

# Melimine-Coated Antimicrobial Contact Lenses Reduce Microbial Keratitis in an Animal Model

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**PURPOSE.** To determine the ability of antimicrobial peptide melimine-coated contact lenses to reduce the incidence of microbial keratitis (MK) in a rabbit model of contact lens wear.

**METHODS.** In vitro antimicrobial activity of melimine-coated contact lenses was determined against *Pseudomonas aeruginosa* by viable count and a radiolabeled assay. The amount of lipopolysaccharide (LPS) associated with bacteria bound to melimine-coated and control lenses was determined. Ocular swabs from rabbit eyes were collected for assessment of ocular microflora. A rabbit model for MK was developed that used overnight wear of contact lenses colonized by *P. aeruginosa* in the absence of a corneal scratch. During lens wear, detailed ocular examinations were performed, and the incidence of MK was investigated. Bacteria associated with worn lenses and infected corneas were determined by viable plate count.

**RESULTS.** Inhibition in viable and total *P. aeruginosa* adhesion by melimine-coated contact lenses was 3.1 log<sub>10</sub> and 0.4 log<sub>10</sub>, respectively. After colonization, the amount of LPS on lenses was approximately the same with or without melimine. Gram-positive bacteria were found in all the ocular swabs followed by fungus (42%). Melimine-coated lens wear was protective and significantly (odds ratio 10.12;  $P = 0.012$ ) reduced the incidence of *P. aeruginosa*-driven MK in the rabbit model. The antimicrobial lenses were associated with significantly ( $P < 0.001$ ) lower ocular scores, indicating improved ocular signs compared with controls.

**CONCLUSIONS.** This study showed that contaminated contact lenses can produce MK without corneal epithelial defect in an animal model. Melimine-coated contact lenses reduced the incidence of MK associated with *P. aeruginosa* in vivo. Development of MK requires viable bacteria adherent to contact lenses, and bacterial debris adherent at the lens surface did not cause keratitis.

Keywords: antimicrobial, contact lens, melimine, microbial keratitis, rabbit, *Pseudomonas*

Microbial keratitis (MK) is a serious ocular condition that has the potential to cause blindness.<sup>1</sup> Risk factors for MK include ocular surface trauma, ocular surface disease, contact lens wear, and hypoxia.<sup>2-5</sup> Trauma and ocular surface diseases remain the major risk factors for MK in developing countries,<sup>6</sup> whereas contact lens wear is the predominant risk factor for MK in developed countries.<sup>2,7</sup> Depending on the study design and location, contact lens wear now accounts for approximately 12% to 66% of all MK events.<sup>8-15</sup> The annualized incidence of MK ranges between 9.3 and 20.9 during overnight wear of lenses and 2.2 and 3.5 per 10,000 wearers during daily wear of lenses.<sup>16-18</sup> With the introduction of high oxygen-permeable silicone hydrogel contact lenses in 1998, it was anticipated that these materials might be able to reduce the incidence rate of MK. However, subsequent studies confirmed that these materials had little effect on absolute and relative risk of MK.<sup>19</sup> Although various microorganisms can cause MK, *Pseudomonas aeruginosa* has been repeatedly reported to be the predominant causative organism.<sup>20,21</sup>

Sankaridurg et al.<sup>1</sup> detailed that the possible sequence of events leading to contact lens-related MK include microbial adhesion to contact lens followed by adhesion to damaged corneal epithelium, then replication and release of toxins and

polymorphonuclear leukocyte (PMN) infiltration. The PMNs and bacteria release enzymes and toxins that lead to collagenolysis of the stroma and suppuration. Because bacterial adhesion to contact lenses is an essential step in the development of MK, it is believed that if adhesion to contact lenses was controlled, the incidence of MK may be reduced.<sup>22,23</sup> There have been various attempts to create antimicrobial contact lenses that can reduce the adhesion of pathogenic microorganisms. Antimicrobials, such as silver,<sup>24</sup> nonsteroidal anti-inflammatory drugs,<sup>25-27</sup> selenium,<sup>28</sup> quorum-sensing inhibitors,<sup>29</sup> poly(ethylene glycol),<sup>30,31</sup> phosphorylcholine,<sup>32</sup> and antimicrobial peptides, have been examined in vitro.<sup>33</sup>

