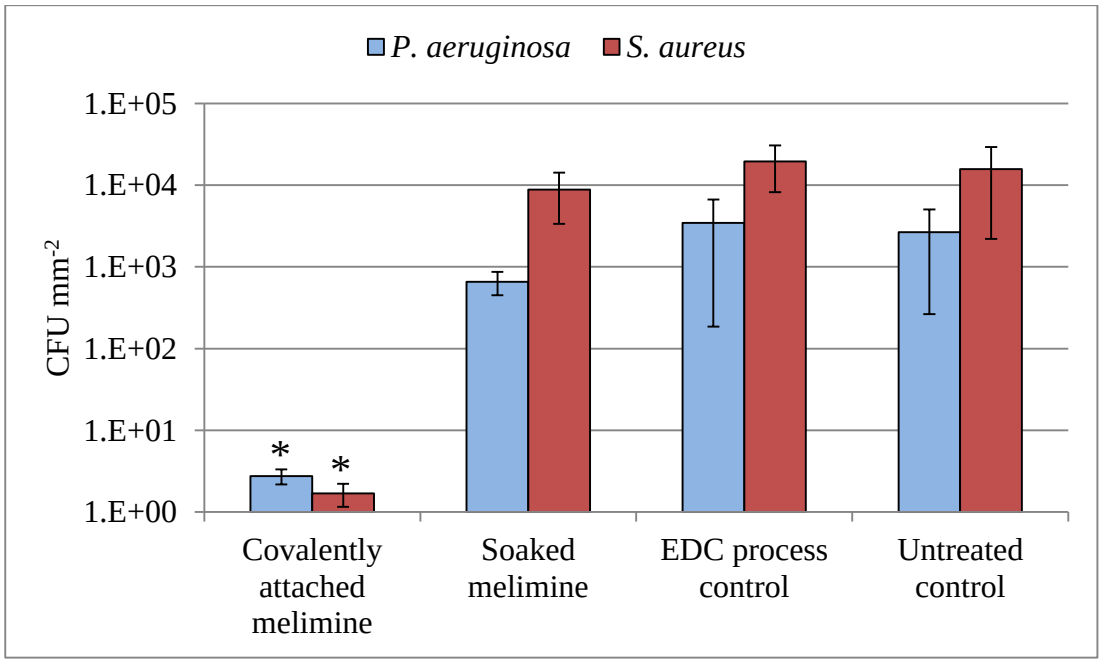

Figure 5-1: Adhesion of *P. aeruginosa* 6294 and *S. aureus* 31 following autoclaving to contact lenses with different treatments.

Asterisks (*) represent significantly ($p < 0.05$) reduced bacterial adhesion compared to contact lenses with soaked melimine, process controls and untreated controls.

5.3.2 Lens Parameter Measurements

The commercially available etafilcon A lenses (with a power of – 3.00 Ds) had an average lens diameter of 13.70 ± 0.01 mm, a central thickness of 57.80 ± 3.11 µm and calculated base curve of 8.26 ± 0.02 mm. After peptide coating there were no statistically significant ($p > 0.05$) changes in lens diameter (13.52 ± 0.02 mm), central thickness (57.80 ± 2.77 µm) or calculated base curve (8.18 ± 0.03 mm).

5.3.3 Lens Hydrophobicity

Figure 5-2 demonstrates that melimine-coating resulted in a significant decrease ($p < 0.05$) in advancing contact angle compared to uncoated lenses. The mean and 95% confidence interval (CI) of the contact angles of the lenses are detailed in Table 5.2. There was less hysteresis in the contact angle after melimine coating; the difference in advancing and receding angle was 5.6 degrees for melimine-coated lenses but 42.7 degrees for uncoated lenses.

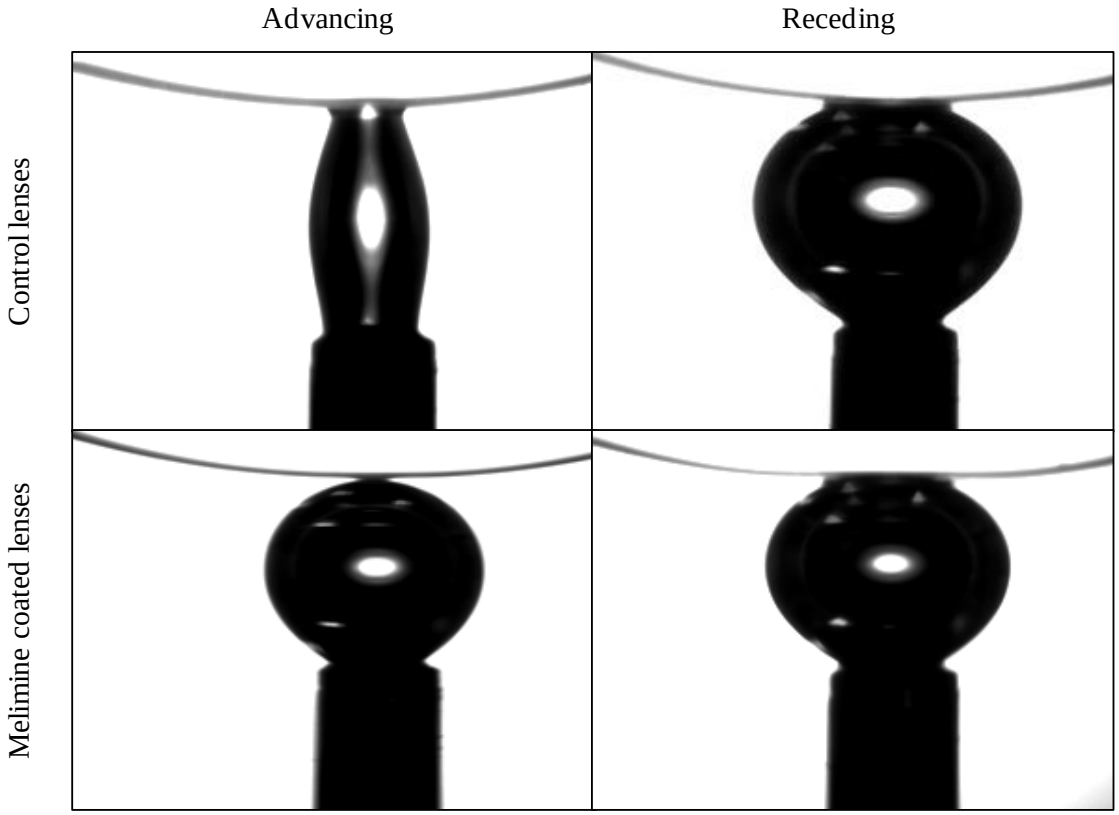

Figure 5-2: Images of air bubbles demonstrating advancing and receding contact angles with melimine-coated contact lenses.

Table 5.2: Contact angle of control and melimine-coated contact lenses

Lens	Advancing (degrees)		Receding (degrees)	
	Mean	95% CI	Mean	95% CI
Control	69.3 ± 14.6	65.9 - 72.6	26.6 ± 6.8	25.0 - 28.2
Melimine	22.7 ± 5.0	21.5 - 24.0	17.1 ± 2.8	16.4 - 17.7

5.3.4 Compatibility of Melimine-Coated Contact Lenses with Contact Lens Care Solutions

5.3.4.1 Retention of Antimicrobial Activity

Figure 5-3 A and B show *P. aeruginosa* 6294 and *S. aureus* 31 adhesion to melimine-coated and uncoated control lenses when soaked in PureMoist, Biotrue, RevitaLens MPDS and PBS after 1 day, 10 days and 30 days. There was minimum bacterial adhesion by either strain to melimine-coated lenses after 1 day soaking in Biotrue and RevitaLens MPDS. This adhesion was significantly lower than uncoated control lenses soaked for 1 day in the same MPDS ($p < 0.05$). Adhesion of both bacterial strains to melimine-coated lenses was further reduced after soaking in Biotrue and Revitalens MPDS for 10 and 30 days. A similar trend was seen with control lenses with decreasing adhesion after 10 days and almost total inhibition after 30 days (compare Figure 5-3 A with B). After 1 day soaking in Biotrue, Revitalens and PureMoist, control lenses showed equivalent adhesion to PBS soaked controls ($p > 0.05$). However, both melimine-coated and control lenses soaked in PureMoist MPDS showed no reduction of *P. aeruginosa* 6294 and *S. aureus* 31 adhesion throughout this experiment and showed comparable adhesion to PBS soaked counterparts including after 30 days soaking. The differences in bacterial adhesion between melimine-coated and uncoated control lenses were prominent after 1 day but over time the differences were reduced. A similar trend was not observed after soaking in PureMoist.

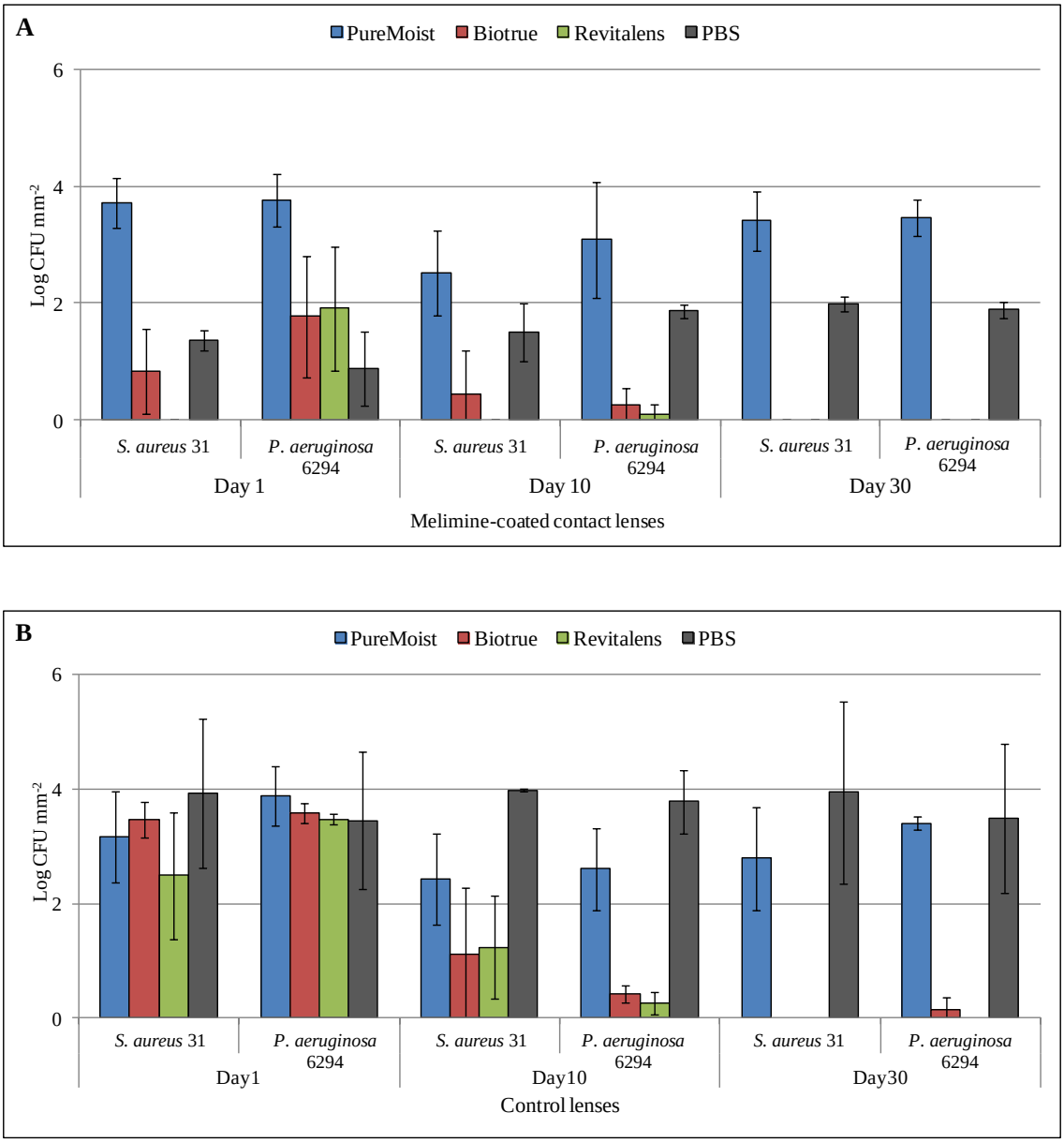

Figure 5-3: Bacterial adhesion to melimine (A) and control lenses (B) after 1 day, 10 days and 30 days soaking in PureMoist, Biotrue, RevitaLens and PBS.

Figure 5-4 shows inhibition of bacterial adhesion by melimine-coated contact lenses compared to uncoated control lenses when soaked in PBS. Untreated melimine-coated lenses gave 2.6 ± 0.4 log and 2.6 ± 0.5 log inhibition of *P. aeruginosa* 6294 and *S. aureus* 31 respectively, when compared to control counterparts. When soaked with PBS for 1 day, 10 days and 30 days, melimine-coated lenses gave 2.5 ±0.6 log, 1.9 ± 0.7 log, 1.7 ± 0.6 log inhibition against *P. aeruginosa* 6294 and 2.5 ± 0.4 log, 2.5 ± 0.4 log, 1.9 ± 0.6 log inhibition against *S. aureus* 31 respectively. On average, there was 0.9 log and 0.6 log reduction in bacterial killing by melimine-coated lenses for *P. aeruginosa* and *S. aureus* respectively over 30 days soaking in PBS and this reached statistical significance for *P. aeruginosa* only ($p = 0.05$ for *P. aeruginosa* and $p = 0.06$ for *S. aureus*).

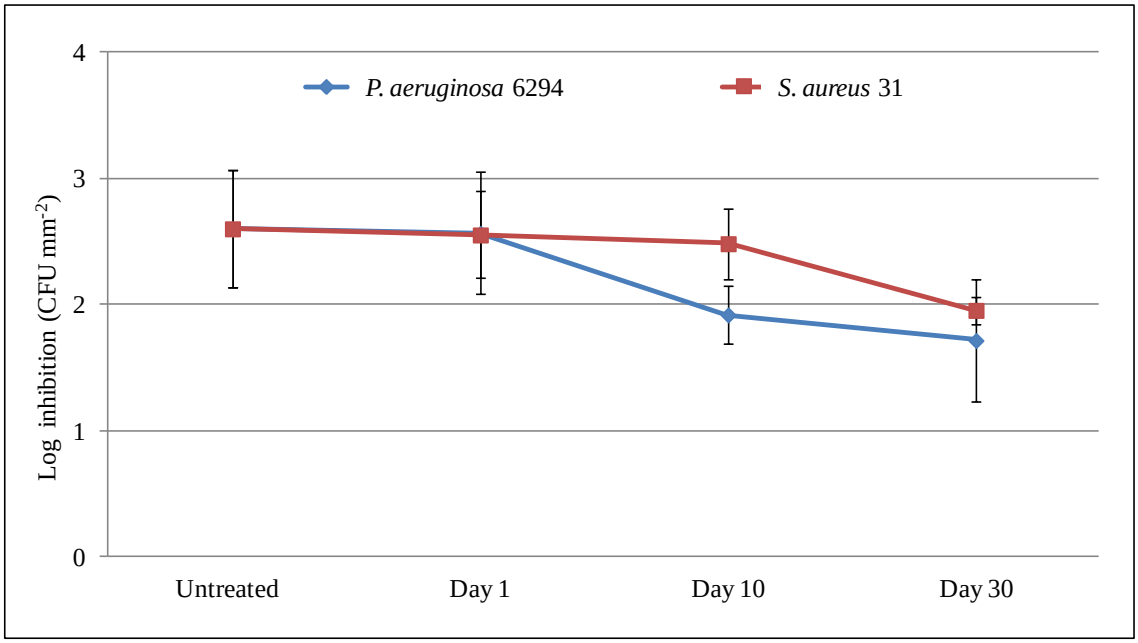

Figure 5-4: Inhibition in *P. aeruginosa* 6294 and *S. aureus* 31 adhesion by melimine-coated contact lenses compared to control lenses after 1 day, 10 days and 30 days soaking in PBS.

5.3.4.2 XPS Characterisation

A higher concentration (%N) of nitrogen ($p < 0.05$) was associated with unsoaked melimine-coated lenses compared with unsoaked control lenses (Table 5.3). In addition, the only binding energy peak in the nitrogen region for all the melimine-coated lenses ranged between 399.56 eV to 400.05 eV, which confirms that the detectable nitrogen was from amine and thus derived from the peptide melimine. There was no other type of nitrogen found with high resolution spectra and so the amount of quaternary ammonia or biguanide based disinfectants, such as PHMB or polyquad could not be directly measured. Figure 5-5 shows the percentage of nitrogen present at the surface of un-soaked and soaked melimine-coated and control lenses determined by XPS analysis. Melimine-coated lenses soaked in Biotrue, RevitaLens and PBS showed similar surface concentrations and type of nitrogen to un-soaked melimine-coated lenses throughout the experiment. There was no significant reduction ($p > 0.05$) in the nitrogen percentage after 1 day, 10 days or 30 days of soaking for these two MPDS. Melimine-coated lens soaked in PBS showed slight reduction in surface nitrogen concentration (0.2% over 30 days) but this was not significant ($p > 0.05$). Longer incubation of melimine-coated lenses in PBS was associated with increasing standard deviations. In contrast, there was a substantial ($p < 0.05$) reduction of detectable nitrogen for all the PureMoist soaked melimine-coated lenses, including after 1 day of soaking. The percentage of nitrogen detected here was very low (~ 0.2%) and was comparable to un-soaked control lenses. Control lenses soaked in the three MPDS had very low percentage of nitrogen similar to un-soaked controls.

Table 5.3: XPS elemental composition of melimine-functionalised and control contact lenses (mean ± SD)

Contact lens samples			Surface analysis		
			%C	%N	%O
Melimine	Un-soaked		68.3 ± 0.4	1.7 ± 0.6	29.8 ± 0.7
	Soaked in PBS	1 day	68.5 ± 0.3	1.6 ± 0.3	29.9 ± 0.4
		10 days	68.8 ± 0.6	1.6 ± 0.4	29.6 ± 0.3
		30 days	68.9 ± 0.6	1.5 ± 0.6	29.6 ± 0.5
Control	Un-soaked		69.5 ± 0.5	0.2 ± 0.1	30.3 ± 0.5

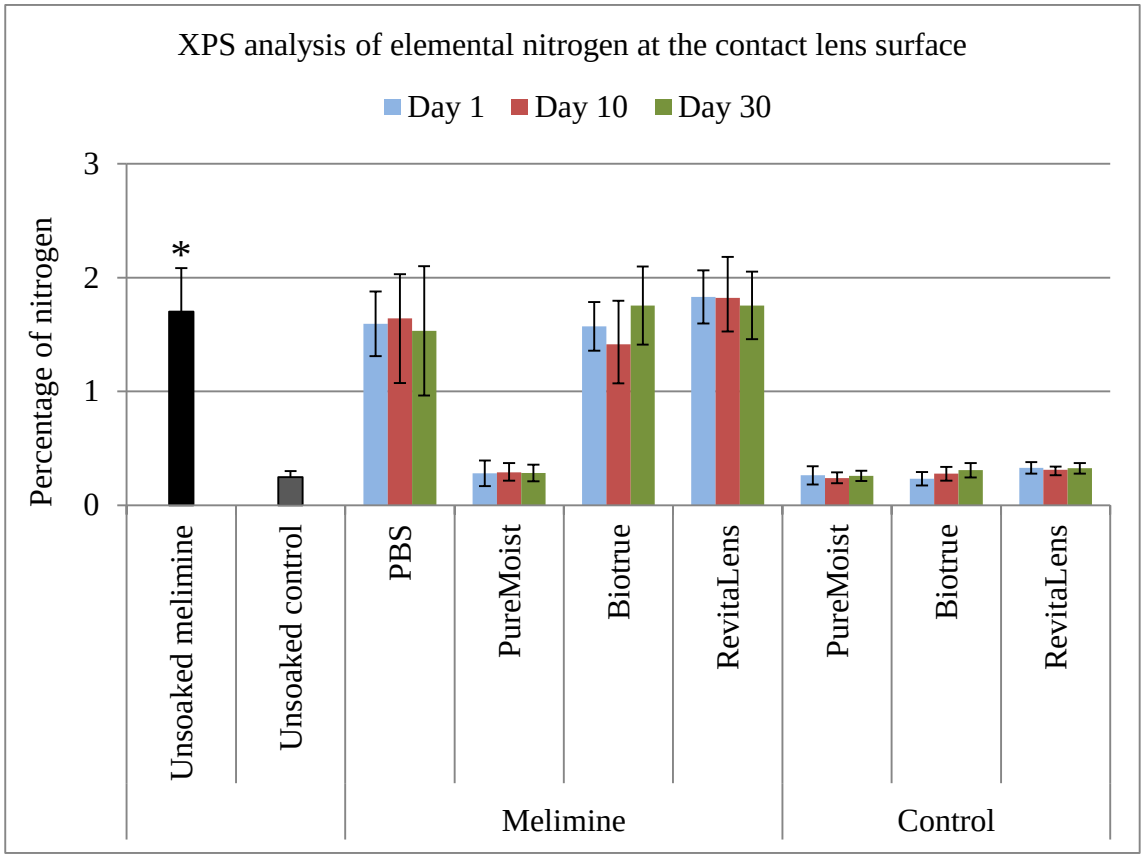

Figure 5-5: XPS analysis results showing percentage of nitrogen at the melimine-coated and control contact lens surfaces before and after soaking.

