Cornea

Broad Spectrum Antimicrobial Activity of Melimine Covalently Bound to Contact Lenses

Debarun Dutta,[1–3] *Nerida Cole,*[*,1,2] *Naresh Kumar,*[4] *and Mark D. P. Willcox*[2]

Purpose. To develop a stable antimicrobial contact lens, which is effective against the International Organization for Standardization (ISO) panel microorganisms, *Acanthamoeba castellanii* and drug resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Methods. Melimine was covalently incorporated into etafilcon A lenses. The amount of peptide present on the lens surface was quantified using amino acid analysis. After coating, the heat stability (121°C), lens surface hydrophobicity (by captive bubble), and in vitro cytotoxicity to mouse L929 cells of the lenses were investigated. Antimicrobial activity against the micro-organisms was evaluated by viable plate count and fluorescence microscopy, measuring the proportion of cell death compared with control lenses with no melimine.

Results. The most effective concentration was determined to be 152 ± 44 μg lens^{-1} melimine on the lens surface. After coating, lenses were relatively hydrophilic and were nontoxic to mammalian cells. The activity remained high after autoclaving (e.g., 3.1, 3.9, 1.2, and 1.0 log inhibition against *P. aeruginosa*, *S. aureus*, *A. castellanii*, and *Fusarium solani*, respectively). Fluorescence microscopy confirmed significantly reduced ($P < 0.001$) adhesion of viable bacteria to melimine contact lenses. Viable count confirmed that lenses were active against all the bacteria and fungi from the ISO panel, *Acanthamoeba* and gave at least 2 log inhibition against all the multidrug resistant *S. aureus* and *P. aeruginosa* strains.

Conclusions. Melimine may offer excellent potential for development as a broad spectrum antimicrobial coating for contact lenses, showing activity against all the bacterial and fungal ISO panel microorganisms, *Acanthamoeba*, and antibiotic resistant strains of *P. aeruginosa* and *S. aureus*. (*Invest Ophthalmol Vis Sci.* 2013;54:175–182) DOI:10.1167/iovs.12-10989

Contact lens wear is a risk factor for the development of microbial keratitis (MK),[1] which is a sight threatening adverse event associated with lens wear. Depending on the study design and location, contact lens wear now accounts for around 12.4% to 66% of all MK events.[2–9] A variety of microorganisms have been implicated in MK, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, coagulase-negative *staphylococci*, *Serratia marcescens*, *Escherichia coli*, *Acanthamoeba castellanii, Fusarium solani*, and *Candida albicans*.[1,10,11] Contact lens related acute red eye (CLARE), contact lens peripheral ulcer (CLPU), and infiltrative keratitis (IK) are also associated with contact lens wear.[12,13] These inflammatory adverse events are associated with microbial (mainly gram negative bacteria or *S. aureus*) colonization of contact lenses.[12,14,15]

Antibiotic resistance among the ocular pathogens has been increasing in parallel with the increase observed in systemic bacterial infections.[16] A significant proportion of ocular infections caused by *S. aureus* and *P. aeruginosa* have been associated with antibiotic resistant strains.[11,17] Rates of resistance to ciprofloxacin, a commonly used first line monotherapy for MK,[18] of ocular isolates of *S. aureus* from cases of MK treated in Florida increased from 3% to 8% of isolates in the early 1990s to 27% to 40% in 2000 to 2001 largely due to the more frequent isolation of methicillin-resistant *S. aureus* (MRSA), which had rates of resistance to ciprofloxacin of between 30% to 97% in the same time period.[19] Whilst resistance to ciprofloxacin of *P. aeruginosa* isolates has remained relatively low in Australasia and the United States, rates of resistance of 19% to 23% have been reported from India,[20–22] Iraq, and China.[23] Microbial keratitis associated with drug resistant bacteria can increase morbidity, treatment cost, and poor prognosis.[24] Furthermore, biofilm formation by clinical isolates of *P. aeruginosa*, *S. aureus*, and *S. marcescens* on contact lenses has been reported to increase resistance to several contact lens disinfecting solutions.[25]

The recent outbreaks of fungal and *Acanthamoeba* keratitis associated with specific multipurpose contact lens disinfecting solutions has highlighted these as causative agents of disease during contact lens wear.[26,27] Although the rate of contact lens related *Fusarium* keratitis slowly decreased after withdrawal of the solution,[10] the overall incidence of *Acanthamoeba* keratitis remained higher than prior to the epidemic.[10,28] The incidence of fungal or amoebal keratitis during lens wear still remains much lower than for bacterial keratitis[1,4] but these continue to be difficult infections to diagnose and manage.[29,30]

Strategies that have been designed to prevent microbial colonization of contact lenses include incorporation of nonsteroidal anti-inflammatory drugs (NSAID),[31,32] phosphorylcholine,[33] fimbrolides,[34] silver,[35] selenium,[36] antimicrobial cationic peptides (AMP),[37,38] or high density poly (ethylene oxide) dialdehyde (PEO(ALD)$_2$).[39] However, disadvantages of these technologies are that NSAIDs and silver need to be released from lenses to retain activity, and so might lose activity during use; PEO(ALD)$_2$ and phosphorylcholine coatings are

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TABLE 1. Details of Organisms

Bacterial Strains	Isolation Site	Resistant To*
P. aeruginosa 6294	MK	Not determined (ND)
S. aureus 31	CLPU – contact lens	ND[46]
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[45,72]		
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.

passive anti-adhesive agents (i.e., do not contain inherent antimicrobial activity). In addition, most strategies have not been tested against *Acanthamoeba*, fungal isolates, or drug resistant bacteria.[27,30]

AMPs are known to have broad spectrum antimicrobial activity.[40,41] Previous studies have confirmed that melimine, prepared by combining active regions of protamine (from salmon sperm) and melittin (from bee venom), is a heat stable antibacterial AMP.[38,42] Furthermore, melimine-coated lenses have been shown to reduce corneal infiltrative events in animal models.[37] The aim of this study was to evaluate melimine-coated lenses for activity against fungi, *Acanthamoeba*, and multi-drug resistant *S. aureus* and *P. aeruginosa*. In addition, we wished to confirm that the melimine-coated lenses were nontoxic to mammalian cells and that addition of melimine did not affect the parameters of lenses.