Antimicrobial peptides (AMPs) are a class of antimicrobial agents that are active against a wide spectrum of microorganisms, such as bacteria, viruses, fungi, protozoa, and parasites, including antibiotic-resistant bacteria.<sup>34,35</sup> Melimine is a synthetic peptide consisting of 29 amino acid residues derived from melittin and protamine.<sup>36</sup> Melimine has high activity, which exceeds that of a mixture of the two parent peptides.<sup>37</sup> Earlier studies have shown that melimine-coated contact lenses can reduce *P. aeruginosa* adhesion to contact lenses<sup>33,35</sup> and can reduce the incidence and severity of noninfectious keratitis

in a Guinea pig model.<sup>38</sup> Melimine is not cytotoxic to mammalian cells *in vitro*, or animals' eyes, and can be worn safely by humans.<sup>35,39</sup>

This study aimed (1) to determine whether there was a significant amount of dead bacteria on melimine-coated contact lenses and whether this could lead to the development of keratitis, and (2) to develop a rabbit model for MK and determine whether melimine-coated contact lenses could reduce the incidence of MK induced by *P. aeruginosa*.

## METHODS

Melimine (T-L-I-S-W-I-K-N-K-R-K-Q-R-P-R-V-S-R-R-R-R-R-G-G-R-R-R) was synthesized by conventional solid-phase peptide synthesis protocols and was obtained from American Peptide Company (Sunnyvale, CA, USA; >95% purity). Melimine was covalently attached to etafilcon A (Johnson & Johnson Vision Care, Inc., Jacksonville, FL, USA; base curve: 8.7 mm, diameter: 14.0 mm, power: -3.00 Diopter) lenses as described earlier.<sup>35</sup> Briefly, melimine was diluted in sterile PBS (NaCl 8 g L<sup>-1</sup>, KCl 0.2 g L<sup>-1</sup>, Na<sub>2</sub>HPO<sub>4</sub> 1.15 g L<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 0.2 g L<sup>-1</sup>, pH 7.4). Contact lenses were washed in PBS, then placed in 0.1 mol L<sup>-1</sup> sodium acetate buffer, pH 5.0, containing 2 mg mL<sup>-1</sup> 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for 15 minutes at 25°C. Lenses were washed three times in PBS and then resuspended in 3 mg mL<sup>-1</sup> 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 10% wt/vol NaCl overnight followed by soaking in PBS for 2 hours and autoclaving (121°C, 15 minutes). Etafilcon A lenses were used because they are commercially available and contain carboxylic acid groups (provided by the incorporation of methacrylic acid during manufacture) that facilitates covalent binding of amine groups on peptides.

## In Vitro Bacterial Adhesion

*Pseudomonas aeruginosa* strain 6294 (isolated from a case of MK) was grown overnight in tryptone soya broth (TSB; Oxoid, Basingstoke, UK). *Pseudomonas aeruginosa* 6294 is an invasive strain and the minimum inhibitory concentration for melimine is 250 µL mL<sup>-1</sup>.<sup>40-43</sup> The adhesion conditions of *P. aeruginosa* 6294 with contact lenses have reported previously.<sup>35,44</sup> Briefly, 1 mL *P. aeruginosa* (1.0 × 10<sup>6</sup> CFU mL<sup>-1</sup>) was added to uncoated control or melimine-coated lenses pre-washed in PBS, and incubated for 18 hours with shaking (120 rpm). Then, contact lenses were washed three times with PBS to remove nonadherent bacterial cells and stirred rapidly in 2 mL fresh PBS containing a small magnetic stirring bar. This PBS was serially diluted (1/10) in Dey Engley neutralizing broth (Becton, Dickinson and Company, Sparks, MD, USA), and a 3 × 50-µL aliquot of each dilution was transferred to tryptone soya agar (Oxoid, Basingstoke, UK) containing Tween 80 and lecithin for recovery of bacterial cells. After 24-hour incubation at 37°C, viable microorganisms were enumerated as CFU per contact lens. Measurements were performed in triplicate on a minimum of three separate occasions.