Asterisk '' represent significantly higher ($p < 0.05$) elemental nitrogen at the un-soaked melimine-coated lens surface compared with unsoaked control lens. Both melimine-coated and control lenses were soaked in PureMoist, Biotrue and Revitalens for 1, 10 and 30 days, whereas melimine-coated lenses were also soaked in PBS for the respective days.*

5.4 DISCUSSION

This chapter validated various aspects of melimine-coated contact lenses. The antimicrobial activity of the lenses was found to be heat stable, extending the previous finding that melimine in solution retains activity when autoclaved (Willcox *et al.*, 2008b). In addition, covalent coupling of melimine to contact lenses decreased lens surface hydrophobicity and was not associated with any change in contact lens parameters. The latter finding is similar to an earlier report of fimbrolide- coated contact lenses (Zhu *et al.*, 2008). Melimine coating transforms the anionic etafilcon A contact lens surface to either a cationic or neutral surface, which may have caused a decreased contact angle resulting in a more hydrophilic surface. Melimine-coated lenses had lower hysteresis, which is believed to be associated with reduced lens surface deposits (Cheng *et al.*, 2004).

Importantly, melimine-coated lenses demonstrated high antimicrobial activity when soaked up to 30 days in PBS, reinforcing the results from section 4.3 that the antimicrobial activity obtained from these lenses was mainly due to surface attached melimine and not released melimine. There was a slight, gradual reduction in activity over 30 days which may be due to release a small amount of unbound melimine from the lenses. This is in agreement with the slight reduction in detectable amide nitrogen at the contact lens surface. Longer incubation in PBS was also associated with larger standard deviations implying greater variation in the surface detected melimine. Overall, melimine-coated lenses showed high activity following soaking in PBS for up to 30 days, which indicates long lasting activity from the melimine-coated lenses.

After soaking in Biotrue and RevitaLens, XPS analysis indicated that the amide nitrogen signal remained on the lens surface demonstrating the melimine could still be

found on the lens surface. This result is supported by reduced viable bacterial adhesion. The XPS results were largely consistent when melimine-coated lenses were soaked for longer durations confirming that melimine-coated lenses are compatible with the two MPDS. Biotrue contains polyquad and PHMB, whereas RevitaLens contains polyquad and alexidine disinfectants. During overnight storage, contact lenses can take up these disinfectants and other constituents of the MPDS. This can reduce bacterial viability upon release (Jones *et al.*, 2013). When both melimine-coated and control lenses were soaked in these MPDSs for 10 or 30 days, substantial uptake of disinfectants was inevitable. Given that XPS could not detect quaternary ammonium as a representative of polyquaternium on the lens surfaces, the resulting antimicrobial activity of cationic lenses is likely due to the release of absorbed rather than adsorbed (i.e. surface bound) disinfectants after 10 and 30 days. This trend was also partly observed with melimine-coated lenses. Bacterial adhesion to melimine-coated lenses after 1 day soaking in these MPDS was similar to unsoaked melimine-coated lenses. This implies that uptake of disinfectants after 1 day might have been negligible. Thus, bacterial adhesion associated with melimine-coated lenses was after 1 day likely due to surface attached melimine. Whereas, the observed inhibitions after 10 and 30 days were the result of both melimine activity and possibly released disinfectants.

Interestingly, soaking lenses in PureMoist produced different results. After soaking in this MPDS, melimine-coated lenses were not able to reduce bacterial adhesion. This is in agreement with the XPS results, which could not detect nitrogen on the lens surface that would indicate the presence of melimine. The detected low concentration of nitrogen was similar to the percentage found in un-soaked control lenses. PureMoist MPDS consists of a reconditioning system which combines TETRONIC 1304 with a proprietary multifunctional linear di-block copolymer

composed of poly(oxyethylene)-poly(oxybutylene) (EOBO; HydraGlyde Moisture Matrix). The EOBO is a large di-block copolymer which acts as a surface active agent (Millar *et al.*, 2010), and when combined with TETRONIC 1304 may accumulate on the surface of the lens (adsorption). This interaction is driven primarily by surface related phenomena such as molecular charge and hydrophobicity and is claimed to increase lens wettability (Campbell *et al.*, 2012). The poly(oxyethylene) component of EOBO is hydrophilic and poly(oxybutylene) is hydrophobic. The melimine coating on the lenses may attract the hydrophilic part of EOBO to form a film by adsorption. This stable layer may have prevented melimine from coming into contact with bacterial cells. The layer made up with di-block copolymers may be more than 5 nm thick (Davis *et al.*, 2010) which prevented melimine characterisation by XPS. It is unlikely that melimine was degraded by any of the PureMoist components. PureMoist also contains the dual disinfectants, Polyquad and Aldox (Campbell *et al.*, 2012). Aldox is a small cationic and relatively hydrophobic biocide (Jones *et al.*, 2013). Powell *et al.* (2010) have shown that Aldox uptake is much lower for ionic hydrogel lenses than PHMB uptake and so at the presence of EOBO uptake of Aldox or even perhaps Polyquad from PureMoist may not have been sufficient to affect bacterial viability.

In summary, this chapter varified various features of the melimine-coated contact lens coating. This coating was heat stable and increased surface wettability. The covalent coupling also did not change contact lens parameters. Melimine coating was stable and compatible with solution components of Biotrue and RevitaLens MPDS, but not with PureMoist. To evaluate *in vivo* biocompatibility, contact lens wear for 22 consecutive days, determining safety and biocompatibility by ocular study using rabbit eyes following ISO 9394 is necessary. Finally, the safety and retention of antimicrobial

activity of the melimine-coated contact lenses in human participants is necessary and will be investigated in subsequent studies.

Chapter 6: Evaluation of *In Vivo* Safety and Ocular Irritation of Melimine-Coated Contact Lenses in Rabbit Eyes

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6.1 INTRODUCTION

Previous chapters have detailed how high antimicrobial activity was achieved against various strains of bacteria, fungi and *Acanthamoeba* through optimisation of the concentration of melimine on the contact lens surface. *In vitro* tests according to the relevant International Organisation for Standardisation (ISO) showed that melimine coating on contact lenses was non-toxic to mammalian cells, did not change contact lens shape or other parameters and was compatible with certain multipurpose disinfection solution (MPDS). Following these tests, evaluation of *in vivo* ocular irritation and biocompatibility of melimine-coated antimicrobial contact lenses is required. Historically, rabbits have been used for *in vivo* evaluation of ocular irritation for human products because of the physiological similarities between rabbit and human corneal tissues (Hayashi *et al.*, 2002). In addition, white rabbits are free of pigments making ocular examination easy. Alternatives to performing this study were explored; however to appropriately evaluate the ocular tolerability of the melimine-coated lenses, a whole-body test system was required.

The objective of this study was to evaluate the ocular irritation and biocompatibility of melimine-coated contact lenses compared to marketed contact lenses when placed in the eye of New Zealand white rabbits. The ISO standard 9394, Ophthalmic Optics — Contact Lenses and Contact Lens Care Products — Determination of Biocompatibility by Ocular Study with Rabbit Eyes (2012) was used to test the degree of irritation to the ocular tissue. The hypotheses of this study were that the melimine-coated contact lenses are biocompatible and do not trigger ocular irritation in rabbit eyes.

6.2 METHODS

This was a prospective, masked, randomised and controlled study, conducted following the guidelines of ISO 9394, Ophthalmic Optics — Contact Lenses and Contact Lens Care Products — Determination of Biocompatibility by Ocular Study with Rabbit Eyes (2012). All animals were treated strictly in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and prior to the study commencement, approval from University of New South Wales Animal Care and Ethics Committee (ACEC; ref # 12/118B) was obtained.

6.2.1 Contact Lenses

Hydrogel contact lenses of etafilcon A material (ACUVUE® 2; Johnson & Johnson Vision Care Inc., Jacksonville, FL; Base curve: 8.3 mm, Diameter: 14.0 mm, Power: -3.00 Diopter) were used. These lenses were covalently coated with melimine (>80% purity; American Peptide Company, Sunnyvale, CA, USA) following the procedures described in section 3.2.2. Uncoated etafilcon A lenses were used as controls. During the trial, lenses were stored overnight in commercially available Biotrue® multipurpose contact lens solution (Bausch + Lomb, Rochester, NY, USA) in contact lens storage cases. The same multipurpose solution was used for pre-lens insertion rinsing.

6.2.2 Rabbits

A total of six female New Zealand white rabbits were allocated for contralateral melimine-coated and control contact lens wear following the protocol described in ISO 9394 (2012).

6.2.2.1 Husbandry and Food

Upon arrival, each animal was weighed and health was assessed. Animals were acclimated for at least 1 week. At the end of the acclimation, the rabbits were again evaluated for general health in accordance with animal welfare requirements described in ISO 10993-5 (2009). The animals had free access to water and commercially available rabbit feed.

6.2.2.2 Environment

The animal housing room temperature and relative humidity were monitored daily. The temperature range for the room was 16-22ºC. The recommended humidity range for the room was 30-70%. The light cycle was controlled using an automatic timer (12 hr light, 12 hr dark). The rabbit pens contained environmental enrichments (i.e. wooden boxes, in which to rest or hide).

6.2.2.3 General Health

All rabbits were monitored daily for any indication of stress by examining their movements, alertness, gait, behaviour, vocalisations, and respiration (Appendix C). Rabbits were weighed at baseline and days 8, 15 and 22 of contact lens wear. Special attention was given to observe any scratching or pawing of eyes, which might indicate ocular irritation.

6.2.2.4 Selection and Allocation

Only rabbits in good general health, weighing more than 3.5 kgs and having eyes free of clinically significant ocular irritation were used in the study. The nictitating membrane was not removed from the rabbits' eyes. Each rabbit was given a permanent ink marking and a unique name. All the rabbits wore contralateral contact lenses of

melimine-coated and control lenses. The test and control eyes were randomly assigned using Excel® (Office; Microsoft®, Redmond, USA), following ISO guidelines.

6.2.3 Lens Wear and Assessments

Rabbits wore contact lenses for 7 to 8 hr daily for 21 consecutive days. Lenses were disinfected overnight in Biotrue® multipurpose disinfecting solution when not being worn. The first day of contact lens wear was designated as day 1. Day 22 was the last day and lenses were worn for at least 4 hr on this day. Before lens insertion, lenses were examined for particulate matter, physical damage and inversion. Detailed slit-lamp ophthalmic examinations were performed prior to study commencement (base line) and immediately after lens removal on Days 8, 15 and 22 following McDonald-Shadduck Score System (Appendix D) by a masked observer. Conjunctival congestion, conjunctival swelling, conjunctival discharge, aqueous flare, iris involvement, corneal cloudiness, vascularisation and fluorescein staining were determined in each observation. Baseline examinations were performed within 24 hr of starting the study. Slit-lamp biomicroscopy was performed using a Nikon photographic slit lamp (Nikon FS-3V, Tokyo, Japan) which provided up to 32x magnification. Detailed anterior segment examination was carried out with direct, indirect and diffuse slit-lamp illumination system including sodium fluorescein (Fluorets ophthalmic strips, 1 mg, Chauvin Pharmaceuticals Ltd, Surry, UK). Wratten # 12 filter (Bausch & Lomb, Rochester, USA) was used in conjunction with cobalt blue filter to excite fluorescence while fluorescein was used to examine the eyes. Scores of "1" for fluorescent retention by the cornea are commonly noted in healthy rabbits' eyes, thus this score was not considered clinically significant. Rabbit eyes were thoroughly washed with preservative-free saline (AstraZeneca, Sydney, Australia) after all ophthalmic evaluations. Rabbits were restrained in a specially designed bag with their head outside

facing slit lamp, allowing them to sit comfortably. Ophthalmic slit-lamp photographs of both diffuse illumination and fluorescent staining were obtained during slit-lamp examination. Contact lenses that fell out of the eye during the treatment period were thoroughly examined, rinsed and reinserted. If the lenses were lost or damaged, back-up fresh lenses were inserted as replacements. A maximum of four replacement lenses were allowed during whole study. Lens retention at the rabbit eyes was checked frequently by visual inspection.

Gross ocular observations were performed immediately before lens insertion and after lens removal on days 1-7, 9-14, and 16-21 following the Draize scale for scoring ocular lesions (Appendix E). There is no pass or fail criteria in Draize scoring. Minimum conjunctival redness, such as score "1", is not considered clinically significant. Details of the treatment group are described in Table 6.1.

Table 6.1: Allocation and treatment group details of rabbits

Rabbit names (Ink identifications)	Allocation of test and control lenses		Treatment days	Contact lens daily wear period	Euthanasia
	OD	OS			
Josef (blue shoulder)	Control	Test	1 - 22	7 - 8 hrs on days 1-21; at least 4 hr on day 22	day 22
Pri (blue left ear)	Control	Test			
Sami (blue right ear)	Test	Control			
Souvik (blue both ear)	Control	Test			
Tamal (blue back)	Test	Control			
Tanima (no blue)	Control	Test			

At the end of 8 hr contact lens wear, the lenses were removed from each eye, inspected for damage, rinsed and soaked overnight in the designated storage cases with solutions.

6.2.4 Euthanasia

On day 22, following the final ophthalmic observations, contact lenses were removed and all rabbits were anesthetised using 5% isoflurane (Cenvet Australia Pty Ltd, Marayong, Australia), induced via induction chamber and anaesthesia was then maintained using a mask. Following sedation, rabbits were euthanised by administration of 100 mg kg^{-1} sodium pentobarbitone (Cenvet Australia Pty Ltd, Marayong, Australia) by intravenous injection.

6.2.5 Histopathology

Twelve corneas of six rabbits were collected in 4% formaldehyde (BDH Chemicals, Victoria, Australia) for histopathology. Corneal samples were placed in cassettes then loaded into a Shandon Excelsior™ ES Tissue Processor (Thermo Fisher Scientific, Waltham, USA) for overnight processing (infiltration with paraffin). Samples

were then removed and embedded in wax moulds on a Shandon Histocentre™ 3 (Thermo Fisher Scientific, Pittsburgh, USA). Wax blocks were trimmed and sections were cut on the Leica RM 2165 Microtome (Leica Microsystems, Rijswijk, The Netherlands) at 4 µm thickness. Slides were placed in a laboratory oven at 56°C for one hour and stained with haematoxylin and eosin (H&E) using a Leica XL Autostainer (Leica Microsystems Inc., Bannockburn, USA). Slides were then coverslipped using the Dako CR 100 Coverslipper (Dako, Produktionsvej, Denmark) and allowed to dry overnight. Processed slides were stored at 4°C prior to microscopic examination.

6.2.6 Data Analysis

Data were analysed using Microsoft® Office Excel® and Statistical Package for the Social Sciences software (SPSS) for Windows software version 21.0 (SPSS, Inc., Chicago, IL, USA). Lens retention was determined as follows: the total possible wear time per rabbit was 172 hr (8 hr per day for 6 rabbits on day 1 to 21 and 4 hr on day 22nd). The total possible wear time for all rabbits was 1032 hr. According to ISO 10993-10 (2002) Annex B, if any treated eye in more than one animal exhibited an irritation response at any observation period, the investigated type of contact lens will be considered an eye irritant. If any lens was found missing during contact lens wear, then the time between that lens check and the preceding check was subtracted from the rabbit wear time. Percent lens retention was calculated as [(actual wear time for duration of study) / (total possible wear time for duration of study)] x 100. Analytical manipulation of the data, such as the sum or frequency of scores, was calculated where appropriate.

6.3 RESULTS

All six animals included in the study maintained good health and no abnormal behaviour was observed during the study. Descriptive statistics on body weight are presented in Table 6.2. One rabbit lost a negligible amount of weight (100 g) during the study, while the remaining rabbits maintained or gained weight.

Table 6.2: Body weights of rabbits during study

Animal names	Body weights (kg)			
	Day 1	Day 8	Day 15	Day 22
Josef	3.5	3.4	3.4	3.4
Pri	3.9	3.9	4.0	3.9
Sami	3.7	3.8	4.2	4.3
Souvik	3.5	3.7	3.8	3.7
Tamal	3.4	3.4	3.5	3.5
Tanima	3.7	3.8	3.9	3.9
Mean ± SD	3.6 ± 0.2	3.7 ± 0.2	3.8 ± 0.3	3.8 ± 0.3

Contact lens loss during the study was infrequent. Data on lens retention are presented in Table 6.3. Lens retention was 94% for melimine-coated contact lenses and 96% for control contact lenses.

Table 6.3: Contact lens retention (%) during study

Rabbits	Control lens	Total study days lens lost / 22 days	Melimine-coated lens	Total study days lens lost / 22 days
Josef	OD	1	OS	1
Pri	OD	0	OS	0
Sami	OS	0	OD	0
Souvik	OD	1	OS	0
Tamal	OS	1	OD	3
Tanima	OD	2	OS	4
Retention (%)		96		94

6.3.1 Ocular Response

6.3.1.1 Gross ocular examination

Gross ocular scores following the Draize scale for scoring ocular lesions are presented in Table 6.4. This observation was performed on study days 1 to 7, 9 to 14, and 16 to 21. Mild conjunctival redness (score 1) was observed twice with melimine-coated lenses. Mild conjunctival discharge (score 1) and redness were observed once with control contact lenses. The remaining eyes appeared normal (score 0) for both melimine-coated and control lenses throughout the study.

Table 6.4: Gross ocular observation by Draize scale.

Lenses	Rabbit	Eye	Response	Grade	Study day
Melimine-coated	Pri	OS	Conjunctival redness	1	9th
	Tamal	OD	Conjunctival redness	1	9th
Control	Josef	OD	Conjunctival discharge	1	14th
	Pri	OD	Conjunctival redness	1	9th
Unless mentioned above all eyes appeared normal; score = 0					

6.3.1.2 Ophthalmic observation by slit lamp biomicrosocopy

Ophthalmic observation by slit lamp biomicroscopy was performed following the McDonald-Shadduck score system prior to study commencement and immediately after lens removal on days 8, 15 and 22. Slit lamp biomicroscopy scores are presented in Table 6.5. Unless detailed in Table 6.5, all eyes appeared normal. At baseline, corneal fluorescein staining (score 1) was observed in two eyes in each treatment group. Mild conjunctival congestion (score 1) and mild corneal fluorescein staining (score 1) were the only two other signs occasionally observed in the study in both the treatment groups.