METHODS

Production of Melimine Coated Contact Lenses

Melimine (>80% purity; American Peptide Company, Sunnyvale, CA) was diluted in sterile 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}). One of the most widely used contact lens materials etafilcon A[43] (Base curve: 8.7 mm, Diameter: 14.0 mm, Power: −3.00 Diopter [D]; Johnson & Johnson Vision Care Inc., Jacksonville, FL) was used for this study. Contact lenses were removed from the manufacturer's vials, and washed three times in 1 mL PBS. Melimine was covalently attached to lenses using a modification of a previously described method.[38] Briefly, lenses were washed twice in 0.1 M l^{-1} sodium acetate buffer (pH 5.0), and soaked in 2 mL 0.1 M l^{-1} sodium acetate buffer (pH 5.0), containing 2 mg ml^{-1} 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for 15 minutes at 25°C. Lenses that reacted only with EDC (no melimine added) served as process controls. For melimine coating, lenses were washed three times in PBS and then suspended in 100 μg ml^{-1}, 500 μg ml^{-1}, 1 mg ml^{-1}, 3 mg ml^{-1}, or 5 mg ml^{-1} of melimine in PBS, and incubated for 2 hours at 37°C with gentle shaking. Subsequently, lenses were washed three times in sterile PBS, and then resuspended in 2 mL of 10% wt/vol NaCl overnight followed by soaking in PBS for 2 hours to extract any dissolved noncovalently attached peptide remaining within the lens matrix. The amount of peptide present on the lens surface was quantified using amino acid analysis as outlined previously.[44] Contact lenses processed only with EDC (without melimine) acted as process controls. A separate batch of lenses prepared by soaking in melimine (same concentration used for EDC coupling) solution for 2 hours without EDC covalent coupling used to determine effectiveness of the covalent attachment. All the lenses were stored in glass vials at 5°C in sterile PBS.

Strains and Adhesion Conditions

All micro-organisms used in this study and their sources are listed in Table 1. Primary evaluation, validation, and screening of the melimine-coated lenses were performed using *P. aeruginosa* 6294 and *S. aureus* 31. Antimicrobial efficacy of lenses containing the lowest concentration of melimine, but highest antibacterial activity were then tested for activity against the drug resistant strains of *P. aeruginosa*[45] and *S. aureus*,[46] ISO panel micro-organisms,[47] and *Acanthamoeba*.

Bacteria were grown overnight in Tryptone Soya Broth (TSB; Oxoid, Basingstoke, UK) and then washed three times in PBS. *S. aureus* strains were resuspended in 1/10 TSB, *S. marcescens* and *P. aeruginosa* strains were resuspended 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 serially diluted (1/10) to 1.0×10^6 CFU ml^{-1} for adhesion assays. Fungal strains were grown on Potato Dextrose Agar (PDA; Oxoid) plates by incubating for 7 to 10 days at 25°C for *F. solani* and for 24 hours at 37°C for *C. albicans*. Both fungal strains then were suspended in sterile PBS and filtered through sterile 70 and 40 μm filters to remove hyphal fragments and finally resuspended to an OD_{660nm} of 2.6 and 1.5 (1.0×10^8 CFU ml^{-1}), respectively. Fungal suspensions were serially diluted to 1.0×10^6 CFU ml^{-1} and used for adhesion assays.

Acanthamoeba castellanii ATCC 50370 was used in this study. Cryopreserved *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} $CaCl_2$, 1g l^{-1} Sodium citrate, $2H_2O$, 20 mg l^{-1} $FE(NH_4)_2$ (SO_4), $6H_2O$, 0.34g l^{-1} KH_2PO_4, 188 mg l^{-1} Na_2HPO_4, 18 g l^{-1} Glucose), and incubated at 32°C for 7 to 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 to 4 days to obtain trophozoites, which were collected by centrifuging for 12 minutes at 1000 rpm and resuspended in Page's saline (0.12g 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 enumerated using a Neubauer haemocytometer and the final inoculum adjusted using Page's saline to approximately 1.0 to 1.5×10^5 cells/mL.

Noncoated control and peptide-coated lenses were washed in PBS and transferred to 1 mL of bacterial, fungal, or acanthamoebal suspensions (prepared above) in wells of 24-well tissue culture plates (CELESTAR; Greiner Bio-One, Frickenhausen, Germany). To allow adhesion of microbial cells, lenses were incubated 18 hours at 37°C for bacteria, 18 hours at 25°C for fungus, and 6 hours at 25°C for amoeba with shaking (120 rpm).

Contact lenses were washed 3 times with PBS to remove nonadherent cells and then stirred rapidly in 2 mL of PBS containing a small magnetic stirring bar. This resulted in a disintegration of the lens. For bacterial strains, following log serial dilutions in Dey Engley neutralizing broth (DE; Becton, Dickson and Company, Sparks, MD), 3 × 50 µL of each dilution were plated on a tryptic soy agar (TSA; Oxoid) containing Tween 80 and lecithin for recovery of cells. For fungal strains, following log serial dilutions in DE neutralizing broth, 100 µL were plated onto PDA for recovery of viable cells. For *Acanthamoeba,* the samples were serially diluted 10-fold in DE broth and quadruplicates of each dilution of cells were plated on to nonnutrient agar (NNA; Oxoid) plates pre-incubated with *Escherichia coli* and incubated at 32°C 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 determined using Reed and Muench computation.[48] After 24 hours incubation at 37°C for bacteria or 2 days incubation at 37°C for *C. albicans* and 4 days incubation at 25°C for *F. solani*, the viable micro-organisms were enumerated as colony forming units per cells per millimeters squared. Results are expressed as the reduction in adherent viable bacteria, fungi or *Acanthamoeba* (compared with the uncoated control lens) of triplicate measurements performed on a minimum of three separate occasions.