To determine the effect of melimine-coated lenses on the total number (viable + nonviable cells) of bacterial adherent cells, *P. aeruginosa* 6294 was grown overnight with 20 µL <sup>3</sup>H-uridine (0.02 mCi; PerkinElmer, Glen Waverley, Australia) in TSB, then centrifuged, washed, resuspended in PBS<sup>44</sup> and incubated with lenses as described for viable cell adhesion. Subsequently, 0.5 mL of the bottle contents was added with 4.5 mL Opti-Fluor scintillation cocktail (Packard Instrument Co., Downers Grove, IL, USA) to 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 minute from each lens were converted to number of bacterial cells mm<sup>-1</sup> of lens surface area based on a standard calibration curve of known numbers of radiolabeled bacteria. Triplicates of each lens type (with and without) were included in each adhesion experiment and the experiment was repeated a minimum of three times.

## Detection of Endotoxin

Melimine-coated and uncoated contact lenses were incubated overnight with *P. aeruginosa* 6294 following the methods described for viable cell adhesion. 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). This solution was diluted 100 times in a pyrogen-free container (Kinesis Australia, Redland Bay, Australia) and endotoxin associated with both the melimine-coated and uncoated contact lenses was determined by Limulus amoebocyte lysate (LAL) assay (Lonza, Walkersville, MD, USA). In brief, 50 µL LAL reagent was added to 50 µL × 100 diluted samples in a 96-well endotoxin-free plate (CELLSTAR, Greiner bio-one, Frickenhausen, Germany); 100 µL prewarmed (37 ± 1°C) chromogenic substrate was added after 10 minutes and 100 µL stop reagent (acetic acid, 25% vol/vol glacial acetic acid; Ajax Finechem Pty Ltd., Taren Point, NSW, Australia) added after 16 minutes. During this time, the side of a 96-well plate was gently tapped to facilitate mixing. Endotoxin units (EU) per lens were calculated after measuring the absorbance on a SpectrafluorPlus microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 405 to 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 (Lonza) of known potency and following manufacturer's guidelines. Endotoxin units per lens were calculated. Measurements were performed in triplicate.

## Animal Model for Assessing the Incidence of MK With Melimine-Coated Contact Lenses

This was a prospective, controlled study conducted to determine whether melimine-coated contact lenses could reduce the incidence of MK caused by adherent *P. aeruginosa* in a rabbit model compared with uncoated control lenses. All animals were treated strictly in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and before study commencement, approval from the University of New South Wales Animal Care and Ethics Committee was obtained. A total of 27 conventionally reared New Zealand white rabbits (Nanowie Animal Facility, Bellbrae, VIC, Australia) were allocated for this study. They were acclimatized for at least 1 week before the study start. Only rabbits in good general health, and having eyes free of clinically significant ocular irritation or staining were used in the study. The nictitating membrane was not removed from the rabbits' eyes.

Aseptic swabs from the right eye upper bulbar conjunctiva and lower lid margins of seven rabbits were taken using sterile calcium alginate swabs (Puritan, Guilford, ME, USA) to determine the rabbit normal ocular microbiota. The swabs were then placed in a vial containing 2 mL sterile PBS and vortexed for 30 seconds. Aliquots of the saline (400 µL) were inoculated onto three chocolate agar plates and one Sabouraud's dextrose agar plate. One of the three chocolate agar

plates was incubated at 35°C in 5% CO<sub>2</sub> (2 days), one in aerobic conditions (2 days), and one under anaerobic conditions (4 days). The Sabouraud's dextrose agar plate was incubated at 25°C for 7 days to culture for yeasts and fungi. Microbial growth on the various media was enumerated and the number of CFUs of each distinct colony type per swab was calculated.<sup>45</sup> Representative bacterial colonies from each plate were Gram stained and identified using Analytical Profile Index (Biomérieux, Grenoble, France) commercially available kits.