Table 6.5: Ophthalmic observation by slit lamp biomicrosocopy

Lenses	Day	Ophthalmic observation	Number of eyes	Score
Melimine-coated	Baseline	Corneal fluorescein staining	2	1
	Day 8	Conjunctival congestion	1	1
		Corneal fluorescein staining	1	1
	Day15	Appeared normal	0	0
	Day 22	Conjunctival congestion	2	1
		Corneal fluorescein staining	2	1
Control	Baseline	Corneal fluorescein staining	2	1
	Day 8	Conjunctival congestion	1	1
		Corneal fluorescein staining	1	1
	Day 15	Conjunctival congestion	2	1
	Day 22	Conjunctival congestion	1	1
Unless mentioned above, all eyes appeared normal (score = 0)				

Diffuse and fluorescent slit lamp photographs of melimine-coated and control contact lenses worn by the same rabbit at baseline, day 8, day 15 and day 22 are shown in Figure 6-1. The fluorescein photographs in Figure 6-1 (pictures 1C to 4C and 1D to 4D) confirm absence of corneal staining of control and test eyes. Neither melimine-coated nor control contact lens wear was associated with any other slit lamp biomicroscopy signs of ocular irritation such as conjunctival chemosis or swelling, discharge, iris changes, corneal cloudiness or vascularisation. Over the study period, observations made by slit lamp biomicroscopy examinations indicated no significant clinical signs that might suggest ocular irritation induced by the melimine coating. None of the rabbits was discontinued from contact lens wear during trial.

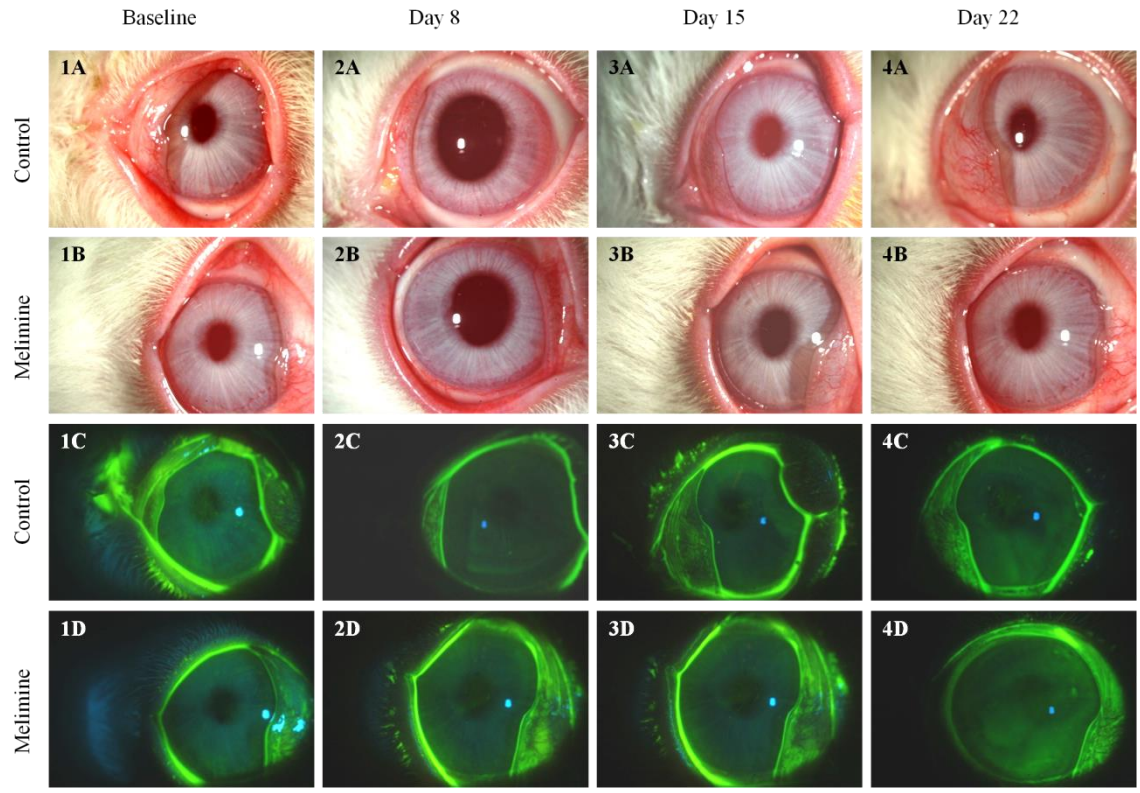

Figure 6-1: Representative images of diffuse and fluorescent slit lamp photographs of one control and melimine-coated contact lens worn rabbit eyes over 22 days.

A & B = Diffuse; C & D = Fluorescent slit lamp photographs; 1 = Baseline, 2 = day 8, 3 = day 15, 4 = day 22 observations. Captured using slit lamp biomicroscope at 32x magnification.

6.3.2 Histopathology

Histopathology of corneal sections stained with H&E indicated no major structural differences between corneas exposed to melimine-coated or control contact lenses. All the sections from 12 corneas showed normal central and peripheral structure. All the three layers of the corneal epithelium (basal layer, intermediate layer and flattened cells) were intact and identical in all sections observed with high (40x objective) magnification. Figure 6-2 shows representative H&E stained light micrographs of corneal sections from rabbit eyes after melimine-coated and control

contact lens wear. The empty spaces in the stroma are artefacts produced during histopathology processing.

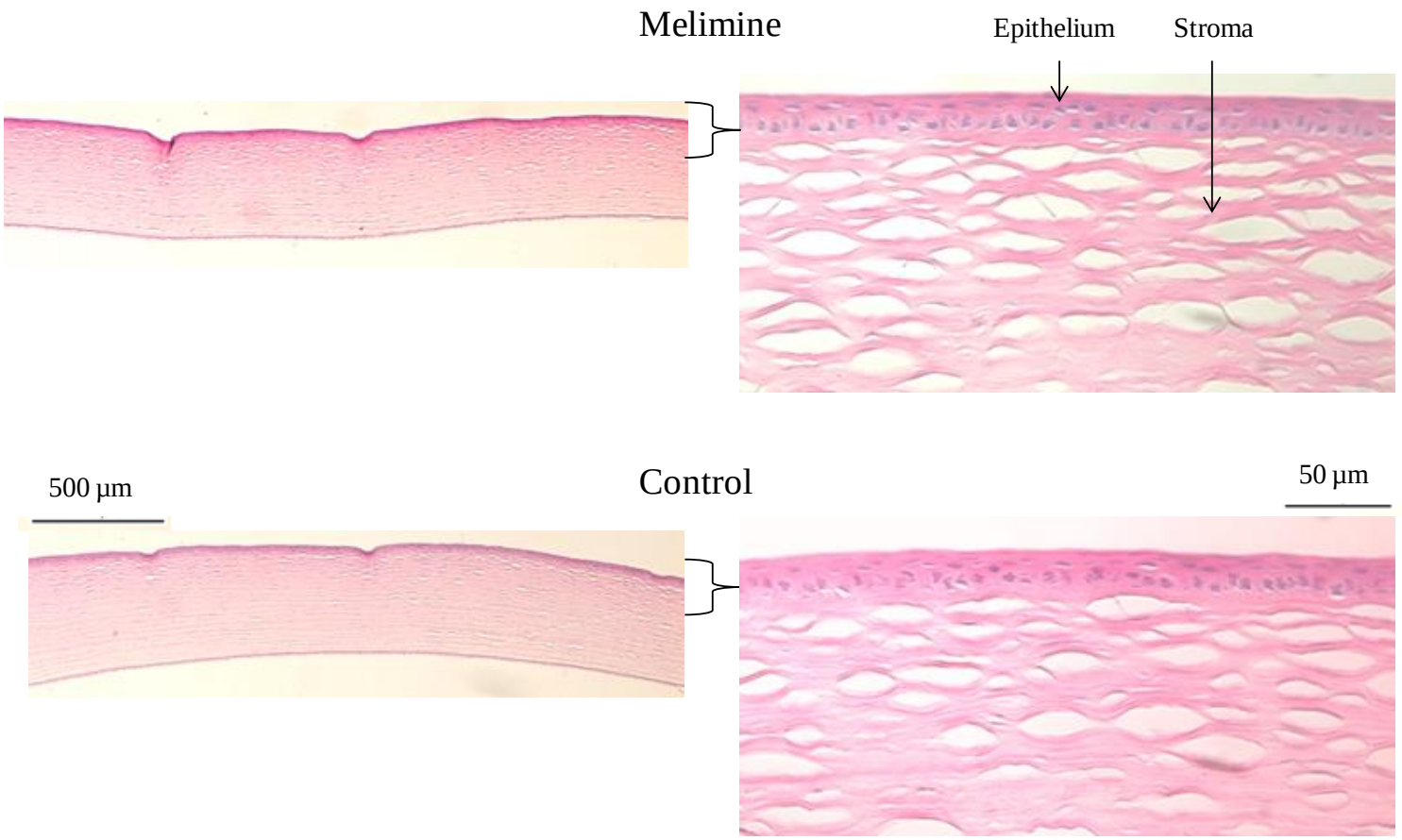

Figure 6-2: Representative light micrographs of comparative rabbit corneal histology sections after melimine-coated and control contact lens wear for 22 days.

6.4 DISCUSSION

Melimine-coated contact lenses did not produce ocular irritation for any rabbits during 22 days of daily lens wear. All the animals during this trial remained healthy and behaved normally, and no ocular irritation-related symptom such as eye scratching or pawing of eyes was observed. Both melimine-coated and control contact lenses were retained in rabbit eyes at an acceptable level (> 90%). Slit lamp and gross ocular observation of cornea, conjunctiva and ocular adnexa confirmed the absence of ocular signs that might indicate irritation. Overall, corneal fluorescein staining indicated no difference between eyes during melimine-coated or control contact lens wear. This fact was supported by histopathological investigation which confirmed the absence of toxicity to corneal tissue, especially epithelium. A previous study has shown that melimine-coated contact lenses were able to reduce the clinical manifestations of CLPU and CLARE, arising from both Gram positive and Gram negative bacterial contamination in rabbit and guinea pig models respectively (Cole *et al.*, 2010). Considering the results presented here and those of previously reported studies it may be concluded that, at least in animal models, melimine-coated contact lenses are safe in the wear modalities that have been investigated.

In summary, the results of this chapter indicate that melimine-coated contact lenses are biocompatible with rabbit eyes in daily wear. The results of this study and the previous *in vitro* toxicity studies described in section 4.3.2 with murine cells indicate that these lenses are non-toxic to mammalian cells. However, there is a need for further investigation evaluating the biocompatibility and retention of antimicrobial activity of melimine-coated contact lenses in human participants.

Chapter 7: Biocompatibility and Retention of Antimicrobial Activity of Melimine-Coated Contact Lenses in a Human Clinical Trial

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7.1 INTRODUCTION

Microbial contamination of contact lenses during wear is closely associated with ocular inflammation such as contact lens-induced acute red eye (CLARE) (Sankaridurg *et al.*, 1996a; Holden *et al.*, 1996), contact lens peripheral ulcer (CLPU) (Wu *et al.*, 2003) and infiltrative keratitis (IK) (Willcox *et al.*, 2011). Although rare, microbial keratitis (MK) is a sight-threatening contact lens-related infection (Willcox *et al.*, 2001b; Dart *et al.*, 1991; Ormerod *et al.*, 1986). Such infection and inflammations continue to be an ongoing problem with contact lens wear for wearers and practitioners alike. A contact lens with high antimicrobial activity may inhibit microbial adhesion and consequently reduce these contact lens related adverse events. Similar to other antimicrobial peptides (AMPs), melimine has antimicrobial activity (Willcox *et al.*, 2008a) and previous chapters have demonstrated that it can be attached to lenses in high density via EDC coupling. The melimine-coated lenses have broad spectrum activity against various strains of bacteria, fungi and *Acanthamoeba in vitro*. In addition, rabbits could wear melimine-coated lenses on a daily wear basis for 22 days with no adverse effects.

Although more than 900 to 1,200 antimicrobial peptides (AMPs) have been discovered (Yount *et al.*, 2006; Brogden, 2005), only a few have been tested on humans (Brandenburg *et al.*, 2012). Most of the clinical trials have studied AMPs as topical applications and therefore no detailed data about systemic toxicology or pharmacokinetic mechanisms are available (Yeung *et al.*, 2011; Mayer *et al.*, 2010). Polymyxin B (Falagas *et al.*, 2005; Bradshaw, 2003), gramicidin S (Bradshaw, 2003), ambicin (nisin) (Cheigh *et al.*, 2005; Hansen, 1994), micafungin (Smith *et al.*, 2010), anidulafungin (Smith *et al.*, 2010), pexiganan (Lipsky *et al.*, 2008; Hancock *et al.*,

1998), omiganan (Yeung *et al.*, 2011) and iseganan (Trotti *et al.*, 2004) are the few polypeptides that have been investigated for human use. In a placebo controlled, double masked, randomised clinical trial Lipsky *et al.* (2008) evaluated pexiganan acetate cream to treat mildly infected diabetic foot ulcers in comparison to systemic ofloxacin, and showed that the topical AMP cream was an effective alternative, and might avoid the risk of developing antibacterial resistance. Another phase III trial demonstrated that use of omiganan was associated with significant reductions in catheter related infections (Yeung *et al.*, 2011). Clinical application of these AMPs has been challenging and several problems have proved difficult to solve. Problems include, toxicity against eukaryotic cells, development of allergies to peptides and *in vivo* reduction of antimicrobial efficacy (Bradshaw, 2003). Redesigning these novel antimicrobial peptides and optimisation of surface attachment have been suggested to overcome these road blocks (Yeung *et al.*, 2011; Costa *et al.*, 2011; Bradshaw, 2003). Melimine bound lenses have shown promising preclinical results *in vitro* and *in vivo* studies (chapter 4 and chapter 6), therefore it is worthwhile to investigate the performance of the melimine-coated lenses in terms of ocular health, subjective responses, and retention of antimicrobial activity in a human clinical trial.

Randomised controlled trials (RCT) are best suited for exploration of safety and biocompatibility of new investigational contact lenses, providing the strongest evidence of association between a study factor and the outcome of interest (Chalmers *et al.*, 1981). This study design has less bias and fewer other potential errors compared to any other study design (Chalmers *et al.*, 1981). Thus, the aims of this chapter were to evaluate the biocompatibility and retention of antimicrobial activity of melimine-coated contact lenses in a human clinical trial. The hypotheses of this study were that the

melimine-coated contact lenses are safe during human wear and retain antimicrobial activity after lens wear.

7.2 METHODS

7.2.1 Contact Lenses

Hydrogel contact lenses of etafilcon A material (ACUVUE® 2; Johnson & Johnson Vision Care Inc., Jacksonville, FL) were used in this study (Table 7.1). The lenses were covalently coated with melimine (>80% purity; American Peptide Company, Sunnyvale, CA, USA) as described in section 3.2.2. Uncoated etafilcon A lenses were used as controls. Ten melimine-coated contact lenses were not used for human trial testing but were used as unworn lenses during testing for retention of antimicrobial activity. To facilitate masking of the contact lens types during lens dispensing, control lenses were carefully removed from the blister packets, washed three times in phosphate buffered saline (PBS) and autoclaved in 5 ml of PBS in a glass vials which are visually identical to the melimine-coated contact lens vial. All the contact lenses were stored in a cold room (5ºC) until required.

Table 7.1: Description of contact lenses and modality for use in the clinical trial

Contact lens	Manufacturer	Materials (coatings)	Mode of wear	Power (Ds)	Diameter (mm)	Base curve (mm)
ACUVUE® 2®	Johnson & Johnson	etafilcon A	Daily wear (one day)	+8.00 to -12.00 Ds (spherical only)	14	8.7
ACUVUE® 2®	Johnson & Johnson	etafilcon A + melimine	Daily wear (one day)	+8.00 to -12.00 Ds (spherical only)	14	8.7

7.2.2 Study Design

7.2.2.1 Type of Investigation

This was a prospective, randomised, double-masked, contralateral, one day clinical trial in which participants' subjective responses and corneal health were evaluated, and the lenses were collected on completion of the study to determine the retention of antimicrobial activity.

7.2.2.2 Study Aims

- Primary aim: Safety of melimine-coated contact lenses in terms of ocular physiology and subjective responses.
- Secondary aim: Retention of antimicrobial activity of collected (worn) lenses when tested in laboratory.

7.2.2.3 Study Endpoints

- Primary: corneal fluorescein staining, and bulbar, limbal and palpebral redness
- Secondary: reduction in viable bacterial adhesion

7.2.2.4 Inclusion Criteria

Participants were healthy and were not taking any medications for general health conditions. Both experienced and non-contact lens wearers were included in the study. However, known contact lens wearers were advised to discontinue lens wear two days prior to the start of the trial. The specific inclusion criteria are listed below:

- Able to read and comprehend English and give informed consent as demonstrated by signing a record of informed consent

- At least 18 years old
- Willing to comply with the wearing and clinical trial visit schedule as directed by the investigator
- Have ocular health findings considered to be "normal" and which would not prevent the participant from safely wearing contact lenses
- Be correctable to 6/12 (20/40) or better in each eye with contact lenses
- Have experienced an ocular adverse event that is not considered to be severe by the investigator (Investigator discretion) or that is temporary in nature

7.2.2.5 Exclusion Criteria

Participants enrolled in the trial must NOT:

- have any pre-existing ocular irritation, injury or condition (including infection or disease) of the cornea, conjunctiva or eyelids that would preclude contact lens fitting and safe wearing of contact lenses
- have any systemic disease (self-reported) that adversely affects ocular health e.g. diabetes, Graves disease, and auto-immune diseases such as ankylosing spondylitis, multiple sclerosis, Sjögrens syndrome and systemic lupus erythematosus. Conditions such as systemic hypertension and arthritis do not automatically exclude prospective participation
- be using or a need for concurrent category S3 and above ocular medication at enrolment and/or during the clinical trial
- be using or a have need for any systemic medication or topical medications up to 12 weeks prior to during the trial that may alter normal ocular findings / are known to affect a participant's ocular health /

physiology or contact lens performance either in an adverse or beneficial manner at enrolment and/or during the clinical trial

- have had eye surgery within 12 weeks immediately prior to enrolment for this trial
- have undergone corneal refractive surgery
- have contraindications to contact lens wear
- be currently enrolled in another clinical trial
- be pregnant or lactating

7.2.2.6 Sample Size Calculation

Sample size was determined based on a previous study (Diec *et al.*, 2012) which used equivalent techniques to those used in this study. Seventeen participants were enrolled in order to demonstrate a statistically significant difference in corneal staining score of 0.5 ± 0.7 at the 5% level of significance and 80% power.

7.2.2.7 Masking Procedure

Both the participants and the investigators were masked to the type of contact lenses dispensed during the clinical trial. The investigators were unmasked during data analysis of the trial.

7.2.2.8 Clinical Trial Randomisation

The randomisation plan was generated as per http://www.randomization.com/. A randomisation list was generated and applied through the Clinic Data Management system. Each participant was randomised to wear a melimine-coated contact lens in one eye and an uncoated contact lenses as a control in the contralateral eye.

7.2.3 Ethics Approval

This study was approved by Human Research Ethics Committee (HREC) of the University of New South Wales (approval ref # HC13087) and followed the tenets of the Declarations of Helsinki 1975 as amended in 2000 including local regulations as applicable such as Therapeutic Goods Administration, Australia (TGA; Appendix F). The clinical trial was conducted under the clinical trial notification (CTN) scheme following the regulations of the Therapeutic Goods (Medical Devices) Regulations 2000. The clinical trial was registered in the publicly accessible Australian New Zealand Clinical Trial Registry (ANZCTR; trial ID: ACTRN 12613000369729).

7.2.3.1 Safety Reporting

A data safety monitoring board (DSMB) was formed with the approval from Human Research Ethics Committee, UNSW to report any unexpected adverse event.

7.2.4 Participant Enrolment and Selection

Subjects were recruited from the subject population at Brien Holden Vision Institute and School of Optometry and Vision Science, UNSW by way of a general email and from the general population by way of HREC approved advertising in UNSW news sources. Participants were screened for general clinical trial suitability following a routine eye examination which included refraction, visual acuity and general eye health. Informed consent (Appendix B) was obtained from all the participants before the trial. Both experienced contact lens wearers and neophytes (participants with no prior lens wear experience) were enrolled. Subjects received a $15 gift card for each scheduled visit in compensation for the expenses incurred by the participants in attending the clinic.