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) according to the manufacturer's guidelines.[49] Microscopic observation and image acquisition was performed with an Olympus FV1000 Confocal Inverted Microscope (Olympus Corporation, Tokyo, Japan). Images obtained from eight representative areas on each of triplicate samples for each surface were analyzed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD) (Rasband 1997–2008). 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.

Effect of Autoclaving on Activity of Melimine-Coated Lenses

Lenses were autoclaved (121°C) in PBS for 15 minutes, after which the lenses were allowed to return to ambient temperature (~20°C). Retention of antimicrobial activity of the autoclaved lenses was measured using *P. aeruginosa* 6294 and *S. aureus* 31 as described above. Three lenses were used for one experiment and were repeated on a minimum of three separate occasions.

Lens Parameter Measurements

To test whether reacting lenses with melimine resulted in any lens parameter changes, five uncoated contact lenses (–3.00 D) were selected for metrologic evaluation before and after being coated with melimine. Lenses were immersed in PBS at ambient temperature (20°C ± 2°C) for 24 hours prior testing. Center thicknesses were measured using a Heidenhain Soft Contact Lens Thickness gauge (Metrology and Quality Services Ltd., St. Albans, UK) following the ISO: 18369-3,[50] 9339-2[51] and American National Standard Institute (ANSI) Z80.20-1998[52] protocol. The diameter of lenses was measured following ISO: 18369-3,[50] 9338[53] protocol in a wet cell using a Nikon profile projector (Nippon Kogaku K.K., Tokyo, Japan) with horizontal x-y table and digital position readout. Sagittal depth was measured by profile projector following ISO:18369-3[50] and ANSI Z80.20-1998[52] protocols. Base curve equivalents were calculated using measured lens diameters, center thickness, and sagittal depth measurements. All procedures were repeated to obtain five independent measurements for each lens and these were then averaged.

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.[54] Contact angle was determined using a contact angle goniometer (Model no. 200-F1; Rame-Hart, Inc NRL, Succasunna, NJ). Melimine treated and control contact lenses were soaked in PBS for 2 to 3 hours at room temperature (25 ± 2°C), then lenses were carefully rested on a custom made holder so that the convex lens surface faced downward, directly into 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 microsyringe. Assessment of the receding and advancing contact angle 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 (Precision Co., Tokyo, Japan). Image J software was used to calculate advancing and receding contact angle. A minimum of eight measurements was made on five samples each contact lens and were averaged.

Cytotoxicity

In vitro cytotoxicity of the contact lenses was determined using a direct contact method as outlined in ISO 10993.[55] Briefly, murine L929 cells were grown in plastic petri dishes to confluence and melimine-coated lenses or noncoated controls were placed directly on the cell monolayer and incubated for 24 hours with fresh medium. After this incubation the cytotoxicity was assessed using bright field and phase contrast microscopy after staining with Trypan blue (Sigma-Aldrich, St. Louis, MO). Cytotoxic responses (i.e., zone of extent of cell damage) were graded on a scale of 0 to 4.[55] Additional controls used were silastic medical grade tubing (Dow Corning Corporation, Midland, MI)) 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 contact lens samples were used for this test.

Statistical Analysis of Data

The adhesion data were $\log_{10}$ (x+1) transformed prior to data analysis where x is the adherent bacteria or fungi in colony forming units per millimeters to the negative two or amoeba in track forming units per millimeters to the negative two. Microbial adhesion and contact lens parameters were analyzed 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 analyzed 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%.

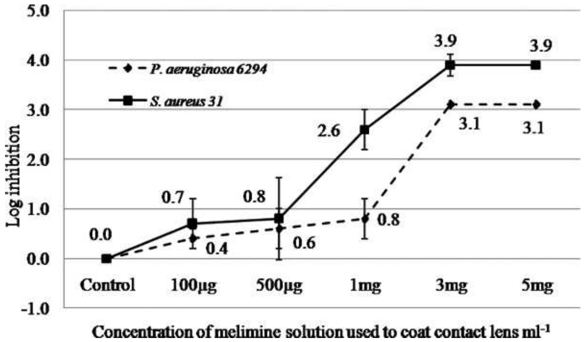

Figure 1. Mean log inhibition of *P. aeruginosa* 6294 and *S. aureus* 31 ($n \geq 9$) adhesion to melimine-coated lenses compared with control noncoated lenses.

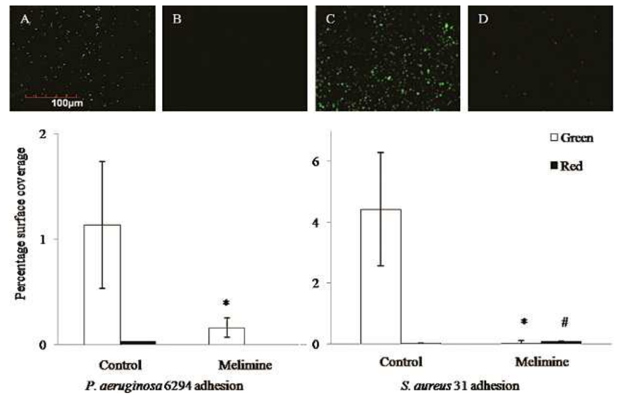

Figure 2. Fluorescence microscopy of bacterial adhesion on contact lens ($n \geq 9$) surfaces in the presence and absence of covalently linked melimine. Bacterial cells with intact membranes stain *green*, while those with permeabilized membranes are *red*. Areas covered by green staining bacteria are represented by *white bars* and the area covered by red-staining bacteria by *black bars*. Captured using a ×20 objective. Magnification = ×200. (**A**) *P. aeruginosa* 6294 adherent to control contact lens. (**B**) *P. aeruginosa* 6294 adherent to melimine lens. (**C**) *S. aureus* 31 adherent to control lens. (**D**) *S. aureus* 31 adherent to melimine lens. 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. The asterisks (*) represents significant ($P < 0.05$) reduction for green stained (*live*) bacteria and # represent significant increase of red stained (*dead*) *S. aureus* 31 on the melimine treated contact lens.