*Pseudomonas aeruginosa* strain 6294 was grown overnight in TSB at 37°C and then washed three times in PBS after centrifugation at 1157g at 18°C for 10 minutes. *Pseudomonas aeruginosa* was resuspended in 10 times diluted TSB in PBS to an OD<sub>660nm</sub> of 0.1 (1.0 × 10<sup>8</sup> CFU mL<sup>-1</sup>). Noncoated control and melimine-coated lenses were washed in PBS and transferred to 1 mL bacterial suspension in wells of 24-well tissue culture plates, and incubated for 18 hours with shaking (120 rpm). Incubated contact lenses were washed once with PBS to remove nonadherent bacterial cells before inserting into the rabbit eyes.

Rabbits wore either melimine-coated or control contact lenses with or without adherent *P. aeruginosa* in their left eyes for 24 hours, while the right eye served as a nonlens control. Before lens insertion, lenses were examined for particulate matter, physical damage, and inversion. Rabbits were reexamined after 6 hours of lens insertion to monitor retention of lens and in case of any lens loss, the respective type of lens with or without adherent bacteria was reinserted. Rabbits were administered subcutaneous 0.01 mg kg<sup>-1</sup> buprenorphine. Contact lens fit was assessed using slit-lamp biomicroscopy, including lens centration, corneal coverage, and tightness. All rabbits had a detailed ocular examination and photographs taken by a single trained optometrist. Slit-lamp biomicroscopy was performed using a Nikon photographic slit lamp (Nikon FS-3V; Tokyo, Japan), which provided up to ×32 magnification. Detailed anterior segment examinations were carried out, including sodium fluorescein (Chauvin Pharmaceuticals Ltd., Surry, UK) staining. Wratten #12 filter (Bausch & Lomb, Rochester, NY, USA) was used in conjunction with cobalt blue filter to excite fluorescence. Photographs were taken and then graded, using the CCLRU grading scale,<sup>46</sup> by a single trained masked optometrist who recorded details of corneal and conjunctival parameters (Table 1). Ocular examinations were performed at baseline and immediately after lens removal at 24 hours and at 48 hours (after 24 hours of no lens wear). The ocular variables assessed and scores given during each examination are listed in Table 1. All rabbits were closely monitored during lens wear for any indication of stress by examining their movements, alertness, gait, behavior, vocalizations, and respiration. After 24 hours, lenses from rabbits' left eyes were aseptically collected in 2 mL sterile PBS and processed to determine viable bacterial count. If MK developed, the animal was immediately euthanized and the left cornea was collected to determine viable bacterial count. All rabbits wore contact lenses for up to three occasions if MK did not develop. If MK developed, the rabbits were euthanized. A minimum of 2 weeks of wash out was provided between each lens wear for rabbits to recover from any effects of lens wear.

After removal from eyes, worn and unworn melimine-coated or control contact lenses were stirred rapidly in 2 mL PBS containing a small magnetic stirring bar to remove bacterial cells. Following log serial dilutions in PBS, 3 × 50 μL of each dilution was plated on nutrient agar (NA; Oxoid, Basingstoke, UK). After 24 hours of incubation at 37°C, viable bacteria were enumerated as CFU lens<sup>-1</sup>. Corneal buttons collected from infected corneas were homogenized (IKA tissue homogenizer; Wilmington, NC, USA) in 4 mL PBS, serially diluted in PBS, and 3 × 50 μL of each dilution was plated on