7.2.5 Study Visits and Clinical Techniques

A baseline visit was conducted to assess the suitability of the participants and baseline measurements were taken for the trial. A total of four visits were undertaken; lens dispensing (visit 1), lens collection after 8 hr (visit 2) and follow ups after 1 week and 4 weeks (visits 3 and 4). As both the follow up visits included no assigned contact lens wear, participants were free to wear their own lenses or glasses if needed. A follow up visit after 4 weeks was recommended by UNSW Human Research Ethics Committee (HREC) to exclude the possibility of delayed toxicity of melimine on the human eye. The study visits and clinical techniques used at each time point are outlined in Table 7.2.

Table 7.2: Clinical techniques and assessment of variables of each visit

Procedures	Base line visit (BL)	Lens dispensing visit	Lens collection visit (8 hr)	1 week follow up visit	4 weeks follow up visit	Unscheduled/ Adverse events
Visit window	N/A	≤10 days from BL	± 2 hr	± 3 days	±1 week	N/A
Informed consent	Y	N	N	N	N	N
Meet inclusion / exclusion criteria	Y	Y	N	N	N	N
Demographics	Y	N	N	N	N	N
History at baseline	Y	N	N	N	N	N
Updated history / any medical problems	N	Y	Y	Y	Y	Y
Ocular symptoms and problems	Y	Y	Y	Y	Y	Y
Baseline information: - visual acuity - spectacle refraction - keratometry	Y	N	N	N	N	N
Recheck visual acuity	N	Y	Y	Y	Y	Y
Wear time, compliance assessment	N	N	Y	N	N	*
Slit-lamp biomicroscopy: Ocular assessment including staining with fluorescein	Y	Y	Y	Y	Y	Y
New lens dispense	N	Y	N	N	N	N
Slit-lamp biomicroscopy: lens fit assessment	N	Y	Y	N	N	*
Photos / video	*	*	*	*	*	Y**
Aseptic lens removal	N	N	Y	N	N	*
Contact lens collection / antimicrobial efficacy assessment	N	N	Y	N	N	**
Questionnaires	N	N	Y	N	N	N
Lid and conjunctiva swabs	N	N	N	N	N	**
Adverse event assessment	Y**	Y**	Y**	Y**	Y**	Y**
Visit summary	Y	Y	Y	Y	Y	Y

Y = Yes, required information, N = No, not required
* *At optometrists discretion*
** *If adverse event detected at time of visit*

7.2.5.1 Ocular Assessments

Visual acuity was measured at each visit using computer letter charts (Ehrmann *et al.*, 2009). Slit lamp biomicroscopy (Zeiss SL-120, Carl Zeiss Meditech, Jena, Germany) was performed by a single masked observer following the schedule in Table 7.2. Bulbar and limbal redness, palpebral redness and roughness, corneal and conjunctival staining were assessed at all visits. Examination of corneal and conjunctival staining and lens induced conjunctival indentation was conducted with fluorescein (Fluorets ophthalmic strips, 1 mg, Chauvin Pharmaceuticals Ltd, Surry, UK) with the help of Wratten # 12 filter (Bausch & Lomb, Rochester, USA) in conjunction with cobalt blue filter. Examination with fluorescein was conducted before and after contact lens wear in the lens dispensing and collection visit respectively. Fluorescein was carefully washed from the eyes prior to the lens insertion. Lenses were inserted and removed using aseptic gloves (DermaClean®Sterile, Ansell Ltd, Richmond, Australia). Lens surface deposits and wetting, back surface debris, centration, tightness, fluting, primary gaze movement and gaze lag, corneal coverage and overall acceptance were assessed at the lens dispensing and collection visits. Slit lamp photographs were taken using a Nikon photographic slit lamp (Nikon FS-3V, Tokyo, Japan) which provided up to 32x magnification. Subjects were asked to rate the comfort of the lenses based on their overall impression of ocular comfort, ocular dryness, lens awareness and lens edge awareness at the time of contact lens collection, using a 1-10 scale using whole number steps (1 = very uncomfortable, dry or aware; 10 = comfortable, not-dry or not-aware). Participants were asked for the preference of either eye (forced preference: either right or left eye) based on contact lens wear experience. After wear, lenses were collected in a glass vial containing 2 ml sterile PBS.

7.2.5.2 Grading Scales

Clinical grading was conducted using the CCLRU (Terry *et al.*, 1993) grading scales (zero to four units) interpolated into 0.1 increments (Appendix G). The scale includes four images increasing in severity of the condition. The photographs are labelled as follows: 1, very slight; 2, slight; 3, moderate; 4, severe.

7.2.6 Retention of Antimicrobial Activity, Strains and Adhesion Conditions

Aseptically collected and unworn contact lenses were processed for evaluation of retention of antimicrobial activity within 48 hr. Reduction in adherent viable bacteria to worn melimine-coated contact lens compared to the worn uncoated control lens was calculated as the retention of antimicrobial activity after lens wear. The bacterial adhesion conditions have been described in section 3.2.4. Briefly, PBS and 10x diluted tryptone soya broth (1/10 TSB; Oxoid, Basingstoke, UK) were used as assay media for the evaluation of antimicrobial activity against *Pseudomonas aeruginosa* 6294 isolated from MK and *Staphylococcus aureus* 31 isolated form CLPU respectively. All contact lenses were incubated with 1 ml bacterial suspension of 10^6 colony forming units (CFU) ml^{-1} at 37ºC for 18 hr.

Following incubation with bacteria, contact lenses were washed three times with PBS to remove non-adherent cells and then stirred rapidly in 2 ml of PBS containing a small magnetic stirring bar. Following log serial dilutions in Dey Engley neutralising broth (DE; Becton, Dickson and Company, USA), 3 x 50 µl of each dilution were plated on a tryptone soya agar (TSA; Oxoid, Basingstoke, UK) containing Tween 80 (0.5%; Sigma-Aldrich, St Louis, USA) and lecithin (0.07%; Sigma-Aldrich, St Louis, USA) for recovery of cells. After 24 hr incubation at 37ºC, the viable microorganisms were

enumerated as CFU mm^{-2} of the lens surface. Results are expressed as the reduction in adherent viable bacteria compared to the uncoated control lens of triplicate measurements performed on a minimum of three separate occasions.

7.2.7 Data Analysis

Data were analysed using Microsoft® Office Excel® and Statistical Package for the Social Sciences software (SPSS) for Windows software version 21.0 (SPSS, Inc., Chicago, IL, USA). Human clinical and subjective ratings were summarised using descriptive statistics. Differences between lens types were determined at each visit using paired *t*-test or Wilcoxon Signed-Rank test based on the type of variable. Frequency and percentage of participants preferring any of the contact lens types were reported for each preference category. The bacterial adhesion data were $\log_{10}(x+1)$ transformed prior to data analysis where x is the number of adherent bacteria in CFU mm^{-2}. Differences in bacterial adhesion were analysed using independent 2-sample *t*-test. Statistical significance was set at 5%.

7.3 RESULTS

A total of 17 participants were enrolled in this study; eight of them were experienced wearers. There were no dropouts during the clinical trial and data from all the enrolled participants were included in the analysis. Table 7.3 shows the gender demographics, mean age and contact wear time while Figure 7-1 displays the ethnic distribution in the study group. Table 7.4 shows refractive error and keratometry readings at the baseline visit (n = 34).

Table 7.3: Lens wear time and gender demographics of study participants.

Number of males	Number of females	Total number of participants	Age (mean years ± SD)	Contact lens wear time (mean hrs ± SD)
7	10	17	30.9 ± 9.4	6.9 ± 0.9

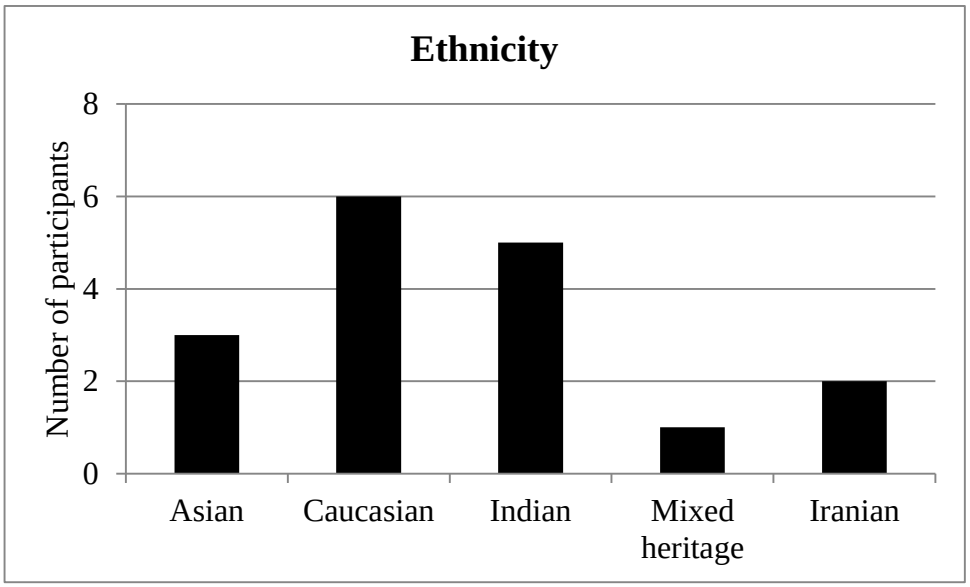


Figure 7-1: The spread of ethnicities among the study participants.

Table 7.4 Refractive error and keratometry readings at baseline for study participants

Variables	Mean	SD
Refractive error - sphere (Ds)	-1.15	1.70
Refractive error - cylinder (Dc)	-0.29	0.43
Keratometry - flat (D)	43.30	1.42
Keratometry - steep (D)	43.86	1.58

7.3.1 Adverse Events

There were no adverse events associated with any lenses during the trial. All participants successfully completed the study. However, one participant developed mild bilateral IK 4 weeks after the study had completed. The participant had no adverse ocular findings at the 1 week follow up visit. The incident was related to participant's habitual wear of contact lenses. The participant was followed up for a further 2 weeks at which time the IK event had resolved.

7.3.2 Lens Surface Characteristics

There were no significant differences ($p > 0.05$) seen in wettability or surface deposition between melimine-coated and control contact lenses during both lens dispensing and collection visits. The levels of debris and deposits were low at each time point. Small (< 0.1 mm diameter), round, grey / brown particles (usually singular) were observed at the anterior surface of two melimine-coated and one control contact lenses. The presence of these particles did not affect subjective comfort or vision and was likely to be related to lens preparation or handling prior to study commencement.

7.3.3 Lens Fit Characteristics

There was no significant difference between melimine-coated and control lenses ($p > 0.05$) in X / Y centration at lens insertion and prior to lens removal. There was no difference ($p > 0.05$) in the amount of lens movement and tightness between the two lens types. Melimine-coated lenses showed clinically acceptable centration, movement and tightness at all times. Overall, fitting acceptance for both the lens types at both time points was rated highly (above 3.0) which indicated complete corneal coverage, good centration, adequate primary gaze movement and acceptable tightness. None of the contact lenses needed to be refitted and no lens loss was reported.

7.3.4 Ocular Physiology

There were no significant differences ($p > 0.05$) in different areas of bulbar redness, limbal redness, palpebral redness and palpebral roughness between the melimine-coated and control lenses. Clinically, one participant with melimine-coated lenses (patient # 4) showed slightly higher conjunctival staining in all four quadrants (average difference in grade 0.7). Overall, melimine-coated lenses did not show any significant difference ($p > 0.05$) in conjunctival indentation and staining when compared to contact lenses. However, melimine-coated contact lens wear was associated with significantly higher levels of extent, depth and type of corneal staining in all areas compared to the control lenses (Table 7.5). Figure 7-2 shows the extent, depth and type (median; mean ± SD) of fluorescein staining associated with melimine-coated and control lenses in all the corneal areas. Both mean and median corneal staining was higher in corneas with melimine-coated lenses than controls. The fluorescein staining observed with melimine-coated lenses was similar to solution-induced corneal staining (SICS) reported to be associated with some silicone hydrogel

contact lens and lens care solutions (Diec *et al.*, 2012; Carnt *et al.*, 2007b; Jones *et al.*, 1997). Melimine-coated contact lenses were associated with higher standard deviation of corneal staining (range: 1.6 to 1.8) than controls (range: 0.4 to 1.3), because 10 participants demonstrated sectoral or diffuse corneal staining but other participants did not have any staining with melimine-coated lenses.

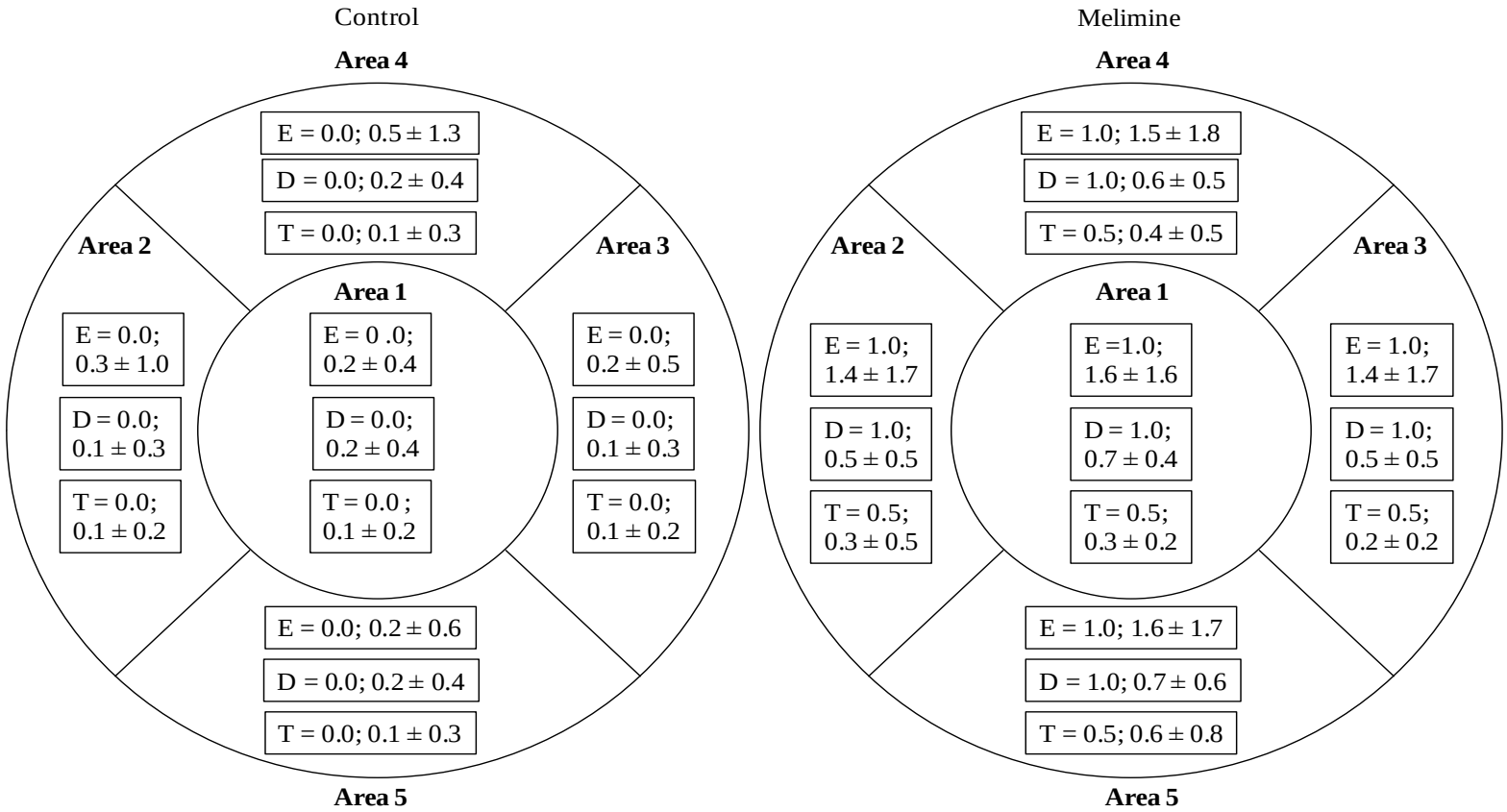


Figure 7-2: Fluorescein staining scores (median; mean ± SD) in melimine-coated and control contact lens corneal areas

Table 7.5: *p*-values for differential staining between melimine-coated and control lenses

Corneal areas	Extent	Depth	Type
1	< 0.01	< 0.01	< 0.01
2	= 0.02	= 0.02	= 0.02
3	= 0.01	= 0.02	= 0.02
4	= 0.01	< 0.01	= 0.02
5	< 0.01	= 0.01	= 0.04

Participant number # 4 had higher conjunctival and corneal staining in all the areas with melimine-coated lens. Figure 7-3 presents slit lamp photographs of this participant with superficial punctuate corneal staining after melimine-coated contact lens wear. None of the participants had any infiltrates or any other ocular signs associated with melimine-coated or control lens wear.

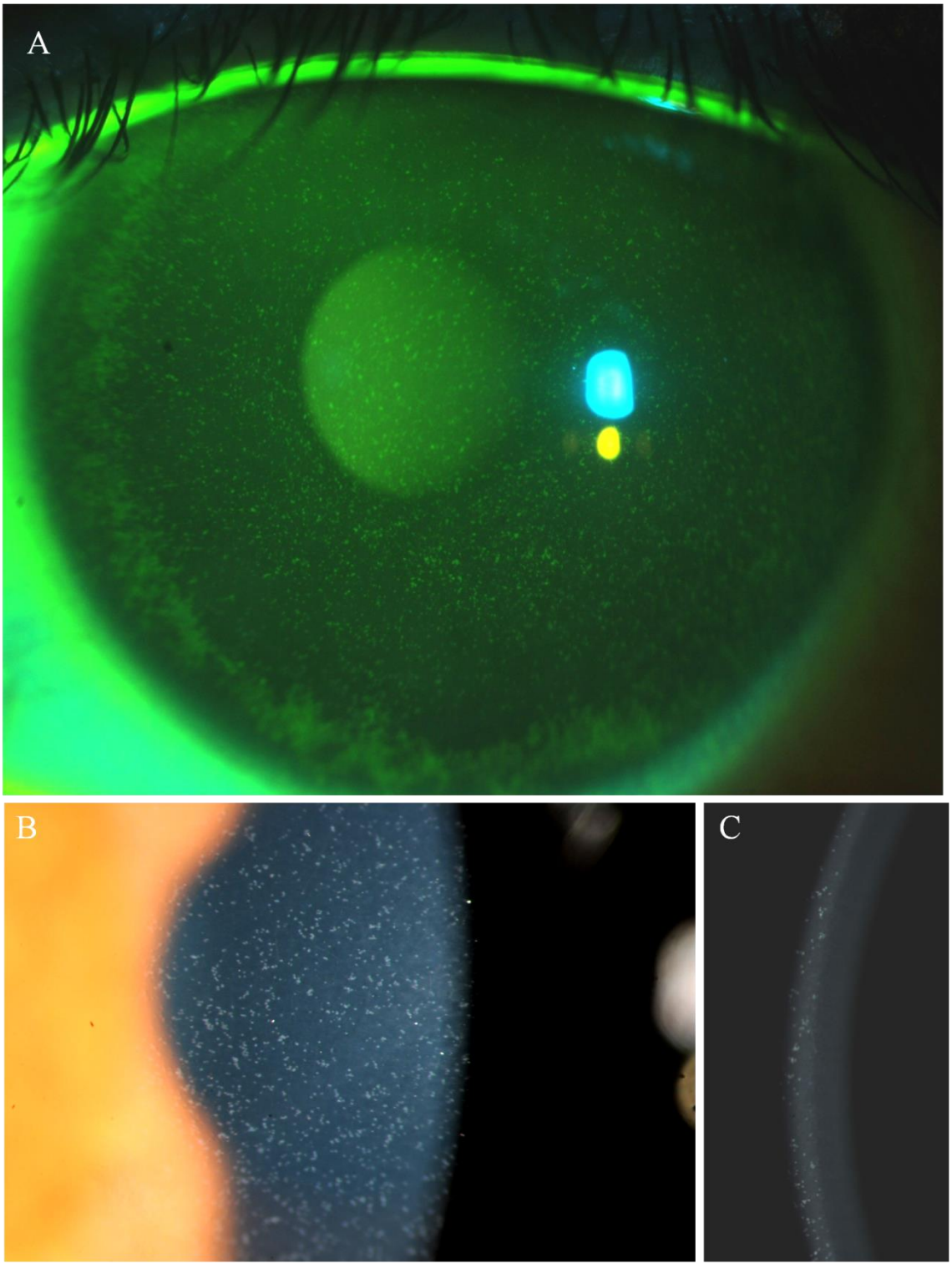


Figure 7-3: Diffuse corneal staining after melimine-coated contact lens wear.