Results

Evaluation of Most Effective Peptide Concentration

Initial studies were to determine the smallest amount of melimine attached to lenses that resulted in the greatest amount of antimicrobial activity. Figure 1 shows the log inhibition of melimine-coated contact lenses compared with control lenses. Increasing the concentration of melimine associated with the lenses resulted higher log inhibition of both *P. aeruginosa* 6294 and *S. aureus* 31. For both bacterial types, lenses prepared by adding 3 mg mL^{-1} melimine gave as great an inhibition of each strain (3.1 ± 0.1 and 3.9 ± 0.2 log inhibition, respectively) as the next highest concentration of melimine (5 mg ml^{-1}). Therefore, for all subsequent experiments, melimine-coated lenses were produced by incubating in 3 mg ml^{-1} of melimine. This resulted in 152 ± 43 µg $lens^{-1}$ melimine associated on the lens surface.

Antimicrobial activity of the selected melimine contact lens was further explored by fluorescence microscopy and image analysis (Fig. 2). For both *P. aeruginosa* 6294 ($P = 0.014$) and *S. aureus* 31 ($P < 0.001$) there was a significant decrease in the numbers of bacteria staining green (indicating intact cell membrane) on the melimine contact lens surfaces compared with control contact lenses. There was no significant difference between areas of the surfaces covered by membrane damaged (red stained) *P. aeruginosa* 6294 ($P = 0.087$) on the melimine contact lenses when compared with control contact lenses. In contrast, red stained *S. aureus* 31 covered a higher percentage area ($P = 0.001$) on melimine lenses than control lenses. Overall, there was significantly ($P < 0.001$) decreased bacterial adhesion (dead and live combined) on melimine contact lenses compared with control lenses.

Effect of Autoclaving and Hypertonic Solution Treatment on Activity of Melimine-Coated Lenses

For both *P. aeruginosa* 6294 and *S. aureus* 31, heat treated melimine contact lenses showed no significant ($P > 0.05$) reduction in antimicrobial activity compared with untreated melimine lenses. Addition of NaCl was performed in order to help remove any noncovalently bound melimine from the contact lenses. Analysis of adhesion of *P. aeruginosa* 6294 or *S. aureus* 31 to lenses that had or had not been treated with NaCl showed no significant effect on inhibition of adhesion (0.01 log, $P > 0.05$), suggesting that very little melimine was adsorbed and noncovalently bound to the 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 or control lenses following the washing steps (Fig. 3), indicating that the washing process had removed most of the adsorbed and noncovalently bound melimine.

Lens Parameters and Hydrophobicity Measurements

The commercially available etafilcon A lenses (with a power of −3.00 D) 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 ($P > 0.05$) change in lens diameter (13.52 ± 0.02 mm), central thickness (57.80 ± 2.77 µm), or calculated base curve (8.18 ± 0.03 mm). The mean and 95% confidence interval (CI) of the contact angles of the lenses are detailed in Table 2. Melimine coating resulted in a significant decrease ($P < 0.001$) in advancing contact angle compared with uncoated lenses.

Cytotoxicity of Melimine-Coated Lenses

In the cytotoxicity assay the responses were graded according to a standard key, which quantifies the zonal extent of cell damage (0–4 maximum). Positive and negative controls worked as expected; the positive and negative controls gave an inhibition of grade 4 and 1, respectively. All the three melimine-coated lenses and commercially available etafilcon A lenses showed a minimal response of grade 1, indicating no cytotoxicity, with only a small annulus of dead cells under the contact area. Thus, the melimine-coated lenses are considered to be nontoxic.

Efficacy of Melimine-Coated Lenses against Drug Resistant Bacteria

Melimine-coated lenses significantly ($P < 0.001$) reduced the viability of all the drug resistant bacteria as well as the high

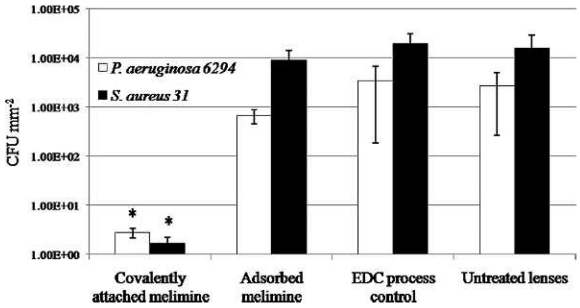

Figure 3. *P. aeruginosa* 6294 and *S. aureus* 31 adhesions to contact lenses following different treatments. *Asterisks* represent significant ($P < 0.001$) reduction in bacterial adhesion compared with contact lenses with adsorbed peptide, process controls, and untreated controls.

biofilm producing strain of *P. aeruginosa* (Fig. 4). Melimine-coated lenses gave at least 2 log inhibition of all the drug resistant bacteria. The viable counts of bacteria associated with melimine-coated lenses ranged from 0 to 16 CFU mm^{-2} compared with controls, which ranged from 3.6×10^2 to 2.0×10^4 CFU mm^{-2}.

Efficacy against ISO Panel Strains

The ability of melimine lenses to inhibit adhesion by ISO panel organisms is shown in Figure 5. Melimine lenses significantly ($P < 0.001$) inhibited the number of live cells adherent to lens surfaces of all the organisms tested. There were 1.0 ± 0.2 log and 1.1 ± 0.2 log inhibition against *F. solani* ATCC 36031 and *C. albicans* ATCC 10231. Antimicrobial activity was least but still significant ($P < 0.001$) against *Serratia marcescens* ATCC 13880 (0.9 ± 0.3 log).