TABLE 1. Scoring of the Rabbit Ocular Response During Contact Lens Wear

Variables	Score
Conjunctival redness	0-4 (0.1) (0 = none, 4 = severe)
Conjunctival chemosis	0-4 (0.1) (0 = none, 4 = severe)
Discharge	0-4 (0.1) (0 = none, 4 = severe)
Aqueous flare	0-4 (0.1) (0 = none, 4 = severe)
Iris involvement	0-4 (0.1) (0 = none, 4 = severe)
Corneal infiltrates	0-4 (0.1) (0 = none, 4 = severe)
Fluorescein staining: type	0.None 1.Micropunctate 2.Macropunctate 3.Coalescent macropunctate 4.Patch
Fluorescein staining: extent	0.None 1.1%-15% 2.16%-30% 3.31%-45% 4.>45%

NA. Following 24 hours of incubation at 37°C, viable bacteria were enumerated as CFU cornea<sup>-1</sup>.

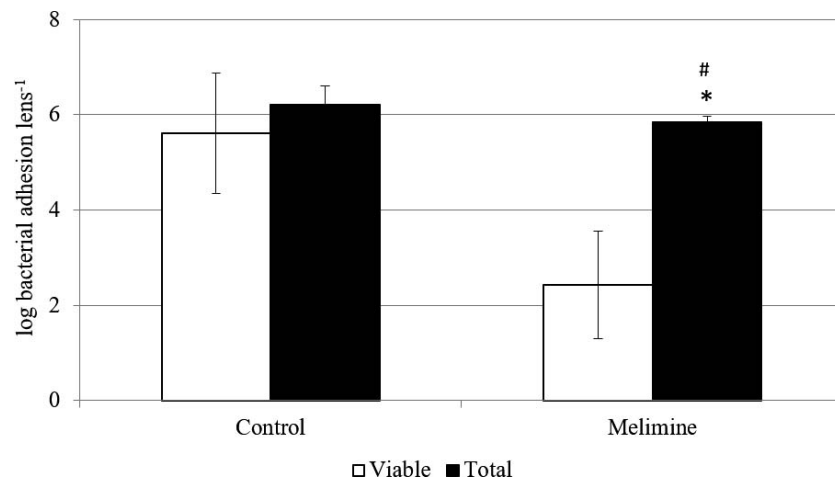
## Data Analysis

Data were analyzed using Excel (Office; Microsoft, Redmond, WA, USA) and Statistical Package for the Social Sciences software (SPSS) for Windows, version 21.0 (SPSS, Inc., Chicago, IL, USA). The in vitro bacterial adhesion data were log<sub>10</sub> (x + 1) transformed before data analysis, where x is the adherent bacteria in CFU lens<sup>-1</sup>. Differences in microbial adhesion and lens-associated endotoxin were analyzed using independent 2-sample t-test. During the animal study, percent lens retention was calculated as ([actual wear time for duration of study]/[total possible wear time for duration of study]) × 100. Data obtained from corneal evaluations in the MK model study were examined for differences using a Wilcoxon sign ranked test for corneal scores and a χ<sup>2</sup> test for corneal infiltrates with MK. All statistical tests were two sided; odds ratio and relative risk for development of MK were determined with test lenses compared with control. Analytical manipulation of the data, such as the sum or frequency of scores, was calculated where appropriate.

## RESULTS

Figure 1 shows total and viable adhesion of *P. aeruginosa* (at the initial inoculum of 1 × 10<sup>6</sup> CFU mL<sup>-1</sup>) to melimine-coated and control lenses. Melimine-coated lenses produced 3.1 ± 0.6 log<sub>10</sub> inhibition in viable adhesion 18 hours after incubation with bacteria. Compared with viable adhesion, there was substantially higher total adhesion by *P. aeruginosa* on melimine-coated lenses (P < 0.05) in the same time frame. Melimine-coated lenses demonstrated a small but statistically significant 0.4 log<sub>10</sub> inhibition in total adhesion for *P. aeruginosa* (P < 0.05) 18 hours after incubation with bacteria. Melimine-coated contact lenses adsorbed 110 ± 11 EU lens<sup>-1</sup> of endotoxin (Fig. 2) over an 18-hour incubation period. Control lenses after adhesion of *P. aeruginosa* had a slightly but not statistically (P = 0.14) higher endotoxin level of 143 ± 32 EU lens<sup>-1</sup> over the same time frame.