Corneal staining was photographed with cobalt blue light in conjunction with a Wratten #12 filter after instillation of sodium fluorescein (A; with 16x magnification), white light using direct illumination (B; with 25x magnification), and optic section (C; with 30x magnification). Photographs A and B detail the type and extent of the staining, whereas photograph C details the depth.

7.3.5 Subjective Ratings

7.3.5.1 Comfort

Average comfort ratings for the subjects wearing the melimine-coated and control lenses are shown in Figure 7-4. Standard deviations of comfort ratings for melimine-coated lenses (range: 1.9 to 2.5) were higher than control lenses (range: 1.7 to 2.0). Distribution of comfort scores during melimine-coated and control contact lens wear is presented at Figure 7-5 using box plots. One participant (number # 4) was uncomfortable with the melimine-coated lens and reported low scores in subjective comfort response; lens awareness and lens edge awareness that are represented as the outliers in that figure. There was no significant difference in overall contact lens comfort (p = 0.07), dryness (p = 0.10), lens awareness (p = 0.06) or lens edge awareness (p = 0.20), although all the scores were slightly worse with melimine-coated lenses.

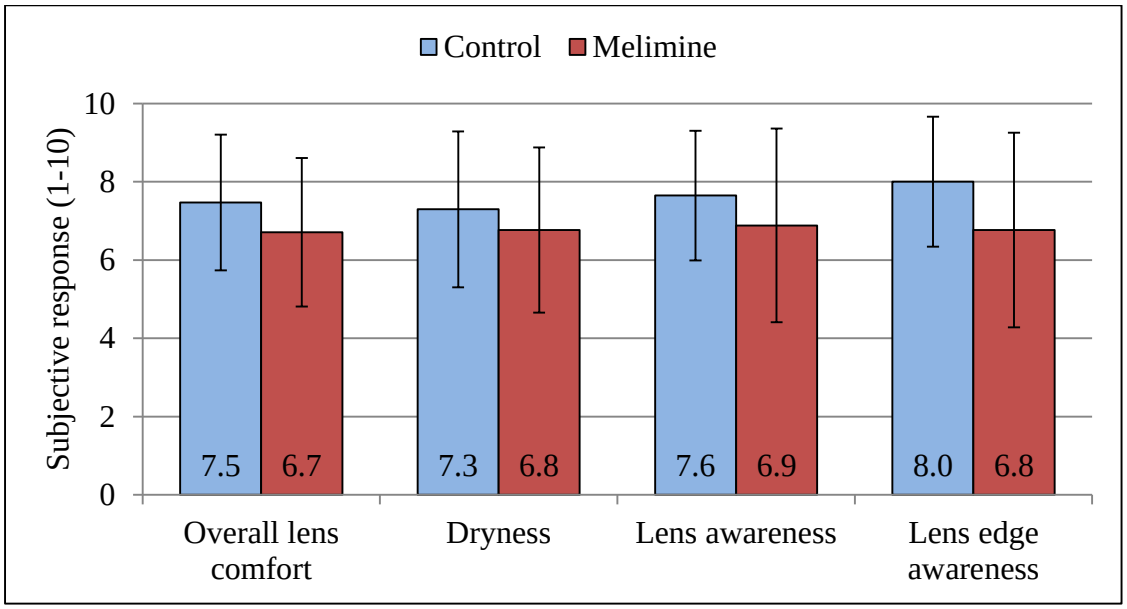


Figure 7-4: Comfort ratings (mean ± SD) for melimine-coated and control lenses.

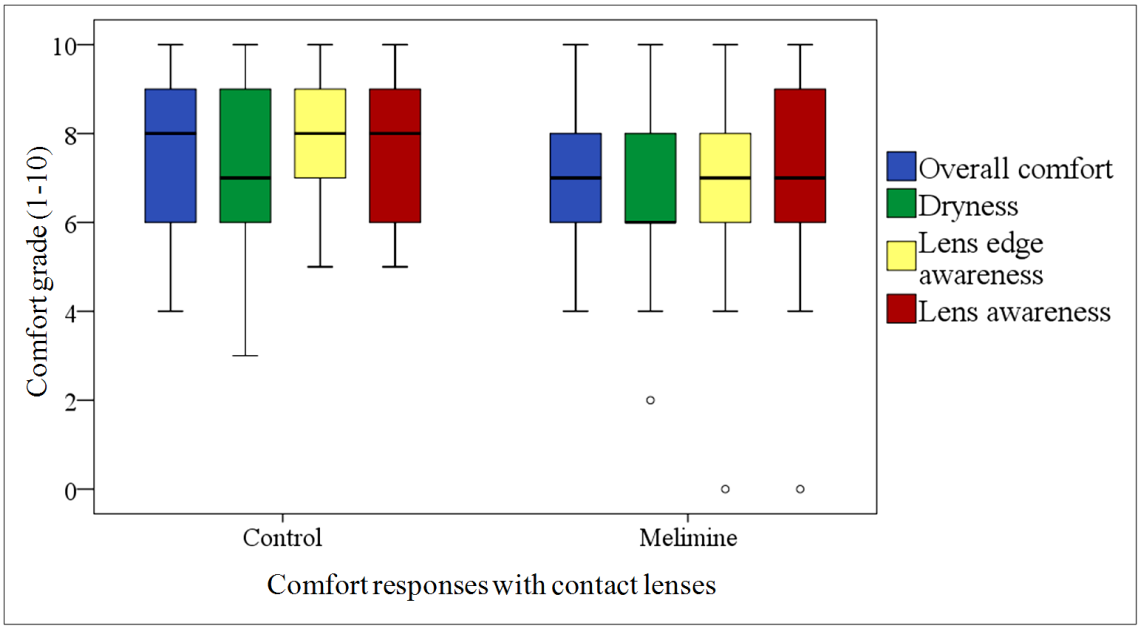


Figure 7-5: Distribution of comfort scores during melimine-coated and control contact lens wear.

Data are presented as box plots showing median, 25th and 75th percentile ranges.

7.3.5.2 Participant preference

Overall, participants preferred the control lenses to the melimine-coated lenses. This study was not powered to evaluate statistical difference in contact lens comfort during melimine-coated and control lens wear using forced preference test and statistical analysis of these data was not undertaken. Figure 7-6 demonstrates percentage of subjects who preferred melimine-coated and control contact lenses.

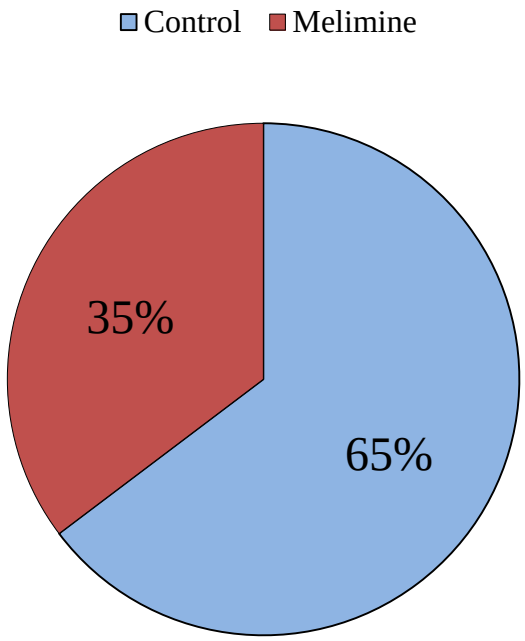


Figure 7-6: Participant preferences for melimine-coated and control contact lenses.

7.3.6 Retention of Antimicrobial Activity

When incubated with *P. aeruginosa* 6294 and *S. aureus* 31, worn melimine-coated contact lenses showed significantly lower adhesion ($p < 0.05$) compared to worn control lenses, resulting in 1.5 ± 0.5 log and 1.5 ± 0.4 log inhibition in adhesion respectively. Worn melimine-coated lenses showed 0.5 ± 0.3 log ($p = 0.05$) and

0.8 ± 0.5 ($p > 0.05$) log higher *P. aeruginosa* 6294 and *S. aureus* 31 adhesion than unworn melimine-coated lenses (Figure 7-7 A and B).

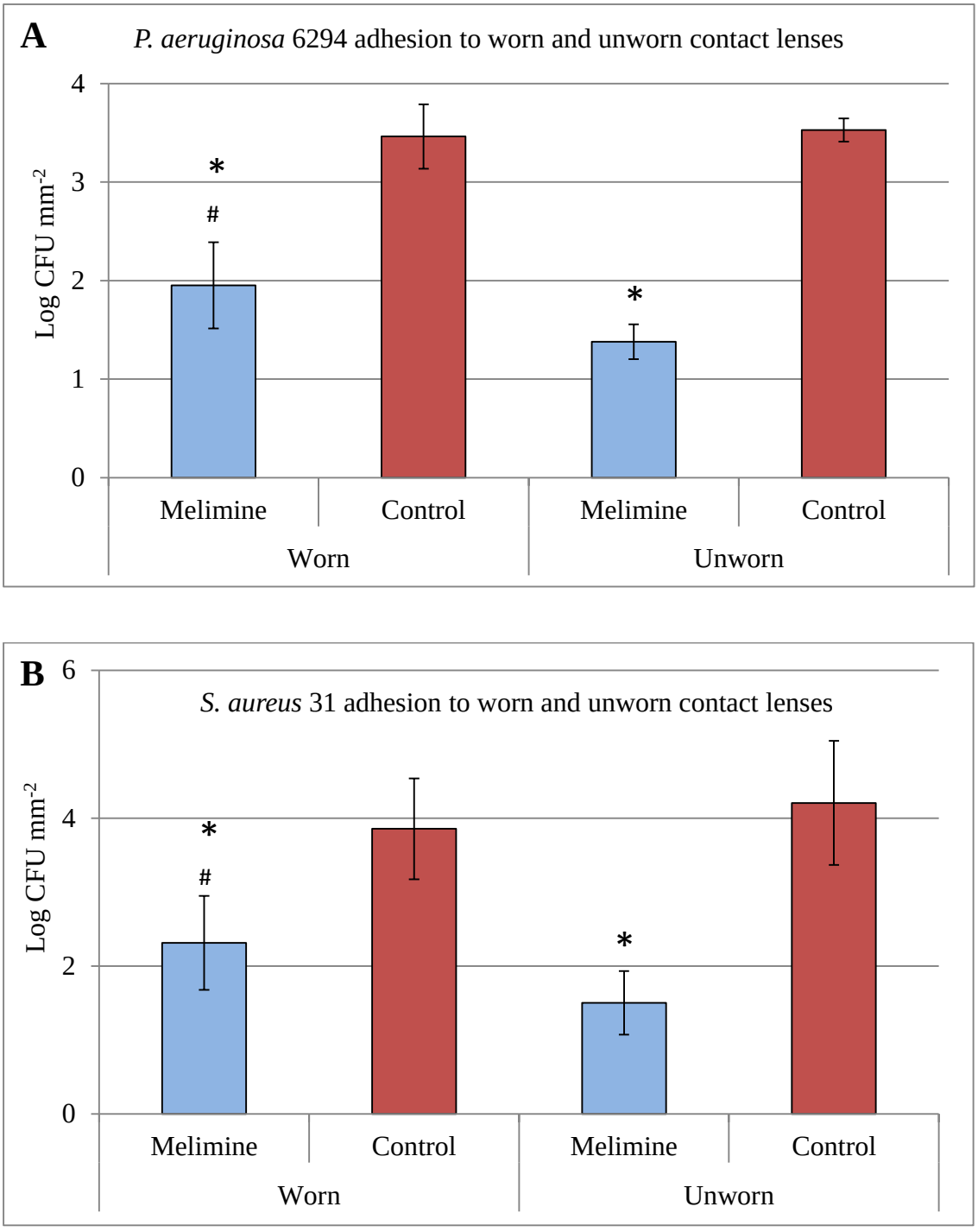
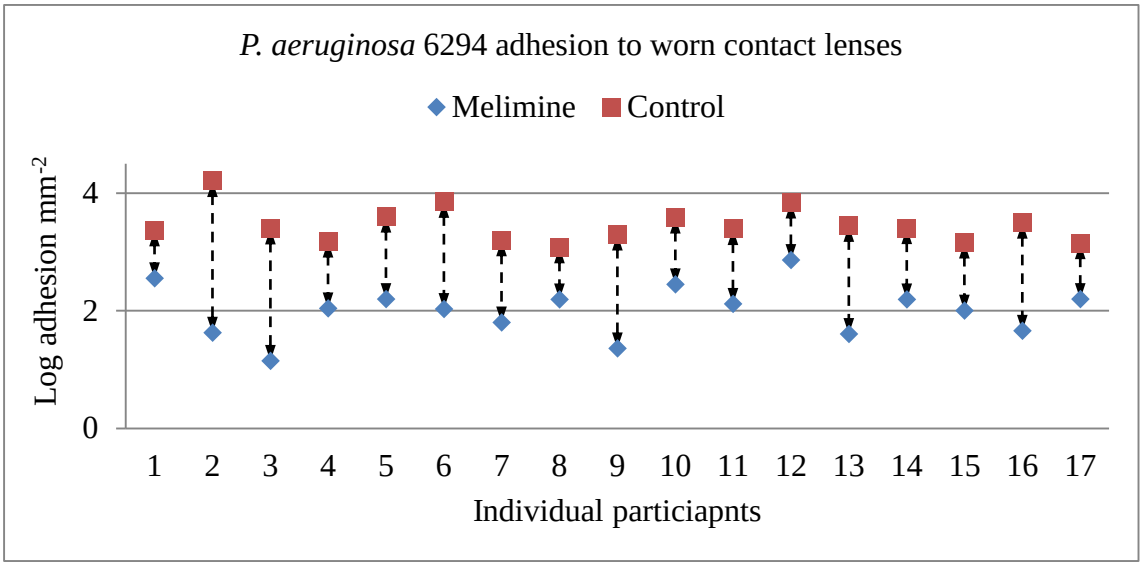


Figure 7-7: Bacterial adhesion to worn melimine-coated and control contact lenses.

The asterisk '' represents significantly reduced adhesion to worn or unworn melimine-coated lenses compared to worn or unworn uncoated lenses, whereas '#' represents significantly higher adhesion to worn melimine lenses compared to unworn melimine lenses.*

P. aeruginosa 6294 and *S. aureus* 31 adhesion to contact lenses collected from each of the 17 participants is presented in Figure 7-8. The figure shows the variation of bacterial adhesion to worn melimine-coated and control contact lenses collected from different individuals. A melimine-coated lens collected from one participant (number # 8) did not show any activity against *S. aureus* but showed more than 1 log inhibition against *P. aeruginosa*.



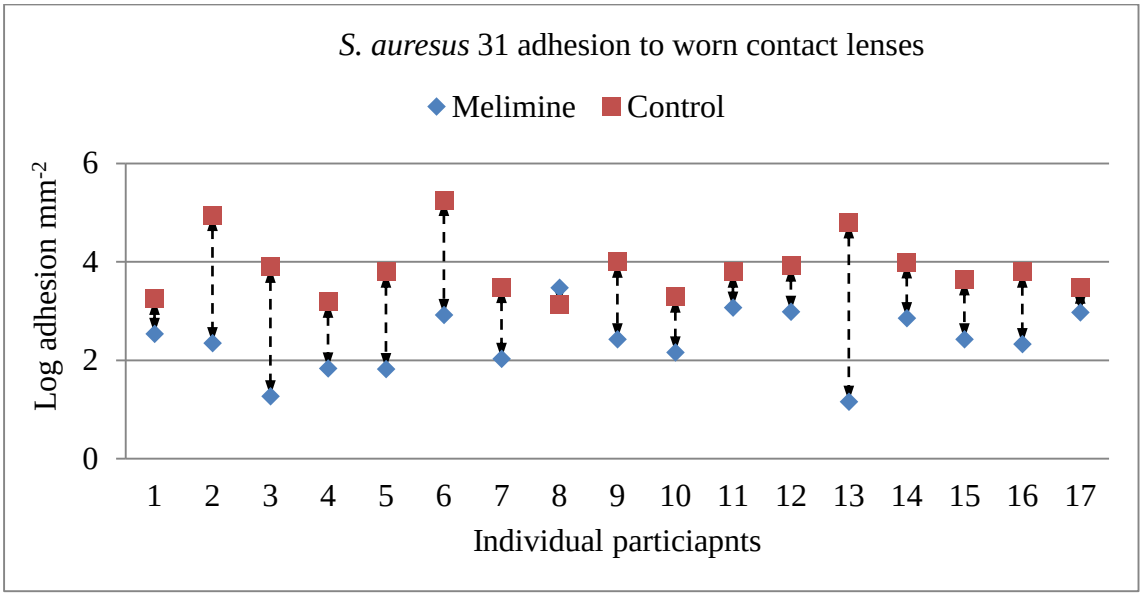


Figure 7-8: Bacterial adhesion to melimine-coated and control contact lenses collected from each participant

The dotted vertical lines show inhibition in bacterial adhesion to melimine-coated lenses when compared to controls after lens wear for each individual.

7.4 DISCUSSION

This study provides the first evidence to indicate antimicrobial peptide-coated contact lenses can be worn by human volunteers without any major side effects. In addition, contact lenses covalently coated with melimine retained antibacterial activity after one day of wear.

Melimine-coated lenses performed almost identically to the control etafilcon A lenses, showing no differences in lens surface characteristics, including deposits and debris. This suggests the melimine covalent binding was not prone to deposits and debris considering the duration of lens wear. Melimine-coated lens surface wetting was satisfactory in both dispensing and collection visits, which is in agreement with the high *in vitro* hydrophilicity of melimine-coated lenses determined in section 5.3.3. Similar to the fimbrolide-coated antimicrobial contact lenses (Zhu *et al.*, 2008), melimine-coated lenses showed acceptable fit with optimum movement, tightness and centration. The fit assessments were similar for melimine-coated and control lenses confirming no clinical implications of melimine-covalent reaction to lens parameters which supports data from section 5.3.2 showing unchanged lens parameters after covalent coupling determined by metrological evaluations.

Previous chapters have shown that melimine-coated contact lenses are non-cytotoxic to mammalian cells *in vitro* and *in vivo* following ISO standards 10993-5 (2009) and 9394 (2012) respectively. This study for the first time investigated biocompatibility of synthetic AMP in human eyes and is one of the few studies that have evaluated human responses of antimicrobial lens in a clinical trial. Melimine-coated lenses were not associated with conjunctival staining, bulbar and limbal redness, and palpebral redness and roughness. The melimine-coated lenses were not associated

with any delayed ocular toxicity. All these results indicate that melimine is unlikely to be toxic to human eyes or mammalian cells. However, when compared with controls, melimine-coated lens wear was associated with significantly higher mean and median corneal fluorescein staining. Ten out of 17 participants wearing melimine-coated lenses had clinically significantly (difference in corneal staining > 0.5 grading) higher corneal staining indicating strong association with the cationic peptide. However, time taken to resolve these staining were not determined with an unscheduled visit and participants were doing well after 1 week.