Efficacy against *Acanthamoeba*

There were on average 1801 mm^{-2} viable *Acanthamoeba* cells adhered to control contact lens surfaces compared with 70 mm^{-2} cells on the melimine-coated contact lens surface. The melimine lenses resulted 1.4 ± 0.2 log inhibition against *A. castellanii* ATCC 50370 ($P < 0.001$).

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 our previous data, 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).[38] Our results showed a significant reduction in the numbers of viable bacteria adherent to melimine-coated contact lenses. There were also significant reductions in the numbers of dead *P. aeruginosa* adherent to melimine 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.[42] Any remaining dead cells on melimine-coated contact lenses are unlikely to be associated with contact lens related inflammatory events as our previous studies confirmed that melimine-coated lenses had the capacity to reduce inflammatory events like CLARE and CLPU in animal models[37] and live *S. aureus* were required to produce a CLPU responses in the animal model.[12] A previous study[37] found that the total count of bacteria did not differ between control and melimine-coated lenses, but this disparity might be either the consequence of the higher concentration of melimine present in etafilcon A lenses (152 μg lens^{-1}) compared with the silicone hydrogel lenses (44 μg lens^{-1}), or due to difference in polymer characteristics of the underlying lens materials used. Moreover, the current study extends our previous finding that melimine in solution retained activity when autoclaved,[38] to

Table 2. Contact Angle of Control and Melimine Contact Lenses

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

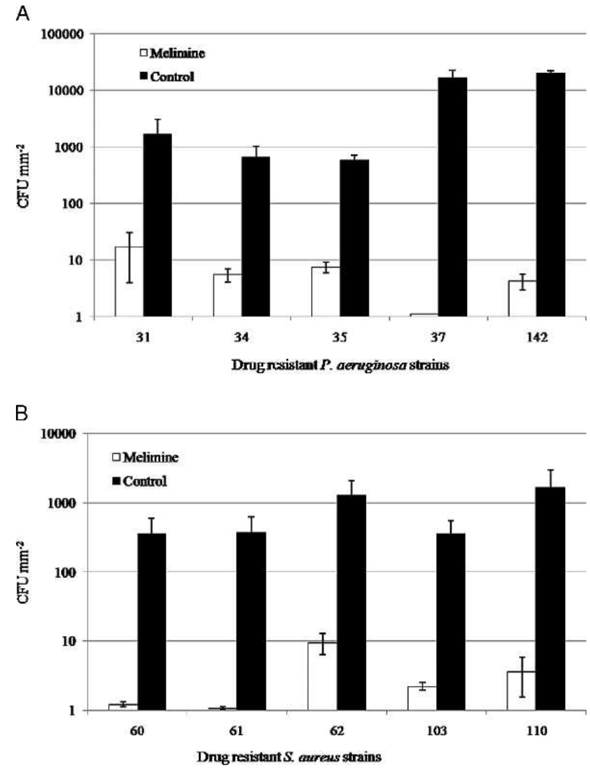

Figure 4. Antibiotic resistant *P. aeruginosa* (**A**) and *S. aureus* (**B**) adhesion to melimine coated and control contact lenses ($n \geq 9$).

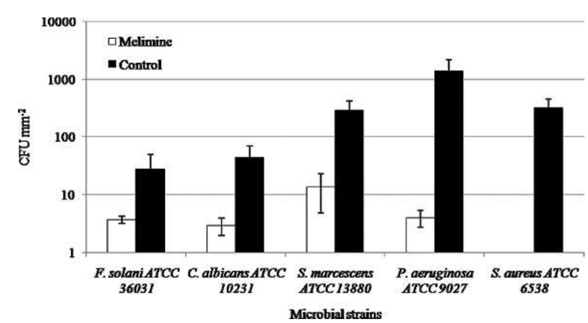

Figure 5. ISO panel bacterial and fungal adhesions to melimine coated and control contact lenses ($n \geq 9$).

show that melimine bound to a surface also retains antimicrobial activity after autoclaving. Because of highly cationic nature of melimine it is very unlikely to form a densely packed layer that interferes with oxygen permeability. However, further investigations might be indicated to evaluate any change in oxygen permeability especially with silicone hydrogel contact lenses. The current study demonstrated that covalently attaching 152 μg melimine on etafilcon A lenses did not alter lens parameters and the surface was not cytotoxic to fibroblasts. The latter finding re-enforces the previously published nonhemolytic activity of melimine in solution.[38]

In this study, we report hydrophilic shift of contact lens surfaces after coating with melimine. This result was evident while measuring the advancing angle in the captive bubble technique. The majority of the melimine is composed of positively charged hydrophilic amino acids, which might have lead to a hydrophilic surface. Evidence suggests that hydrophobic surfaces generally result in higher protein–surface adsorption than hydrophilic surfaces.[56] However, this is not always the case, and the high negative charge associated with methacrylic acid in etafilcon A lenses is well known to encourage deposition of the cationic protein lysozyme from tears.[57,58] The addition of the cationic peptide melimine to lenses is likely to result in an increased positive charge on the lens surface. In the tear film, the protein lipocalin is relatively negatively charged[59] and it might be expected that lipocalin or other negatively charged proteins interact with surface bound melimine, and perhaps affect its activity. However, when unattached melimine has been incubated with tears there is no loss of antimicrobial activity[60] and this may indicate a low likelihood of reduction in activity during wear. This indicates that there is unlikely to be ionic interactions with anionic proteins such as lipocalin in tears that reduce activity. Furthermore, these initial studies suggest that the proteases in tears may also not affect the activity of melimine.