Table 2 details the percentage of rabbit eyes that were colonized with microorganisms and the type of microorganisms. Twenty-eight percent of lower lid and 57% of upper bulbar conjunctiva were sterile. Gram-positive bacteria were



**FIGURE 1.** Number of total and viable *P. aeruginosa* 6294 adherent to melimine-coated and control contact lenses following 18 hours of incubation. \*Significantly higher total bacterial adhesion than viable bacterial adhesion to melimine-coated lenses. #Significantly lower total bacterial adhesion to melimine-coated lenses than uncoated control lenses.

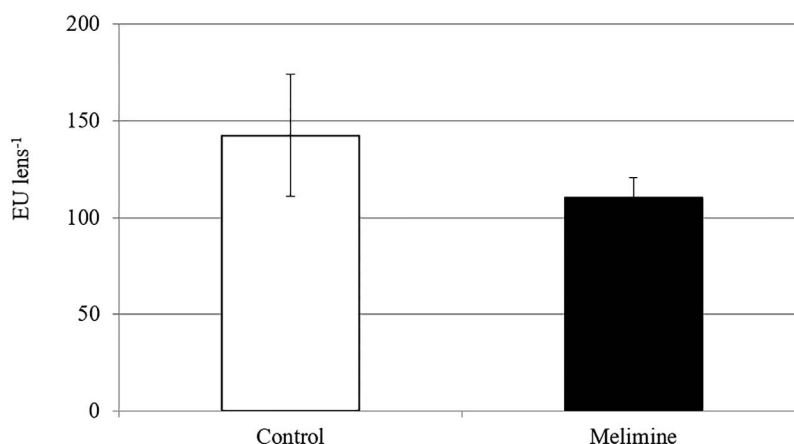
found in all the other ocular swabs. Fungus was found in 42% of both lower lid and upper bulbar conjunctiva. *Moraxella urethralis* was the only gram-negative bacteria that was isolated, from 14% of rabbit lower lid and upper bulbar conjunctiva.

Table 3 demonstrates the demographics of contact lens wear for rabbits, including number of rabbits that retained contact lenses during this study. A total of 22 and 19 eyes were exposed to control and melimine-coated *P. aeruginosa* preincubated contact lenses, respectively, and 59% and 58% of eyes retained lenses after 24 hours of wear, respectively (Table 3). Twenty-two and 13 eyes were exposed to sterile control and melimine-coated lenses, and lens retention was 95% and 92%, respectively. All the animals included in the study maintained good health and no abnormal behavior was observed during the study. All the lenses had acceptable contact lens fit indicated by good centration, 360° corneal coverage, and optimal lens tightness. Sixty-nine percent of rabbit eyes that wore *P. aeruginosa*-colonized control lenses for 24 hours developed MK compared with 18% for melimine-coated lenses ( $P = 0.012$ ). A score for the response of the rabbit eyes was generated from measurement of parameters, including conjunctival redness, conjunctival edema, discharge, and focal and diffuse infiltrates (summarized as “ocular score”). Table 4 summarizes these findings from the eyes that retained

lenses for 24 hours, indicating that the ocular scores associated with *P. aeruginosa*-colonized melimine-coated contact lenses were significantly ( $P < 0.05$ ) lower than *P. aeruginosa*-colonized control lenses. The relative risk of developing MK during melimine-coated lens wear was 0.26 (95% confidence interval [CI] 0.71-0.96;  $P = 0.044$ ) and the odds ratio was 10.12 (95% CI 1.46-69.93;  $P = 0.018$ ) when compared with control lens wear, indicating the melimine-coated lens wear was protective against developing MK.