SICS generally presents as diffuse corneal staining in at least four of the five regions (Carnt *et al.*, 2007b). Similarly, depth, extent and type of fluorescein staining associated with melimine-coated lenses were greater in all the five corneal areas. Uptake of cationic biocides including polyhexamethylene biguanide (PHMB), other quaternary ammonium compounds such as benzalkonium chloride (BAK), and polyquaternary ammonium compounds such as polyquaternium-1 (Jones *et al.*, 2013) have been strongly associated with the incidence of SICS (Lipener, 2009; Stiegemeier *et al.*, 2006; Lebow *et al.*, 2003; Jones *et al.*, 1997). However, the exact mechanism of fluorescein interaction with corneal epithelial cells during SICS is not well understood (Fonn *et al.*, 2010; Morgan *et al.*, 2009; Ward, 2008; Stiegemeier *et al.*, 2006). Fluorescein pooling (Morgan *et al.*, 2009; Ladage *et al.*, 2002), ionic interaction with negatively charged fluorescein (Bright *et al.*, 2012), uptake by apoptotic cells (Bandamwar, 2011), staining of dead or damaged cell contents with compromised membranes (Morgan *et al.*, 2009), and accumulations in the intercellular space on the ocular surface (Feenstra *et al.*, 1992) are various theories that have sought to explain this. However, Bandamwar (2011) has shown that accumulation of fluorescein solutions in the voids on the ocular surface or in the intracellular space is unlikely to be the

mechanism of corneal staining. Given that melimine is covalently coupled and not released from lenses, other hypotheses such as ionic interactions with cationic surfactants bound to epithelial cells and fluorescein molecules, or adhesion of cationic compounds to cell membranes are unlikely to be applicable here. Suggested mechanisms for fluorescein staining of dead cells is controversial and a few studies have shown that dead necrotic cells were actually responsible for lowest staining intensities (Bandamwar, 2011). In addition, Morgan *et al.* (2009) suggested that corneal staining cannot be explained due to uptake by damaged epithelial cells. Apoptotic cells have demonstrated much higher fluorescein staining than live or dead cells (Bandamwar, 2011). The responses of the surface corneal cells could be seen clearly with white light prior to the addition of fluorescein (Figure 7-3). This indicates that the surface-bound melimine must directly contact with these surface corneal epithelial cells and that the fluorescein binds these cells. Perhaps, the bound melimine is inducing apoptosis in these cells. However, this effect was not seen during *in vitro* cytotoxicity assay (section 4.3.2). It should be noted that the *in vitro* assay used mouse fibroblast cells and not human corneal epithelial cells.

SICS associated with the use of PHMB and polyquad based MPDS has been associated with higher corneal infiltrative events than daily lens wear (Carnt *et al.*, 2007a). Whether melimine-coated lenses would be associated with inflammation due to the SICS-like responses cannot be ruled out and needs further exploration. However, Szczotka-Flynn *et al.* (2010a) showed that corneal staining is frequent during continuous contact lens wear and not associated with the development of corneal infiltrative events. This was a contradictory finding with the previous work by the same investigators (Szczotka-Flynn *et al.*, 2007) and was a consequence of different

fluorescein staining grades being used in the earlier study that under-reported corneal staining.

The median comfort scores indicated that control lenses were associated with marginally higher comfort when compared with melimine-coated lenses. Mean grades of overall comfort scores, lens related dryness, lens awareness and lens edge awareness were also slightly higher with control lenses, but the differences with melimine-coated lenses were not significant. This finding is in agreement with the fact that clinical relevance of SICS is not known and often not associated with patient symptoms (Stiegemeier *et al.*, 2006; Garofalo *et al.*, 2005; Jones *et al.*, 2002). Comfort results of melimine-coated contact lens wear was consistent with the results from fimbrolide-coated antimicrobial contact lens trial (Zhu *et al.*, 2008). Fimbrolide-coated lenses were also slightly less comfortable, had slightly increased dryness, lens edge and lens awareness. It is expected that different factors will influence comfort of individuals and comfort might not correlate with slit lamp signs (Diec *et al.*, 2012; Pritchard *et al.*, 2003). The standard deviations of the melimine-coated contact lens comfort scores were larger than control lenses, indicating greater differences in comfort responses. Although this study was not designed to evaluate statistical difference in participants' preference, 65% participants preferred control lenses, indicating 15% more participants ($p = 0.22$) felt better with control lenses than hypothesised (50%). It is difficult to draw conclusions from these subtle differences in comfort score as the melimine covalent coupling procedure involved several additional laboratory steps that could have affected the comfort or preference responses.

Zhu *et al.* (2008) have shown that fimbrolide-coated antimicrobial contact lenses are safe in humans; however they did not evaluate retention of antimicrobial activity.

The current study showed that melimine-coated lenses retained 1.5 log inhibition against *P. aeruginosa* and *S. aureus* after contact lens wear. This is in agreement with the previous assumption (section 1.6) that a minimum of 1.5 log inhibition *in vitro* could be required to reduce contact lens related adverse events. However, a small number of worn melimine-coated contact lenses showed less than one log inhibition against the bacteria tested. The differences seen in *ex vivo* antimicrobial activity with the worn melimine-coated lenses were not observed with unworn *in vitro* results earlier and possibly the consequence of uptake of tear components during wear. When compared to unworn melimine-coated lenses, there was increased bacterial adhesion to worn melimine-coated lenses, but the difference was not statistically significant. The opposite trend was seen with control lenses, which showed 0.4 ± 0.2 ($p > 0.05$) log higher *S. aureus* adhesion to unworn than worn lenses, but that was not the case for *P. aeruginosa*. The level of bacterial adhesion to worn contact lenses can vary between wearers (Miller *et al.*, 1988). Comparative *ex vivo* bacterial adhesion to worn and unworn etafilcon A lenses varies considerably between studies (Borazjani *et al.*, 2004; Boles *et al.*, 1992). Negatively charged methacrylic acid of etafilcon A lenses encourage *S. aureus* adhesion (Arciola *et al.*, 1995) and deposition of the cationic protein lysozyme from tears (Vijay *et al.*, 2012; Senchyna *et al.*, 2004; Bruinsma *et al.*, 2002). However, the attachment of the cationic peptide melimine is likely to result in an increased positive charge on the lens surface, perhaps making the surface either positive or neutral. Bacterial interactions to uncoated negatively charged etafilcon A lenses are certainly different than bacterial interactions with melimine-coated cationic surfaces. The human tear film consists of various negatively charged components such as phospholipid (Rohit *et al.*, 2013) (approximately 14%), mucin and mucin-like proteins such as lubricin (Schmidt *et al.*, 2013) or the protein lipocalin (Greiner *et al.*, 1996;

Berta *et al.*, 1986) which might interact with the surface bound melimine, and perhaps may affect activity. There is a possibility that after lens wear the melimine coating was partly covered with tear proteins, lipids or mucins which worked as a barrier during *ex vivo* bacterial assay allowing bacteria to adhere in higher numbers. In that case, if the lenses were rubbed thoroughly with PBS before assay, this might have changed the adhesion results.

Susceptibility of AMPs to *in vivo* proteolytic degradation poses a challenge and might limit the pharmacokinetics and functions of AMPs (McDermott, 2007; Jenssen *et al.*, 2006). These interactions might render AMPs unsuitable for certain applications. Trotti *et al.* (2004) investigated an AMP called iseganan in a mouth wash to reduce oral mucosis during radiotherapy treatment for head and neck cancer. The peptides failed to effectively reduce ulcerative events and subsequent morbidity when compared to placebo. The presence of various proteases and enzymes in the oral cavity might have reduced the activity of the AMP. An effective way to increase the stability of AMPs against degradation by proteolytic enzymes is to modify the *C*-terminus by amidation (Svenson *et al.*, 2008). Surface attached melimine has been shown to retain activity after exposure to the proteolytic enzyme trypsin (Rasul, 2010), indicating lens surface immobilised melimine might be resistant to proteases at the ocular surface. In addition, section 5.3.4.1 showed that melimine-coated lenses retained activity after 30 days of daily wash with PBS. Coupled with this, the current study showed melimine-coated contact lenses are active even following human lens wear, indicating melimine-coated contact lenses are likely to have a stable activity. Whether melimine will reduce contact lens related adverse events during wear, especially extended wear, requires more extensive clinical trials. Given the incidence of CLARE, CLPU and IK, prospective clinical trials with melimine-coated lenses might be able to demonstrate a reduction.

However, MK is relatively rare and post market studies may be required to demonstrate a reduction in incidence and severity.

In summary, this chapter has shown that melimine-coated contact lenses can be safely worn by humans without any major side-effects and any delayed toxic reactions. Although, melimine-coated lenses were less preferred, subjective comfort scores were broadly comparable to uncoated control lenses. Melimine-coated lens wear was associated with higher corneal staining and retained antibacterial activity against *P. aeruginosa* and *S. aureus* after wear. In conclusion, within the short period of human contact lens wear, melimine-coated lenses were biocompatible and retained antibacterial activity.

Chapter 8: Summary and Conclusions

8.1 SIGNIFICANCE OF RESEARCH

It is estimated that approximately 140 million people use contact lenses worldwide (Subbaraman *et al.*, 2013), and the global contact lens market in 2012 was estimated at USD 6.8 billion with an annual growth rate of 4% (Nichols, 2012). Contact lens wear is the most commonly identified risk factor for development of microbial keratitis (MK) (Keay *et al.*, 2006b) and depending on the study design and location, contact lens wear accounts for approximately 12% to 66% of all MK events (Keay *et al.*, 2006a; Schein *et al.*, 2005; Fong *et al.*, 2004). Contact lens related inflammation is around 150 times more common than corneal infection, affecting approximately 7% of lens wearers, and although not sight threatening, can lead to increased treatment time and temporary or permanent discontinuation of lens wear (Stapleton *et al.*, 2008).

The published literature has indicated that varieties of microorganisms are responsible for contact lens-related keratitis (Willcox, 2007; Willcox *et al.*, 2001b). Recent outbreaks of fungal and *Acanthamoeba* keratitis associated with specific contact lens care solutions have highlighted these microorganisms as causative agents of disease during contact lens wear. In addition, a significant proportion of the ocular infection caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with antibiotic resistant strains (Haas *et al.*, 2011; Willcox, 2011). Previous studies have shown that antimicrobial peptide (AMP) attachment to biomaterials including contact lenses can result in inhibition of adhesion of Gram negative and Gram positive bacteria (McDermott *et al.*, 2010; Chen *et al.*, 2009; Willcox *et al.*, 2008a; Gabriel *et al.*, 2006).

This thesis has made significant contributions to the literature through *in vitro* studies by exploring the broad spectrum antimicrobial activity of melimine covalently bound to contact lenses against strains of fungi, *Acanthamoeba* and multidrug-resistant bacteria. Reducing the adhesion and contamination of these microorganisms to contact lenses might eventually reduce contact lens related adverse events. Further validation of the melimine immobilised lenses included testing of contact lens parameters, surface hydrophobicity, heat stability of melimine-coatings, compatibility with contact lens care solutions and *in vitro* cytotoxicity. This thesis also evaluated *in vivo* biocompatibility of the lenses in an animal model and provided the first evidence to indicate safety and retention of antimicrobial activity of melimine-coated contact lenses in a human clinical trial.

8.2 THESIS SUMMARY

8.2.1 Setting an Appropriate *In Vitro* Assay to Evaluate Antimicrobial Activity of Contact Lenses

Chapter 2 showed that various *in vitro* conditions such as type of bacteria, the size of initial inoculum, contact lens material, nutritional content of media, and incubation period influence bacterial adhesion to contact lenses. Given the multifactorial influence, a minor change in bacterial assay can result in significant differences in bacterial adhesion. *S. aureus* is a fastidious bacterium and required nutritional support in an experimental assay especially when incubated for longer periods. This chapter defined a set of assay conditions most appropriate for measuring the adhesion of *P. aeruginosa* and *S. aureus*. These were using an inoculum size of 10^6 colony forming units (CFU) ml^{-1} with a 18 hr incubation period and cells suspended

in either PBS (*P. aeruginosa*) or 1/10 TSB (*S. aureus*). This set of assay conditions was used for further evaluation of antimicrobial activity of AMP-coated contact lenses.

8.2.2 Evaluation of the Antimicrobial Activity of Covalently Attached Melimine and Cathelicidin onto Contact Lenses

Chapter 3 explored covalent attachment of peptides such as melimine and LL-37 to contact lenses to obtain high antimicrobial activity (more than 1.5 log inhibition) via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) coupling. Increasing concentrations of peptides were used to immobilise peptides onto contact lens surfaces. Although the EDC reaction attached high concentrations of melimine to contact lenses (up to 168 µg $lens^{-1}$), it was unable to attach LL-37 at sufficient concentration to demonstrate adequate inhibition in *S. aureus* adhesion. This chapter reported the optimisation of the melimine concentration on the contact lens surface and lenses prepared in 3 mg ml^{-1} melimine resulted in 152 µg melimine attachment on the lenses. These lenses showed high inhibition of adhesion for both *P. aeruginosa* and *S. aureus* strains. Therefore, for further studies, this concentration of melimine was used to produce antimicrobial contact lenses.

8.2.3 Broad Spectrum Antimicrobial Activity of Melimine-Coated Contact Lenses

Chapter 4 demonstrated for the first time the antimicrobial activity of melimine-coated contact lenses against a range of microorganisms such as fungi, *Acanthamoeba*, ISO panel strains (ISO 14729, 2001) and multidrug-resistant bacterial strains, extending the previous data of activity (Willcox *et al.*, 2008a). Confocal microscopy revealed significant reduction in adherent live and dead bacteria on the melimine-coated surface, whereas total bacterial adhesion obtained by a radio-labelled procedure did not support

the confocal microscopy results and incubation of melimine-coated lenses was associated with endotoxin of Gram negative bacteria. The radio-labelling procedure includes uptake of radioactive-uridine by bacterial RNA, and the radioactivity determined on melimine-coated lenses may be due to the adhesion of bacterial radioactive-RNA to melimine-coated cationic lens surface following disruption of bacterial cytoplasmic membranes. Repeated use of melimine-coated contact lens during planned replacement schedule may collect endotoxin and bacterial RNA/DNA on the melimine-coated lenses. Accumulation of these may activate toll like receptor-4 (TLR) and TLR-9, expressed on corneal epithelium, stromal fibroblasts and bulbar conjuctival cells. TLRs are the part of innate immune system at the ocular surface and may trigger ocular inflammation. This chapter also showed that melimine-coated lenses were non-toxic to murine cells *in vitro*. Melimine-coated contact lenses showed more than 1 log inhibition of viable fungi and *Acanthamoeba* strains. These lenses also showed more than 2 log inhibition against five *P. aeruginosa* and five *S. aureus* strains which were resistant to commonly used antibiotics such as ciprofloxacin, gentamicin, moxifloxacin and tobramycin. This, coupled with the previous demonstration of the ability of melimine-coated lenses to control adverse events in animal models (Cole *et al.*, 2010), indicated that melimine immobilisation is potentially ideal as an antimicrobial coating.

8.2.4 Validation of Melimine-Coated Contact Lenses

Chapter 5 validated several aspects of melimine-coated contact lenses. Covalently immobilised melimine was heat sterilisable and did not change the physical parameters of contact lenses such as diameter, base curve and thickness. Covalently attached melimine also increased lens surface hydrophilicity. When soaked up to 30 days in PBS, melimine-coated lenses retained high activity against *P. aeruginosa* and *S. aureus*, indicating that the melimine-coating was stable and its activity was

predominantly due to surface bound melimine rather than unbound absorbed melimine. This result was supported by detection of amide nitrogen at the contact lens surface by X-ray photoelectron spectroscopy (XPS) throughout 30 days of soaking in PBS. Melimine-coating was compatible with solution components of Biotrue™ and RevitaLens OcuTec® multipurpose disinfection solutions (MPDS). Soaking in OPTIFREE PureMoist® MPDS possibly resulted in a layer of EOBO (EOBO-41™-Polyoxyethylene-Polyoxybutylene) binding to the lens surface, which may then have restricted access to the surface bound melimine and thus prevented any inhibition of bacterial adhesion.

8.2.5 Biocompatibility of Melimine-Coated Lenses with Rabbit Eyes

Chapter 6 showed that melimine-coated contact lenses do not produce ocular irritation in rabbits during 22 days daily lens wear. All the six animals remained healthy and no ocular irritation related symptoms such as eye scratching and pawing was observed. Slit lamp biomicroscopy and histopathology of cornea confirmed the absence of any ocular signs that may indicate irritation or toxicity to ocular tissues respectively.

8.2.6 Biocompatibility and Retention of Antimicrobial Activity of Melimine-Coated Lenses in Human Clinical Trial

Chapter 7 for the first time showed that melimine-coated contact lenses could be worn by humans without any significant ocular event. The melimine-coated lenses performed similarly to the marketed etafilcon A lenses, showing acceptable lens fit and no differences in surface characteristics. Melimine-coated lenses were associated neither with bulbar and limbal redness, or palpebral redness and roughness, nor with any delayed ocular toxicity. However, higher corneal staining was associated with melimine-coated lens wear. This staining might be due to the apoptosis of epithelial

cells following the stress produced by cationic melimine attached onto the contact lens surface. Implications of the corneal staining with melimine-coated contact lenses are difficult to predict and needs further investigation in a larger clinical trial, especially in the face of uncertainty to the causative mechanism of any type of corneal staining during contact lens wear. During *ex vivo* investigations, melimine-coated lenses retained more than 1.5 log inhibition against both *P. aeruginosa* and *S. aureus* after lens wear.

8.3 LIMITATIONS

There are a few limitations in this thesis and these are mentioned as follows:

- Up to 48% of reusable and 14% daily disposable contact lenses worldwide are made by silicone hydrogel materials (Efron *et al.*, 2013), which have higher oxygen permeability than hydrogel lenses. However, this thesis investigated antimicrobial activity of melimine coating on hydrogel etafilcon A lenses and did not use silicone hydrogel lenses.
- EDC coupling, which could attach adequate melimine, was unable to bind LL-37 peptide to a suitable concentration on the contact lenses to provide high inhibition in *S. aureus* adhesion. Alternative method of covalently attaching LL-37 might have been useful.
- The *in vitro* antibacterial activities of melimine-coated lenses against *P. aeruginosa* 6294 and *S. aureus* 31 reported in this thesis have varied slightly from one chapter to another (range: 3.1 log to 2.6 log for *P. aeruginosa* and 3.9 log to 2.6 log for *S. aureus*). This is possibly due to separate batches of synthesised melimine used during different studies. This highlights the need to use peptides with identical purity and peptide contents and if possible, made in the same batch.
- Compatibility of melimine-coated lenses with hydrogen peroxide (H_2O_2) based disinfection system was not investigated in this thesis. H_2O_2 is a strong oxidising agent and can degrade peptides and proteins. It was expected that the melimine antimicrobial coating would not be compatible with this cleaning system and thus it was not tested.

- During the human clinical trial, participants wore contact lenses for 7 hr. However, safety and retention of antimicrobial activity of the melimine-coated lenses could have been further investigated following overnight or extended lens wear. Melimine-coated lenses were associated with significantly higher corneal staining than control lenses. Further implications of the corneal staining, such as association with corneal infiltrative events and contact lens comfort were not determined here.

8.4 FUTURE RESEARCH

EDC covalent coupling was used to bind peptides onto contact lens surface. Peptides can be immobilised onto contact lens surface via various linkers such as 4-azidobenzoic acid (ABA), 4-fluoro-3-nitrophenyl azide (FNA) (Chen *et al.*, 2009) and succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) (Williams *et al.*, 2010). The modified covalent attachment technique could also be applied to silicone hydrogel contact lenses. This thesis has used 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) and LL-37 (L-L-G-D-F-F-R-K-S-K-E-K-I-G-K-E-F-K-R-I-V-Q-R-I-K-D-F-L-R-N-L-V-P-R-T-E-S) peptides. Further truncated versions of the peptides such as Mel-4 (K-N-K-R-K-R-R-R-R-R-R-G-G-R-R-R-R) (Rasul, 2010) or LL-25 (L-L-G-D-F-F-R-K-S-K-E-K-I-G-K-E-F-K-R-I-V-Q-R-I-K) (McDermott *et al.*, 2010; Kolar *et al.*, 2010) could be used to optimise the surface concentration, antimicrobial activity and *in vivo* cytotoxicity.

Further work is needed to investigate the mechanism of antimicrobial activity of AMPs, especially the antimicrobial mechanism of surface attached AMPs. Being a hybrid peptide, melimine does not produce well formed α–helical tertiary structure in solution (Willcox *et al.*, 2008a) like other naturally occurring AMPs (e.g. melittin and

LL-37). In addition, the mechanism of activity against eukaryotic pathogenic cells such as fungus and *Acanthamoeba* needs further research.