Contact lens related fungal keratitis is a rare, but severe form of infectious keratitis generally associated with poor prognosis.[61] The incidence has progressively increased even after the recent *Fusarium* keratitis epidemic.[62] Fungi can be resistant to the activity of several contact lens multipurpose disinfecting solutions.[63] 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 hours,[64] and only one lens case showed limited activity (0.5 log) against *Fusarium solanii*.[43] In this study we have demonstrated that the melimine-coated lenses produced at least one log inhibition against both *Candida* and *Fusarium* strains, indicating the possibility of controlling colonization 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.[28,29,65] Keratitis caused by *Acanthamoeba* often has limited treatment options, significantly higher duration of hospital admission, and unpredictable outcome.[29] Many commonly used contact lens disinfecting solutions have only limited amoebicidal efficacy.[66] The recent outbreak of contact lens related *Acanthamoeba* keratitis associated with use of Complete MoisturePlus contact lens disinfecting solution[10] and persistent elevated numbers of events, even after removal of this solution from sale,[10,28] 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.[34]

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.[1,16,45,67] Here we have reported at least 2 log inhibition of adhesion by melimine-coated lenses for 10 *P. aeruginosa* and *S. aureus* strains, which were resistant against commonly used antibiotics such as ciprofloxacin, gentamicin, moxifloxacin, and tobramycin. This combined with our previous finding of the inability of bacterial strains to become resistant after repeated exposure to sub-inhibitory concentration of melimine is a promising finding toward controlling these resistant bacteria.

Naturally occurring AMPs such as beta defensin 3 (hBD-3) and cathelicidin LL 37 have been found in tears and have broad spectrum antimicrobial activity.[41] Melimine is a synthetic cationic peptide designed to have maximum activity in its bound state. The minimal inhibitory concentrations (MIC) of naturally occurring AMPs (between 1–100 μg ml^{-1})[68] in their free state are lower than melimine. There have been successful attempts to achieve antimicrobial activity by covalently attaching AMPs over different surfaces such as polymide resin, cellulose, glass coverslips, and so on.[69] Covalent immobilization of cathelicidin LL 37 on titanium surfaces gives bactericidal activity against *E. coli*.[70] However, retention of antimicrobial activity of these naturally occurring AMPs onto surfaces, such as hydrogel and silicon hydrogel, has not yet been investigated, nor has their resistance to autoclaving. In this study, we were able to optimize and demonstrate very high antimicrobial activity against gram negative and gram positive bacteria by attaching high concentration of melimine onto the contact lens surface by EDC covalent coupling. It would be worth investigating using similar technology onto the attachment of the naturally occurring AMPs over contact lens or lens case surface that might lead to novel antimicrobial surface development strategy.

Future work that is necessary prior to further development of melimine-coated lenses would be to investigate the interaction of melimine-coated contact lenses with commercially available multipurpose disinfection solutions, and perhaps any interactions with the commercially available silver antimicrobial contact lens cases. Contact lens wear for consecutive 22 days, determining safety and biocompatibility by ocular study using rabbit eyes following ISO 9394 is necessary.[71] In summary, this study demonstrated that melimine-coated contact lenses have broad spectrum antimicrobial activity. They are also nontoxic, the binding of melimine does not alter lens parameters, and the coated lenses are heat stable. This, coupled with our previous demonstration of the ability of melimine-coated lenses to control adverse events in animal models,[37] makes melimine-coated lenses potentially ideal as an antimicrobial coating for preventing initiation of MK and other microbially-driven adverse events during contact lens wear.