Figure 3 details the ocular response scores for rabbit eyes wearing lenses with and without adherent *P. aeruginosa* for both control and melimine-coated lenses. When lenses without adherent *P. aeruginosa* were worn, there were no significant differences between melimine-coated and control lenses ( $P > 0.05$ ) for any ocular variables, including total ocular score. *Pseudomonas aeruginosa*-colonized melimine-coated lenses showed similar scores compared with counterparts without adherent bacteria ( $P > 0.5$ ) except for the total scores (Fig. 3;  $P = 0.03$ ). For *P. aeruginosa*-colonized control lenses, the mean total score was  $14.3 \pm 8.4$ , whereas *P. aeruginosa*-colonized melimine-coated lenses had mean total scores of  $4.2 \pm 2.1$  ( $P = 0.001$ ).

Figure 4 is a bubble chart that details the total ocular scores of rabbit eyes following wear of *P. aeruginosa*-colonized control and melimine-coated lenses after 24 hours and 48



**FIGURE 2.** Amount of endotoxin associated with control and melimine-coated lenses after incubation with *P. aeruginosa* for 18 hours.

**TABLE 2.** Types of Microorganisms Isolated From Rabbit Eyes Before Contact Lens Wear

Microorganisms	Percentage of Eyes	
	Lower Lid	Upper Bulbar Conjunctiva
Gram positive		
<i>Staphylococcus epidermidis</i>	14	14
<i>Staphylococcus schleiferi</i>	14	0
<i>Staphylococcus saprophyticus</i>	14	14
<i>Streptococcus viridans</i>	14	0
<i>Protonibacterium</i> sp.	28	14
Gram negative		
<i>Moraxella urethralis</i>	14	14
Fungus	42	42
Sterile	28	57

hours. The size of the bubbles indicates the number of rabbit eyes with that score. For instance, after 48 hours of wear of *P. aeruginosa*-colonized melimine-coated lenses, nine rabbits showed total ocular scores of 1.0 as represented by a large bubble, whereas only two rabbits showed an ocular score of 1.0 with *P. aeruginosa*-colonized control lenses after 48 hours, and that is represented by a small bubble. The black bubbles indicate eyes that developed MK during lens wear. After 24 hours, three rabbits that wore *P. aeruginosa*-colonized control lenses had total ocular scores of more than 5, whereas only one rabbit wearing *P. aeruginosa*-colonized melimine-coated lenses had a similar score; other rabbits had lower scores in *P. aeruginosa*-colonized melimine-coated lenses. After 48 hours, nine rabbit eyes wearing *P. aeruginosa*-colonized control lenses showed ocular scores of 10 or more, indicating a severe adverse event, compared with only one rabbit eye wearing *P. aeruginosa*-colonized melimine-coated lenses.

Figure 5 illustrates representative appearances of rabbit eyes seen with slit-lamp biomicroscopy. Figure 5A shows a healthy eye with normal redness and other ocular parameters following wear of sterile control lenses, whereas Figure 5B shows fluorescein staining with full-thickness loss of epithelium (black arrow) observed following 24 hours of wear of a *P. aeruginosa*-colonized control lens. Figure 5C shows conjunctival redness (arrow) observed following 24 hours of wear of *P. aeruginosa*-colonized control contact lens, and Figure 5D shows a case of MK observed during wear of a *P. aeruginosa*-colonized control contact lens. The MK was characterized by a large, dense, irregular infiltrate (white arrow) with diffuse conjunctival swelling and hyperemia (white arrow).