During human contact lens wear the interactions between surface attached cationic melimine and epithelial cells triggered corneal staining. Site-directed attachment of peptides through the thiol (-SH) group of a single cysteine residue might optimise the cationic charge on the lens surface and further reduce the corneal staining. Attachment of cationic peptides onto a contact lens surface might also attract anionic tear components such as lubricin and lipocalin, forming deposits on contact lenses following lens wear. Increased deposits onto contact lenses have been associated with increased microbial adhesion (Butrus *et al.*, 1990) and therefore further investigations are needed to explore that hypothesis. However, incorporation of passive non-cidal agents such as poly(ethylene glycol) or immobilisation of peptides via covalent linkers may be able to reduce surface deposits. The effectiveness of antimicrobial contact lenses in reducing lens related adverse events during daily lens wear, especially extended wear needs to be explored with a larger clinical trial.

8.5 CONCLUSIONS

This thesis contributed to the knowledge of activity and biocompatibility of surface immobilised antimicrobial peptides onto contact lenses. AMP attachment to contact lenses was shown to be an effective method to develop an antimicrobial contact lens. As hypothesised, melimine covalently attached to contact lenses via EDC coupling showed high antimicrobial activity against a range of microorganisms implicated in contact lens related adverse events, such as fungi, *Acanthamoeba*, and various bacteria including multidrug-resistant *P. aeruginosa* and *S. aureus* strains. For clinical applications, various aspects of the melimine-coated contact lenses were evaluated

which showed that the coating was readily heat sterilisable, non-toxic to mammalian cells *in vitro*, did not change contact lens parameters and formed a wettable surface. This coating was also compatible with PHMB-based commonly used contact lens care solutions. The antimicrobial coating was biocompatible and did not show any signs or symptoms of ocular irritation during animal and human lens wear except higher human corneal staining. Following human contact lens wear melimine-coating retained more than 1.5 log inhibition against *P. aeruginosa* and *S. aureus* indicating that covalent immobilisation of peptides onto contact lenses is a safe and efficient technology to develop antimicrobial contact lenses.

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Appendix A: Publications and Presentations

Publications

Accepted:

- ***Dutta D***, Willcox MD. Antimicrobial strategies for contact lenses and lens cases: A review. *Eye and Contact Lens.* Accepted for publication (22/05/2014)

- ***Dutta D***, Ozkan J, Willcox MD. Biocompatibility and retention of antimicrobial activity of melimine contact lenses in a human and rabbit trial. *Optometry and Vision Science* 2014; 91(5):570-81

- ***Dutta D***, Willcox MD. A laboratory assessment of factors that affect bacterial adhesion to contact lenses. *Biology*. 2013;2(4):1268-1281

- ***Dutta D***, Cole N, Kumar N, Willcox MD. Broad spectrum antimicrobial activity of melimine covalently bound to contact lenses. *Investigative Ophthalmology and Visual Science* 2013;54(1):175-82

- ***Dutta D***, Rao HL, Addepalli UK, Vaddavalli PK. Corneal thickness in keratoconus: comparing optical, ultrasound, and optical coherence tomography pachymetry. *Ophthalmology* 2013; 120(3);475-6

- ***Dutta D***, Cole N, Willcox MD. Factors influencing bacterial adhesion to contact lenses. *Molecular Vision* 2012;18:14-21

- Invited article written for the mivision magazine on "Antimicrobial strategies for contact lenses and lens cases" February, 2014

Presentations

Oral:

- ***Dutta D***, Willcox MD. (2013) Antimicrobial efficiency of covalently attached melimine onto contact lenses and *in vivo* Safety in rabbit eyes. *Optom Vis Sci AAO*, Seattle, USA. E-Abstract 130728

- ***Dutta D***, Willcox MD. (2013) Antimicrobial efficiency of covalently attached melimine onto contact lenses and *in vivo* safety in rabbit eyes. *International Society for Contact Lens Research*, Kyoto, Japan

- ***Dutta D***, (2013) Invited presentation: Antimicrobial contact lenses; Understanding the possibilities. *Cornea and Contact Lens Society of Australia* (CCLSA: Victoria division), Melbourne, Australia

- ***Dutta D***, Willcox MD. (2012) Development of an antimicrobial contact lens. *UNSW Faculty of Science Research Competition*, Sydney, Australia

Poster:

- ***Dutta D***, Willcox MD (2014). Biocompatibility and retention of activity of melimine antimicrobial contact lenses in a human clinical trial. Submitted to *Association for Research in Vision and Ophthalmology (ARVO)*, Orlando, USA

- Willcox MD, ***Dutta D*** (2014). Compatibility of melimine antimicrobial contact lenses with contact lens care solutions**.** Submitted to *Association for Research in Vision and Ophthalmology (ARVO)*, Orlando, USA

- ***Dutta D,*** Willcox MD. (2013) Antimicrobial efficiency of covalently attached melimine onto contact lenses and *in vivo* Safety in rabbit eyes. *Optom Vis Sci AAO*, Seattle, USA. E-Abstract 130728

- ***Dutta D***, Zhu H, Willcox MD (2013). Antimicrobial activity of multipurpose disinfection solution soaked contact lenses. *Tear Film and Ocular Society*, Taormina, Italy. Page 24, E-Abstract 54

- ***Dutta D***, Willcox MD. (2013) Antimicrobial efficiency of covalently attached melimine onto contact lenses and *in vivo* safety in rabbit eyes. *International Society for Contact Lens Research*, Kyoto, Japan

- ***Dutta D***, Willcox MD (2013). Antimicrobial activity of melimine or cathelicidin bound to contact lenses. *Invest Ophthalmol Vis Sci, ARVO*. Seattle, USA. 54: E-Abstract 507

- ***Dutta D***, Cole N, Willcox MD (2012). The material characteristics that affect bacterial adhesion to contact lenses, and affect of lens wear. *Cont Lens Anterior Eye, BCLA*. Birmingham, UK. 35, E-Abstract 1 (10-11)

- ***Dutta D***, Cole N and Willcox MD (2012). Antimicrobial efficacy of melimine covalently bound to contact lenses. *Invest Ophthalmol Vis Sci, ARVO.* Fort Lauderdale, USA. 53: E-Abstract 6085

- ***Dutta D***, Cole N, Willcox MD (2012). Broad spectrum antimicrobial efficacy of the cationic peptide melimine. *Molecular Microbiology Meeting*, Sydney, Australia

GRANTS AND AWARDS

2014 **Association for Research in Vision and Ophthalmology**
ARVO international travel grant

2013 **American Academy of Optometry, supported by Bausch and Lomb**
William C. Ezell fellowship

2013 **American Academy of Optometry, supported by Vistakon**
Student travel grant towards oral presentation at annual conference, Seattle (self-declined, being a recipient of Ezell fellowship)

2013 **International Association for Contact Lens Research**
Student scholarship, towards presentation at biennial conference, Kyoto

2012 **Optometric Vision Research Foundation, Graduate Research School, University of NSW**
Maki Shiobara Scholarship

2012 & 11 **Post-graduate Conference, University of NSW and Brien Holden Vision Institute, Sydney**
Best oral presenter in 2012 and runner up in 2011

2010 **University of New South Wales, Sydney**
University International Postgraduate Award

2010 **Brien Holden Vision Institute, Sydney**
Post graduate research scholarship

Appendix B: Participant Information Statement and Consent Form

The University of New South Wales and The Brien Holden Vision Institute

Biocompatibility and Retention of Activity in a Human Clinical Trial (Approval no. HC13087)

Research Study - Participant Information Sheet	
Title:	Evaluation of the clinical performance and retention of antimicrobial activity of melimine coated contact lenses.
Trial Location:	Clinical trial and Research Centre, Brien Holden Vision Institute
Protocol number	CRTC-2013-01
Version Date:	2, 24/04/2013
Investigator:	Prof. Mark DP Willcox
Contact Number/s:	Business Hours: +612 9385 7516 / 7517 / 6176; After Hours: +614 25136301

1. Invitation

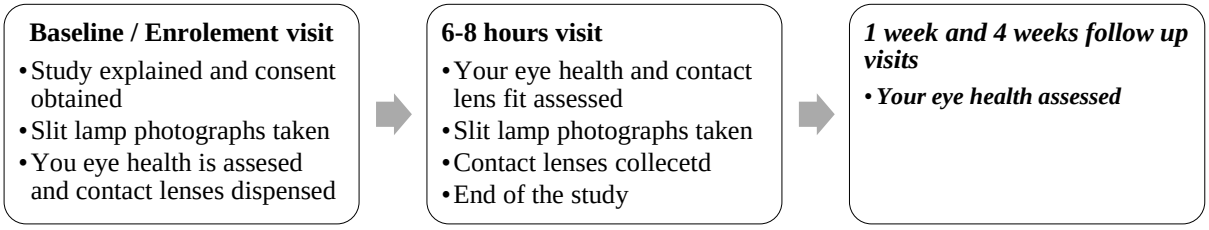
You are invited to take part in this PhD research study because you fulfil the initial study criteria. being healthy, over the age of 18 years, and able to wear contact lenses. Taking part in this research study is voluntary and your relationship with Brien Holden Vision Institute will not be affected in any way if you choose not to participate. Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it involves. Please take the time to read the following information carefully, ask any questions, and discuss it with others if you wish.

2. What is the purpose of this research?

This study aims to find out the clinical performance and retention of antimicrobial activity of melimine coated contact lenses. ACUVUE 2 contact lenses coated and uncoated with melimine are used in this study. The melimine coated lenses have the ability to reduce bacterial contamination. To achieve this, we wish to monitor your eyes' response to the products and what you think of them. Approximately **17** people will take part in this study.

3. What does the study involve?

If you decide to take part in this study, you will be asked to attend the School of Optometry and Vision Science, Brien Holden Vision Institute for an initial visit (the Baseline visit) and two scheduled visits. You will be wearing melimine coated ACUVUE 2 contact lenses in one eye and untreated ACUVUE 2 contact lenses in the other eye. The lenses will be randomly allocated. The baseline visit aims to see if the study is right for you and if it is, the dispensing visit will follow either immediately or within 10 days. At this visit, the study contact lenses will be fitted. If the study is not suitable for you, the reasons will be discussed with you.
You will be asked to wear 1 pair of contact lenses for consecutive **6-8 hours**. The contact lenses that you will be given will be allocated from a computer generated list according to your unique study number. This is called randomisation and is a standard method researchers use to help make sure the results of the study are reliable. You have an equal chance of getting each contact lens types in your eyes and neither you nor your Study Optometrist will be able to influence the choice. Once the study is finished, you will be advised for follow up visits after 1 week and 4 weeks.
This study is a double-masked study. This means neither you nor your Study Optometrist will know which type of contact lenses you have been given until after the study is finished. Masking is another way researchers help protect the reliability of the research.



4. Will taking part in this study cost me anything?

All study procedures and products connected with this study will be given to you free of charge. Limited free parking is available. Additional costs such as travel and time costs will NOT be reimbursed, however Coles/Myer gift vouchers to the value of $15 per scheduled visit ($**60** total) will be given to each of the study participants. The baseline visit and the dispensing visit are considered one scheduled visit, even when they occur on different days. You will only be entitled to vouchers for finishing the study, even if you withdraw/are withdrawn from the study for any reason

5. Are there any benefits to participating?

This study will not directly benefit you. However, you may benefit from the extra optometric attention and products that you will receive free of charge during the study, and the knowledge that information gained from this research may help others in the future. Contact lenses offer improved side vision and the convenience of not wearing spectacles.

6. What will happen to my test samples?

Your samples will be collected at the end of the study for laboratory testing of antibacterial activity.

7. What are the side effects, risks or inconveniences involved?

All contact lenses and contact lens care products have potential side effects, The most serious complication that contact lenses can cause is an infection of the cornea (the transparent membrane that covers the coloured part of the eye and the pupil), also known as microbial keratitis. The rate of presumed microbial keratitis is 25 per 10,000 wearers per year if wearing lenses during sleep the possible outcomes of such an event are severe pain, hospitalisation, corneal scarring and possible loss of vision. If you are observed to have a complication such as a suspected infection or inflammation, we may take swabs of the eye surface and lid margin. These swabs are analysed to determine if micro-organisms such as bacteria are present, and to also identify the type of micro-organism. This analysis may prove useful should treatment be required. Taking swabs of the eye and lid margin is harmless
You do not need to remove contact lenses during the 2 weeks contact lens wear. If you feel dryness in the eyes you can use sterile saline drops. Your optometrist will provide you these salines. You should remove your lenses and contact us immediately (Business Hours: +612 9385 7516 After Hours: +61425136301) if you experience any of the side effects, risks or inconveniences, eye symptoms that concern you.

8. How will my confidentiality be protected?

Your records relating to this study and any other information received will be kept strictly confidential except as required by law. By providing consent to participate in this study you authorise release of your study data for further research, analysis or teaching purposes. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your permission..

9. What are my rights and protections if I participate?

You will be informed as soon as possible of any new information arising that could affect your safety or choice to participate and do not waive any legal rights by signing this consent form.
In accordance with relevant Australian and/or State privacy and other relevant laws, you have the right to access the information collected and stored by the researchers about you.
Should you experience any injuries or complications as a result of this study, you should contact the Study Optometrist as soon as possible, who will assist you in arranging appropriate treatment. The parties to this study agree to follow the Medicines Australia

Guidelines for 'Compensation for Injury Resulting from Participation in an Industry-Sponsored Clinical Trial'.

Should you suffer any injuries or complications resulting from this study, you may have the rights to take legal action to obtain compensation.
These guidelines are available for your inspection on the Medicines Australia Website (www.medicinesaustralia.com.au) under Issues/Information Clinical Trials Indemnity & Compensation Guidelines. Alternatively, your study optometrist can provide you with a hard-copy of the guidelines. It is the recommendation of the independent ethics committee responsible for the review of this trial that you seek independent legal advice before taking any steps towards compensation for injury.

10. Are there alternative treatments?

Other treatments include marketed contact lenses and care products, spectacles and refractive surgery. However, should these be required, you must purchase them at your own expense from another source and in consultation with your primary eye health care provider.

11. Could this research be stopped unexpectedly?

Your Study Optometrist, the Regulatory Agencies may stop your participation in this study (at any time) if it is believed to be in your best interest, if you do not follow the study instructions, if it is discovered that you do not meet the study requirements, or if the study is cancelled. The reasons will be discussed with you, final safety checks may need to be performed and you will be asked to return all study products.

12. How is this study being paid for?

This study is being funded by the Institute.

13. Who can I contact?

You should contact your Study Optometrist immediately if you experience any eye discomfort, problems with your vision, are concerned with how your eyes appear, or have any questions during the study.

14. Complaints?

The UNSW Human Ethics Research Committee (HREC) has reviewed this study, and their primary interests are the safety, welfare and rights of participants in this study. The ethics committee members are independent of the Study Sponsor and Study Team.

Enquiries about your rights as a research study participant and complaints concerning this research project may be directed to: The Ethics Secretariat, UNSW Research Services, Level 3, South Wing, Rupert Myers Building Gate 14 Barker Street, UNSW, Sydney 2052, Australia (phone (02) 9385 4234 or email ethics.sec@unsw.edu.au).

SUMMARY OF INFORMED CONSENT	
Title:	Evaluation of the clinical performance and retention of antimicrobial activity of melimine coated contact lenses.
Trial Location:	Clinical trial and Research Centre, Brien Holden Vision Institute
Protocol number	CRTC-2013-01
Version Date:	26/03/2013
Investigators:	Prof. Mark DP Willcox
Contact Number/s:	**Business Hours:** +612 9385 7516 / 7517 / 6176; **After Hours**: +614 25136301

By signing this Informed Consent form, I agree to the following:

- I have read the attached information sheet, which describes the purpose and nature of the research, the products and procedures to be followed, reasonably known risks and discomforts, benefits to be expected, alternative treatments, release of my health records, possible involuntary removal from the study, and treatment for study-related physical injury.

- My Study Optometrist has discussed each of these with me, and I understand what the study involves.

- I have had the opportunity to ask questions, and have received answers to my satisfaction.

- I authorise release of my health information to the School of Optometry and Vision Science, Brien Holden Vision, its authorised representatives, and the Ethics Committee. I understand that the Australian Therapeutic Goods Administration (TGA), agencies may access my records if needed.

- I authorise release of my study data for current and further research, analysis or teaching purposes.

- I will follow instructions on use of the products and other study procedures, and return the study products at the end of my participation.

- I will try to complete the study period and follow the required visits and procedures, but I understand that I am free to discontinue participation at any time without penalty by notifying the Study Optometrist.

- I voluntarily agree to participate in this study, and by signing this consent I have not waived any of my legal rights.

- I consent to my GP being notified of any clinically relevant information noted during this trial.

- I understand I will be given a signed copy of this Participant Information/Informed Consent Form.

NOTE: All parties signing the consent must date their own signature				
Participant Signature:			**Date:**	
	(Signature)	*(Print Name)*		*(dd/mon/yyyy)*

I have explained the nature and purpose of the study to the named participant above, and have given the opportunity to ask questions, and the time to decide whether to participate.

Investigator Signature:			**Date:**	
	(Signature)	*(Print Name)*		*(dd/mon/yyyy)*

REVOCATION OF CONSENT

SUMMARY OF REVOCATION OF CONSENT	
Title:	Evaluation of the clinical performance and retention of antimicrobial activity of melimine coated contact lenses.
Title:	A prospective, randomised, double masked clinical trial to investigate the clinical performance and retention of antimicrobial activity of melimine coated contact lenses.
Trial Location:	Clinical trial and Research Centre, Brien Holden Vision Institute
Protocol number	CRTC-2013-01
Version Date:	26/03/2013
Principal Investigator:	Prof. Mark DP Willcox
Contact Number/s:	**Business Hours:** +612 9385 7516 / 7517 / 6176; **After Hours**: +614 25136301

By signing this Revocation of Consent form, I agree to the following:

- I hereby wish to **WITHDRAW** my consent to participate in the research proposal stated above and understand that such withdrawal **WILL NOT** jeopardise any treatment or any relationship with School of Optometry and Vision Science, Brien Holden Vision Institute and University of New South Wales.

NOTE: All parties signing the revocation must date their own signature				
Participant Signature:			**Date:**	
	(Signature)	*(Print Name)*		*(dd/mon/yyyy)*

I have explained all the necessary questions named participant above, and have given the opportunity to ask questions, and the time to decide for revocation.

Investigator Signature:			**Date:**	
	(Signature)	*(Print Name)*		*(dd/mon/yyyy)*

Appendix C: Daily Monitoring Sheet for Rabbits

Daily Monitoring Sheet

Ethics approval No.	Species and sex:
Experimental Code:	Animal number:

Date							
Procedure							
Day							
Distant observation							
Activity							
Hydration							
Appearance							
Eye Appearance							
Clinical Signs							
Eating/Drinking/ Urinating/Defecating							
On stimulation							
Activity							
Hydration							
Appearance							
Clinical Signs							
Body weight (g) % change from maximum							
Nothing abnormal (please tick)							
Signature:							

Scores for abnormal conditions (**Euthanasia if cumulative score is equal to or greater than 5**)

Activity	**Score**		**Score**
Slow moving, decreased alertness	2	Decreased activity, fearful	4
Abnormal gait, isolated, hyperactive	4	Severe self-mutilation	5
Immobile, shaking, vocalizations	5	Unresponsive when stimulated	5
Hydration			
Dry eyes	1	Sunken eyes	3
Listless skin tenting (1 sec)	4		
Appearance			
Decreased grooming	2	Alopecia, minor abrasion	2
Soiled/rough coat	3	Nasal/Ocular discharge	3
Ulcerated skin, coat ruffled, hunched	5	Paresis/paralysis of limbs	5
Clinical Signs			
Increased respiration, effort, circling	1	Soft stools	3
Shallow/laboured respirations	3	Irregular/gasping respirations	5
Seizures	5		
Tumours			
Subcutaneous, small	1	Diameter 1-1.5 erythema	3
Tumour > 1.5cm or ulcerated	5		

Appendix D: McDonald-Shadduck score system (Slit lamp) for Animal Study

A.1 Conjunctival congestion

0 = Normal. May appear blanched to reddish pink without perilimbal injection (except at 12:00 and 6:00 o'clock positions) with vessels of the palpebral and bulbar conjunctiva easily observed.

+1 = A flushed, reddish colour predominantly confined to the palpebral conjunctiva with some perilimbal injection but primarily confined to the lower and upper parts of the eye from the 4:00 to 7:00 and 11:00 to 1:00 o'clock positions.