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References

1. Green M, Apel A, Stapleton F. Risk factors and causative organisms in microbial keratitis. *Cornea*. 2008;27:22–27.

2. Bourcier T, Thomas F, Borderie V, Chaumeil C, Laroche L. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. *Br J Ophthalmol*. 2003;87:834–838.
3. Fong CF, Tseng CH, Hu FR, Wang IJ, Chen WL, Hou YC. Clinical characteristics of microbial keratitis in a university hospital in Taiwan. *Am J Ophthalmol*. 2004;137:329–336.
4. Keay L, Edwards K, Naduvilath T, et al. Microbial keratitis predisposing factors and morbidity. *Ophthalmology*. 2006; 113:109–116.
5. Mela EK, Giannelou IP, Koliopoulos JX, Gartaganis SP. Ulcerative keratitis in contact lens wearers. *Eye Contact Lens*. 2003;29:207–209.
6. Wong T, Ormonde S, Gamble G, McGhee CN. Severe infective keratitis leading to hospital admission in New Zealand. *Br J Ophthalmol*. 2003;87:1103–1108.
7. Gebauer A, McGhee CN, Crawford GJ. Severe microbial keratitis in temperate and tropical Western Australia. *Eye*. 1996;10:575–580.
8. Rattanatam T, Heng WJ, Rapuano CJ, Laibson PR, Cohen EJ. Trends in contact lens-related corneal ulcers. *Cornea*. 2001; 20:290–294.
9. Baier RE. Comments on cell adhesion to biomaterial surfaces: conflicts and concerns. *J Biomed Mater Res*. 1982;16:173–175.
10. Tu EY, Joslin CE. Recent outbreaks of atypical contact lens-related keratitis: what have we learned? *Am J Ophthalmol*. 2010;150:602–608.
11. Haas W, Pillar CM, Torres M, Morris TW, Sahm DF. Monitoring antibiotic resistance in ocular microorganisms: results from the Antibiotic Resistance Monitoring in Ocular micRorganisms (ARMOR) 2009 surveillance study. *Am J Ophthalmol*. 2011; 152:567–574.
12. Wu P, Stapleton F, Willcox MD. The causes of and cures for contact lens-induced peripheral ulcer. *Eye Contact Lens*. 2003; 29:S63–66.
13. Willcox M, Sharma S, Naduvilath TJ, Sankaridurg PR, Gopinathan U, Holden BA. External ocular surface and lens microbiota in contact lens wearers with corneal infiltrates during extended wear of hydrogel lenses. *Eye Contact Lens*. 2011;37:90–95.
14. Holden BA, La Hood D, Grant T, et al. Gram-negative bacteria can induce contact lens related acute red eye (CLARE) responses. *CLAO J*. 1996;22:47–52.
15. Sankaridurg PR, Sharma S, Willcox M, et al. Bacterial colonization of disposable soft contact lenses is greater during corneal infiltrative events than during asymptomatic extended lens wear. *J Clin Microbiol*. 2000;38:4420–4424.
16. Sharma S. Antibiotic resistance in ocular bacterial pathogens. *Indian J Med Microbiol*. 2011;29:218–222.
17. Willcox MD. Review of resistance of ocular isolates of *Pseudomonas aeruginosa* and *Staphylococci* from keratitis to ciprofloxacin, gentamicin and cephalosporins. *Clin Exp Optom*. 2011;94:161–168.
18. Willcox MD. Management and treatment of contact lens-related *Pseudomonas* keratitis. *Clin Ophthalmol*. 2012;6:919–924.
19. Marangon FB, Miller D, Muallem MS, Romano AC, Alfonso EC. Ciprofloxacin and levofloxacin resistance among methicillin-sensitive *Staphylococcus aureus* isolates from keratitis and conjunctivitis. *Am J Ophthalmol*. 2004;137:453–458.
20. Kunimoto DY, Sharma S, Garg P, Rao GN. In vitro susceptibility of bacterial keratitis pathogens to ciprofloxacin - emerging resistance. *Ophthalmology*. 1999;106:80–85.
21. Garg P, Sharma S, Rao GN. Ciprofloxacin resistant *Pseudomonas* keratitis. *Ophthalmology*. 1999;106:1319–1323.
22. Bharathi MJ, Ramakrishnan R, Shivakumar C, Meenakshi R, Lionalraj D. Etiology and antibacterial susceptibility pattern of community-acquired bacterial ocular infections in a tertiary eye care hospital in south India. *Indian J Ophthalmol*. 2010; 58:497–507.
23. Zhang C, Liang Y, Deng S, Wang Z, Li R, Sun X. Distribution of bacterial keratitis and emerging resistance to antibiotics in China from 2001 to 2004. *Clin Ophthalmol*. 2008;2:575–579.
24. French GL. Clinical impact and relevance of antibiotic resistance. *Adv Drug Deliv Rev*. 2005;57:1514–1527.
25. Szczotka-Flynn LB, Imamura Y, Chandra J, et al. Increased resistance of contact lens-related bacterial biofilms to antimicrobial activity of soft contact lens care solutions. *Cornea*. 2009;28:918–926.
26. Ahearn DG, Zhang S, Stulting RD, et al. *Fusarium* keratitis and contact lens wear: facts and speculations. *Med Mycol*. 2008; 46:397–410.
27. Patel A, Hammersmith K. Contact lens-related microbial keratitis: recent outbreaks. *Curr Opin Ophthalmol*. 2008;19: 302–306.
28. Yoder JS, Verani J, Heidman N, et al. *Acanthamoeba* keratitis: the persistence of cases following a multistate outbreak. *Ophthalmic Epidemiol*. 2012;19:221–225.
29. Otri AM, Fares U, Al-Aqaba MA, et al. Profile of sight-threatening infectious keratitis: a prospective study [published online ahead of print August 3, 2012]. *Acta Ophthalmol*. doi:10.1111/j.1755-3768.2012.02489.x.
30. Thomas PA, Geraldine P. Infectious keratitis. *Curr Opin Infect Dis*. 2007;20:129–141.
31. Arciola CR, Montanaro L, Caramazza R, Sassoli V, Cavedagna D. Inhibition of bacterial adherence to a high-water-content polymer by a water-soluble, nonsteroidal, anti-inflammatory drug. *J Biomed Mater Res*. 1998;42:1–5.
32. Bandara BMK, Sankaridurg PR, Willcox MDP. Non-steroidal anti inflammatory agents decrease bacterial colonisation of contact lenses and prevent adhesion to human corneal epithelial cells. *Curr Eye Res*. 2004;29:245–251.
33. Selan L, Palma S, Scoarughi G, et al. Phosphorylcholine impairs susceptibility to biofilm formation of hydrogel contact lenses. *Am J Ophthalmol*. 2009;147:134–139.
34. Zhu H, Kumar A, Ozkan J, et al. Fimbrolide-coated antimicrobial lenses: their in vitro and in vivo effects. *Optom Vis Sci*. 2008;85:292–300.
35. Willcox MD, Hume EB, Vijay AK, Petcavich R. Ability of silver-impregnated contact lenses to control microbial growth colonisation. *J Optom*. 2010;3:143–148.
36. Mathews SM, Spallholz JE, Grimson MJ, Dubielzig RR, Gray T, Reid TW. Prevention of bacterial colonization of contact lenses with covalently attached selenium and effects on the rabbit cornea. *Cornea*. 2006;25:806–814.
37. Cole N, Hume EB, Vijay AK, Sankaridurg P, Kumar N, Willcox MD. In vivo performance of melimine as an antimicrobial coating for contact lenses in models of CLARE and CLPU. *Invest Ophthalmol Vis Sci*. 2010;51:390–395.
38. Willcox MD, Hume EB, Aliwarga Y, Kumar N, Cole N. A novel cationic-peptide coating for the prevention of microbial colonization on contact lenses. *J Appl Microbiol*. 2008;105: 1817–1825.
39. Thissen H, Gengenbach T, du Toit R, et al. Clinical observations of biofouling on PEO coated silicone hydrogel contact lenses. *Biomaterials*. 2010;31:5510–5519.
40. Kolar SS, McDermott AM. Role of host-defence peptides in eye diseases. *Cell Mol Life Sci*. 2011;68:2201–2213.
41. McDermott AM. The role of antimicrobial peptides at the ocular surface. *Ophthalmic Res*. 2009;41:60–75.
42. Rasul R, Cole N, Balasubramanian D, Chen R, Kumar N, Willcox MD. Interaction of the antimicrobial peptide melimine

with bacterial membranes. *Int J Antimicrob Agents*. 2010;35: 566–572.

43. Efron N, Morgan PB, Woods CA. Survey of contact lens prescribing to infants, children, and teenagers. *Optom Vis Sci*. 2011;88:461–468.
44. Kaspar H, Dettmer K, Gronwald W, Oefner PJ. Advances in amino acid analysis. *Anal Bioanal Chem*. 2009;393:445–452.
45. Conibear T. *A Study of Virulance Factors Associated with Pseudomonas Aeruginosa Clinical Isolates*. Sydney, Australia: University of New South Wales; 2006.
46. Schubert TL. *Increasing the Ability of Antibiotics to Control S. aureus keratitis*. Sydney, Australia: University of New South Wales; 2008. Thesis.
47. International Organization for Standardization. ISO14729. *Ophthalmic Optics—Contact Lens Care Products—Microbiological Requirements and Test Methods for Products and Regimens for Hygienic Management of Contact Lenses*. Geneva: ISO; 2001.
48. Buck SL, Rosenthal RA. A quantitative method to evaluate neutralizer toxicity against *Acanthamoeba castellanii*. *Appl Environ Microbiol*. 1996;62:3521–3526.
49. Chen R, Cole N, Willcox MD, et al. Synthesis, characterization and in vitro activity of a surface-attached antimicrobial cationic peptide. *Biofouling*. 2009;25:517–524.
50. International Organization for Standardization. ISO 18369-3. *Ophthalmic Optics—Contact Lenses—Part 3: Measurement Methods*. Geneva: ISO; 2006.
51. International Organization for Standardization. ISO 9339-2. *Optics and Optical Instruments—Contact Lenses—Determination of thickness—Part 2: Hydrogel Contact Lenses*. Geneva: ISO; 1998.
52. American National Standards Institute. ANSI Z80.20. *Ophthalmics: Contact Lenses—Standard Terminology, Tolerances, Measurements and Physicochemical Properties*. Merrifield: 1998..
53. International Organization for Standardization. ISO 9338. *Optics and Optical Instruments-Contact Lenses-Determination of the Diameters*. Geneva: ISO; 1996.
54. Read ML, Morgan PB, Kelly JM, Maldonado-Codina C. Dynamic Contact Angle Analysis of Silicone Hydrogel Contact Lenses. *J Biomater Appl*. 2011;26:85–99.
55. International Organization for Standardisation. ISO 10993-5. *Biological Evaluation of Medical Devices-Part 5: Tests for In Vitro Cytotoxicity*. Geneva: ISO; 2009.
56. Israelachvili JN. *Intermolecular and Surface Forces with Applications to Colloidal and Biological Systems. 2 ed*. City: Academic Press; 1992.
57. Garrett Q, Garrett RW, Milthorpe BK. Lysozyme sorption in hydrogel contact lenses. *Invest Ophthalmol Vis Sci*. 1999;40: 897–903.
58. Jones L, Senchyna M, Glasier MA, et al. Lysozyme and lipid deposition on silicone hydrogel contact lens materials. *Eye Contact Lens*. 2003;29:S75–79; discussion (suppl 83)-74:S192–194.
59. Molloy MP, Bolis S, Herbert BR, et al. Establishment of the human reflex tear two-dimensional polyacrylamide gel electrophoresis reference map: new proteins of potential diagnostic value. *Electrophoresis*. 1997;18:2811–2815.
60. Willcox M, Hume E, Cole N, Aliwarga Y, Zanini D, inventors; Johnson and Johnson Vision Care (US), assignee. Biomedical devices with antimicrobial coatings. US patent US2004126409/A2. October 12, 2005.
61. Tuli SS, Iyer SA, Driebe WT, Jr. Fungal keratitis and contact lenses: an old enemy unrecognized or a new nemesis on the block? *Eye Contact Lens*. 2007;33:415–417.
62. Gorscak JJ, Ayres BD, Bhagat N, et al. An outbreak of *Fusarium* keratitis associated with contact lens use in the northeastern United States. *Cornea*. 2007;26:1187–1194.
63. Retuerto MA, Szczotka-Flynn L, Ho D, Mukherjee P, Ghannoum MA. Efficacy of care solutions against contact lens-associated *Fusarium* biofilms. *Optom Vis Sci*. 2012;89:382–391.
64. Dantam J, Zhu H, Stapleton F. Biocidal efficacy of silver-impregnated contact lens storage cases in vitro. *Invest Ophthalmol Vis Sci*. 2011;52:51–57.
65. Por YM, Mehta JS, Chua JL, et al. *Acanthamoeba* keratitis associated with contact lens wear in Singapore. *Am J Ophthalmol*. 2009;148:7–12.
66. Boost MV, Shi GS, Lai S, Cho P. Amoebicidal effects of contact lens disinfecting solutions. *Optom Vis Sci*. 2012;89:44–51.
67. Mayo MS, Cook WL, Schlitzer RL, Ward MA, Wilson LA, Ahearn DG. Antibiograms, serotypes, and plasmid profiles of *Pseudomonas aeruginosa* associated with corneal ulcers and contact lens wear. *J Clin Microbiol*. 1986;24:372–376.
68. McDermott AM. Defensins and other antimicrobial peptides at the ocular surface. *Ocul Surf*. 2004;2:229–247.
69. Costa F, Carvalho IF, Montelaro RC, Gomes P, Martins MC. Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces. *Acta Biomater*. 2011;7:1431–1440.
70. Gabriel M, Nazmi K, Veerman EC. Nieuw Amerongen AV, Zentner A. Preparation of LL-37-grafted titanium surfaces with bactericidal activity. *Bioconjug Chem*. 2006;17:548–550.
71. International Organization for Standardization. ISO 9394. *Ophthalmic Optics—Contact Lenses and Contact Lens Care Products—Determination of Biocompatibility by Ocular Study Using Rabbit Eyes*. Geneva: ISO; 1998.
72. Schubert TL, Hume EB, Willcox MD. *Staphylococcus aureus* ocular isolates from symptomatic adverse events: antibiotic resistance and similarity of bacteria causing adverse events. *Clin Exp Optom*. 2008;91:148–155.