+2 = Bright red colour of the palpebral conjunctiva with accompanying perilimbal injection covering at least 75 % of the circumference of the perilimbal region.

+3 = Dark, beefy red colour with congestion of both the bulbar and the palpebral conjunctiva along with pronounced perilimbal injection and the presence of petechia on the conjunctiva. The petechia generally predominate along the nictitating membrane.

A.2 Conjunctival swelling

0 = Normal or no swelling of the conjunctival tissue.

+1 = Swelling above normal without eversion of the lids (can be easily ascertained by noting that the upper and lower eyelids are positioned as in the normal eye), swelling generally starts in the lower cul-de-sac near the inner canthus, which needs slit-lamp examination.

+2 = Swelling with misalignment of the normal approximation of the lower and upper eyelids; primarily confined to the upper eyelid so that in the initial stages the misapproximation of the eyelids begins by partial eversion of the upper eyelid. In this stage, swelling is confined generally to the upper eyelid, although it exists in the lower cul-de-sac (observed best with the slit-lamp).

+3 = Definite swelling with partial eversion of the upper and lower eyelids essentially equivalent. This can be easily ascertained by looking at the animal head-on and noticing the positioning of the eyelids; if the eye margins do not meet, eversion has occurred.

+4 = Eversion of the upper eyelid is pronounced with less pronounced eversion of the lower eyelid. It is difficult to retract the lids and observe the perilimbal region.

A.3 Conjunctival discharge

Discharge is defined as a whitish, gray precipitate, which should not be confused with the small amount of clear, inspissated, mucoid material that can be formed in the medial canthus of a substantial number of rabbit eyes.

0 = Normal. No discharge.

+1 = Discharge above normal and present on the inner portion of the eye but not on the lids or hairs of the eyelids. One can ignore the small amount that is in the inner and outer canthus.

+2 = Discharge is abundant, easily observed, and has collected on the lids and around the hairs of the eyelids.

+3 = Discharge has been flowing over the eyelids so as to wet the hairs substantially on the skin around the eye.

A.4 Aqueous flare

The intensity of the Tyndall phenomenon is scored by comparing the normal Tyndall effect observed when the slitlamp beam passes through the lens with that seen in the anterior chamber. The presence of aqueous flare is presumptive evidence of breakdown of the blood-aqueous barrier.

0 = The absence of visible light beam light in the anterior chamber (no Tyndall effect).

+1 = The Tyndall effect is barely discernible. The intensity of the light beam in the anterior chamber is less than the intensity of the slit beam as it passes through the lens.

+2 = The Tyndall beam in the anterior chamber is easily discernible and is equal in intensity to the slit beam as it passes through the lens.

+3 = The Tyndall beam in the anterior chamber is easily discernible; its intensity is greater than the intensity of the slit beam as it passes through the lens.

A.5 Iris involvement

In the following definitions the primary, secondary and tertiary vessels are utilized as an aid to determining a subjective ocular score for iris involvement. The assumption is made that the greater the hyperaemia of the vessels and the more the secondary and tertiary vessels are involved, the greater the intensity of iris involvement.

0 = Normal iris without any hyperaemia of the iris vessels. Occasionally around the 12:00 to 1:00 o'clock position near the pupillary border and the 6:00 to 7:00 o'clock position near the pupillary border there is a small area around 1 mm to 3 mm in diameter in which both the secondary and tertiary vessels are slightly hyperaemic.

+1 = Minimal injection of secondary vessels but not tertiary. Generally, it is uniform, but may be of greater intensity at the 1:00 or 6:00 o'clock position. If it is confined to the 1:00 or 6:00 o'clock position, the tertiary vessels must be substantially hyperaemic.

+2 = Minimal injection of tertiary vessels and minimal to moderate injection of the secondary vessels.

+3 = Moderate injection of the secondary and tertiary vessels with slight swelling of the iris stroma (this gives the iris surface a slightly rugose appearance, which is usually most prominent near the 3:00 and 9:00 o'clock positions).

+4 = Marked injection of the secondary and tertiary vessels with marked swelling of the iris stroma. The iris appears rugose; may be accompanied by hemorrhage (hyphaemia) in the anterior chamber.

A.6 Cornea

The scoring scheme measures the severity of corneal cloudiness and the area of the cornea involved. Severity of corneal cloudiness is graded as follows:

0 = Normal cornea. Appears with the slit-lamp as having a bright gray line on the epithelial surface and a bright gray line on the endothelial surface with a marble-like gray appearance of the stroma.

+1 = Some loss of transparency. Only the anterior half of the stroma is involved as observed with an optical section of the slit-lamp. The underlying structures are clearly visible with diffuse illumination, although some cloudiness can be readily apparent with diffuse illumination.

+2 = Moderate loss of transparency. In addition to involving the anterior stroma, the cloudiness extends all the way to the endothelium. The stroma has lost its marble-like appearance and is homogeneously white. With diffuse illumination, underlying structures are clearly visible.

+3 = Involvement of the entire thickness of the stroma. With optical section, the endothelial surface is still visible. However, with diffuse illumination the underlying structures are just barely visible (to the extent that the observer is still able to grade flare, iritis, observe for pupillary response, and note lenticular changes).

+4 = Involvement of the entire thickness of the stroma. With the optical section, cannot clearly visualize the endothelium. With diffuse illumination, the underlying structures cannot be seen. Cloudiness removes the capability for judging and grading aqueous flare, iritis, lenticular changes, and pupillary response.

The surface area of the cornea relative to the area of cloudiness is divided into five grades from 0 to +4.

0 = Normal cornea with no area of cloudiness.

+1 = 1 % to 25 % area of stromal cloudiness.

+2 = 26 % to 50 % area of stromal cloudiness.

+3 = 51 % to 75 % area of stromal cloudiness.

+4 = 76 % to 100 % area of stromal cloudiness.

Pannus is vascularization or the penetration of new blood vessels into the corneal stroma. The vessels are derived. from the limbal vascular loops. Pannus is divided into three grades.

0 = No pannus.

+1 = Vascularization is present but vessels have not invaded the entire corneal circumference. Where localized vessel invasion has occurred, they have not penetrated beyond 2 mm.

+2 = Vessels have invaded 2 mm or more around the entire corneal circumference.

The use of fluorescein is a valuable aid in defining epithelial damage. The area of staining can be judged on a 0 to +4 scale using the same terminology as for corneal cloudiness.

0 = Absence of fluorescein staining.

+1 = Slight fluorescein staining confined to a small focus. With diffuse illumination the underlying structures are easily visible. (The outline of the pupillary margin is as if there were no fluorescein staining.)

+2 = Moderate fluorescein staining confined to a small focus. With diffuse illumination the underlying structures are clearly visible, although there is some loss of detail.

+3 = Marked fluorescein staining. Staining may involve a larger portion of the cornea. With diffuse illumination the underlying structures are barely visible but are not completely obliterated.

+4 = Extreme fluorescein staining. With diffuse illumination the underlying structures cannot be observed. The lens should be evaluated routinely during ocular evaluations and graded as either N (normal) or A (abnormal). The presence of lenticular opacities should be described and the location noted as defined below:

Anterior capsule

Anterior subcapsule

Anterior cortical

Nuclear

Posterior cortical

Posterior subcapsule and Posterior capsule

Appendix E: Draize Scale for Scoring Ocular Lesions in Animal Study

B.1 Cornea

(A) Opacity-degree of density (area most dense taken for reading)
No opacity 0
Scattered or diffuse area, details of iris clearly visible 1
Easily discernible translucent areas, details of iris slightly obscured..... 2
Opalescent areas, no details of iris visible, size of pupil barely discernible..... 3
Opaque, iris invisible..... 4

(B) Area of cornea involved
One quarter (or less) but not zero 1
Greater than one quarter, but less than half..... 2
Greater than half, but less than three quarters..... 3
Greater than three quarters, up to the whole area..... 4
Score equals A x B x 5 ..... Maximum = 80

B.2 Iris

(A) Values
Normal 0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive)..... 1
No reaction to light, hemorrhage, gross destruction (any or all of these) 2
Score equals A x 5..... Maximum = 10

B.3 Conjunctivae

(A) Redness (refer to palpebral and bulbar conjunctivae excluding cornea and iris)
Vessels normal 0
Vessels definitely injected above normal 1
More diffuse, deeper crimson red, individual vessels not easily discernible..... 2
Diffuse beefy red 3

(B) Chemosis
No swelling 0
Any swelling above normal (includes nictitating membrane) 1
Obvious swelling with partial eversion of lids 2
Swelling with lids about half closed 3
Swelling with lids half closed to completely closed..... 4

(C) Discharge
No discharge..... 0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)..... 1
Discharge with moistening of the lids and hairs adjacent to lids 2
Discharge with moistening of the lids and hairs, and considerable area around the eye 3
Score equals (A + B + C) x 2 .....Maximum = 20

B.4 Maximum total score

The maximum total score is the sum of all scores obtained for the cornea, iris and conjunctivae. Total maximum score possible = 110 per eye.

Appendix F: Clinical Trial Notification (CTN) for New Human Trial

Australian Government

Department of Health and Ageing
Therapeutic Goods Administration

Mr Debarun Dutta – Co-Investigator
Level 5, Brien Holden Institute
Rupert Myers Building, North Wing
Barker Street, Gate 14, UNSW
KENSINGTON NSW 2033

Dear Mr Dutta,

CLINICAL TRIAL NOTIFICATION FOR A NEW TRIAL

Title: A prospective, randomised, double masked, contra lateral clinical trial to investigate the clinical performance and retention of antimicrobial activity of melamine coated contact lenses.

Protocol: CRTC-2013-01

Product name(s): Antimicrobial peptide melamine covalently coated over surface of commercially available Acuvue 2 contact lenses.

Your notification to conduct the above-mentioned clinical trial under the Clinical Trial Notification (CTN) scheme, pursuant to Schedule 4 of Regulation 7.1 of the Therapeutic Goods (Medical Devices) Regulations 2000, has been received by the Market Authorisation Group.

The clinical trial has been allocated **CTN Number 157/2013.** Please quote this number in any subsequent correspondence about this trial.

It is noted that the trial will be conducted at the following site(s):
Clinical Research & Trial Center, Brien Holden Institute, Kensington, NSW

It is also noted that:

i. approval for the goods for this trial was given in accordance with Item 2.3 of Schedule 4 of the Therapeutic Goods (Medical Devices) Regulations 2000 by the body or organisation conducting the trial at each site; and

ii. the representative of the ethics committee for each site has certified that the committee is constituted and operates in accordance with the NHMRC National Statement on Ethical Conduct in Research Involving Humans, has considered this clinical trial, and has provided advice to the body or organisation conducting the trial.

PO Box 100 Woden ACT 2606 ABN 40 939 406 804
Phone: 02 6232 8995 Fax: 02 6232 8112 Email: eps@tga.gov.au www.tga.gov.au

TGA Health Safety Regulation

The Therapeutic Goods Administration (TGA) has not carried out an assessment of the quality, safety and efficacy of this product in connection with this or any other notification.

In the event that the Secretary of the Australian Government Department of Health and Ageing becomes aware that to undertake or continue the trial would be contrary to the public interest, the Secretary has the authority to direct that the use of the products for this clinical trial must cease.

If a trial is discontinued for any reason, the Market Authorisation Group should be notified using the TGA's CTN and CTX Trial Completion Advice form enclosed with this acknowledgment.

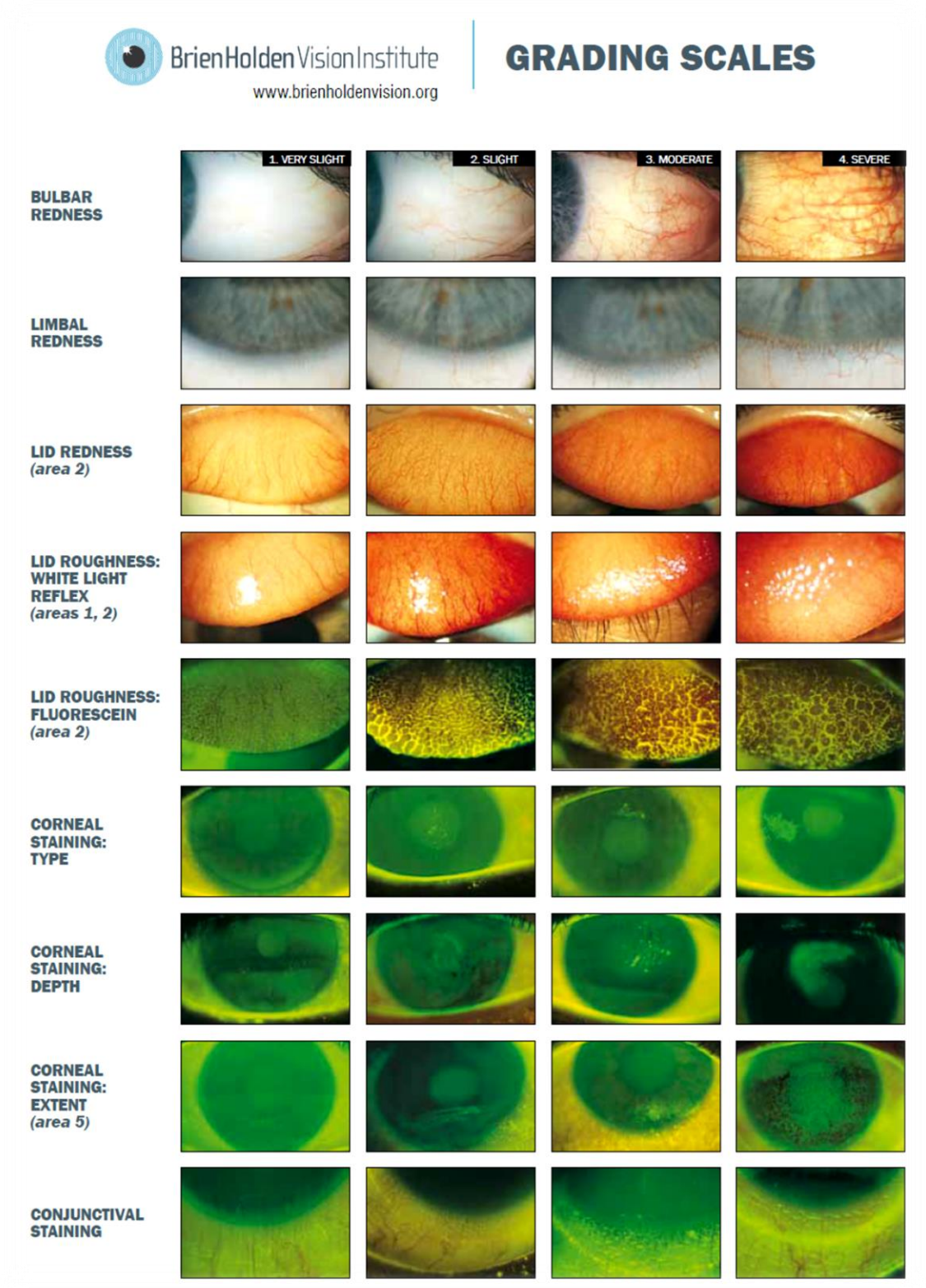
Yours sincerely

Riannon Cuschieri
Administrative Officer
Market Authorisation Group

17 June 2013

CTN2013 157

Appendix G: CCLRU-Brien Holden Vision Institute Grading Scales



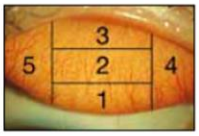
BrienHoldenVisionInstitute
www.brienholdenvision.org

GRADING SCALES

APPLICATION OF GRADING SCALES

- Patient management is based on how much the normal ocular appearance has changed.
- In general, a rating of slight (grade 2) or less is considered within normal limits (except staining).
- A change of one grade or more at follow up visits is considered clinically significant.

PALPEBRAL CONJUNCTIVAL GRADES



- The palpebral conjunctiva is divided into five areas to grade redness and roughness.
- Areas 1, 2 and 3 are most relevant in contact lens wear.

ADVERSE EFFECTS WITH CONTACT LENSES

CLPC CONTACT LENS PAPILLARY CONJUNCTIVITIS

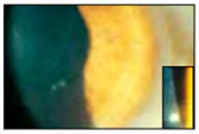
Inflammation of the upper palpebral conjunctiva

Signs
- Redness
- Enlarged papillae
- Excess mucus

Symptoms
- Itchiness
- Mucus strands
- Lens mislocation
- Intolerance to lenses

INFILTRATES

Accumulation of inflammatory cells in corneal sub-epithelial stroma.
Inset: high magnification view

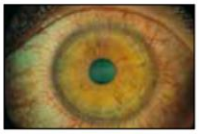


Signs
- Whitish opacity (focal) or grey haze (diffuse)
- Usually confined to 2-3mm from limbus
- Localized redness

Symptoms
- Asymptomatic or scratchy, foreign body sensation
- Redness, tearing and photophobia possible

CLARE CONTACT LENS ACUTE RED EYE

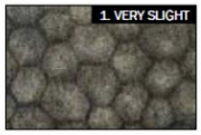
An acute corneal inflammatory episode associated with sleeping in soft contact lenses

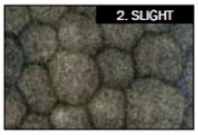


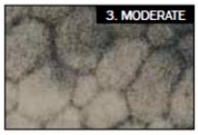
Signs
- Unilateral
- Intense redness
- Infiltrates
- No epithelial break

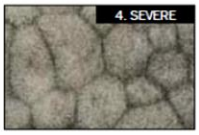
Symptoms
- Wakes with irritation or pain
- Photophobia
- Lacrimation

POLYMEGETHISM





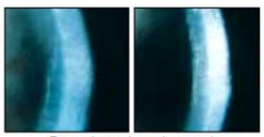




VASCULARIZATION

Vessel extension beyond translucent limbal zone is recorded (mm)

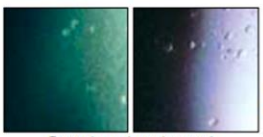
STROMAL STRIAE and FOLDS



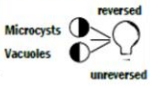
One striae = 5% edema
One fold = 8% edema
(each additional striae or fold indicates 1% more edema)

Record number observed

MICROCYSTS and VACUOLES



Located in epithelium. Identified by side showing brightness.



Record number observed

CORNEAL STAINING GRADES

- Staining assessed immediately after single instillation of fluorescein using cobalt blue light and wratten 12 (yellow) filter over the slit lamp objective.
- The cornea is divided into five areas. The type, extent and depth of staining are graded in each area.

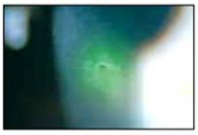
Type
1 Micropunctate
2 Macropunctate
3 Coalescent macropunctate
4 Patch

Extent Description	Depth Description*
1 ≤5%	1 Superficial epithelium
2 6-15%	2 Deep epithelium, delayed stromal glow
3 16-30%	3 Immediate localized stromal glow
4 >30%	4 Immediate diffuse stromal glow

** Based on penetration of fluorescein and slit lamp optic section*

EROSION

Full thickness epithelial loss over a discrete area

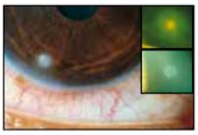


Signs
- No stromal inflammation
- Immediate spread of fluorescein into stroma

Symptoms
- Can be painful
- Photophobia
- Lacrimation

CLPU CONTACT LENS PERIPHERAL ULCER

Round, full thickness epithelial loss with inflamed base, typically in the corneal periphery which results in a scar. Insets: with fluorescein, scar

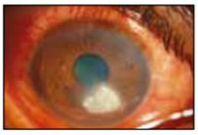


Signs
- Unilateral, "white spot"
- Localized redness
- Infiltrates
- Post healing scar

Symptoms
- Varies from foreign body sensation to pain
- Lacrimation and photophobia may occur

INFECTED ULCER

Full thickness epithelial loss with stromal necrosis and inflammation, typically central or paracentral



Signs
- Intense redness
- "White patch" (raised edges)
- Infiltrates
- Epithelial and stromal loss
- Anterior chamber flare
- Conjunctival and lid edema

Symptoms
- Pain, photophobia
- Redness, mucoid discharge
- ↓ VA (if over pupil)