Contact lenses analyzed before insertion into rabbit eyes showed that after incubation with  $1 \times 10^8$  CFU mL<sup>-1</sup> the number of bacterial cells on control lenses was  $7.3 \pm 0.5$  log<sub>10</sub> CFU lens<sup>-1</sup>, whereas on the melimine-coated lenses, there was

**TABLE 4.** Ocular Responses Following Wear of Contact Lenses Preincubated With *P. aeruginosa* in Rabbits

Ocular Characteristics	Control Lens	Melimine-Coated Lens	P Value
Conjunctival redness	3.0 ± 1.4	1.2 ± 0.6	<0.001
Conjunctival chemosis	2.5 ± 1.6	0.1 ± 0.3	<0.001
Discharge	2.2 ± 1.3	0.2 ± 0.6	0.000
Corneal infiltrate	2.7 ± 1.9	0.4 ± 1.2	0.012
Corneal staining-type	3.2 ± 1.0	1.8 ± 1.5	0.000
Total ocular score	14.3 ± 11.3	4.2 ± 3.4	<0.001
Incidence of MK	69.2%	18.2%	0.012

$6.3 \pm 0.4$  log<sub>10</sub> CFU lens<sup>-1</sup> and this difference was statistically significant ( $P < 0.001$ ). After lens wear, the number of *P. aeruginosa* cultured from control lenses was  $5.4 \pm 0.4$  log<sub>10</sub> CFU lens<sup>-1</sup> and from melimine-coated lenses was  $4.8 \pm 0.2$  CFU log<sub>10</sub> lens<sup>-1</sup> ( $P < 0.001$ ). The corneas of eyes that developed MK were processed to determine the number of *P. aeruginosa* associated with each eye. Corneas that had been exposed to control or melimine-coated lens wear were associated with  $6.8 \pm 0.4$  log<sub>10</sub> or  $6.8 \pm 0.8$  log<sub>10</sub> cultivable *P. aeruginosa*, respectively ( $P > 0.05$ ).

## DISCUSSION

This study for the first time has demonstrated that melimine-coated contact lenses can reduce the incidence of MK associated with *P. aeruginosa* infection in an animal model. The animal model developed was an acute MK model in rabbit eyes wearing bacteria-colonized contact lenses without any epithelial scratch or defect. Bacterial debris was associated with melimine-coated contact lenses, but this debris did not result in MK and caused only a low grade of ocular response during the 24 hours of lens wear.

Frequency of MK in eyes that wore lenses for 24 hours with control lens wear was 69%, whereas when wearing melimine-coated contact lenses for 24 hours, the frequency of MK was only 18%. Furthermore, the ocular response to melimine-coated lens wear in the presence of adherent *P. aeruginosa* was also significantly reduced. These results are similar to the results obtained in a Guinea pig model in which melimine-coated lenses were able to reduce the incidence and severity of noninfectious keratitis.<sup>38</sup> Previous models of MK with *P. aeruginosa* have either used injection of viable bacteria into the cornea of rabbits,<sup>47</sup> or used rats that had worn lenses for a continuous period of 72 hours before wearing contact lenses soaked in  $1 \times 10^{11}$  *Pseudomonas* mL<sup>-1</sup> followed by topical application of  $1 \times 10^{10}$  *P. aeruginosa* 19660 bacterial cells, and an additional topical application of the same concentration for up to 3 days for MK to be obtained.<sup>48</sup> Another model used rats wearing contact lenses with  $10^6$  or  $10^5$  *P. aeruginosa* PAO1 (green fluorescent protein-producing mutant) adherent to the lenses and lens wear for up to 10 days before MK developed.<sup>49</sup>

**TABLE 3.** Number of Rabbits Wearing Contact Lenses and That Developed MK

Demographics of Rabbits	Lenses Preincubated With <i>P. aeruginosa</i>		Lenses Without Adherent Bacteria	
	Control	Melimine-Coated	Control	Melimine-Coated
Total number of rabbits fit with lenses	22	19	22	13
Number of rabbits that had contact lenses on eye for 24 h (%)	13 (59)	11 (58)	21 (95)	12 (92)
Number of rabbits that developed MK (% of rabbits wearing lenses for 24 h)	9 (69)	2 (18)	0 (0)	0 (0)

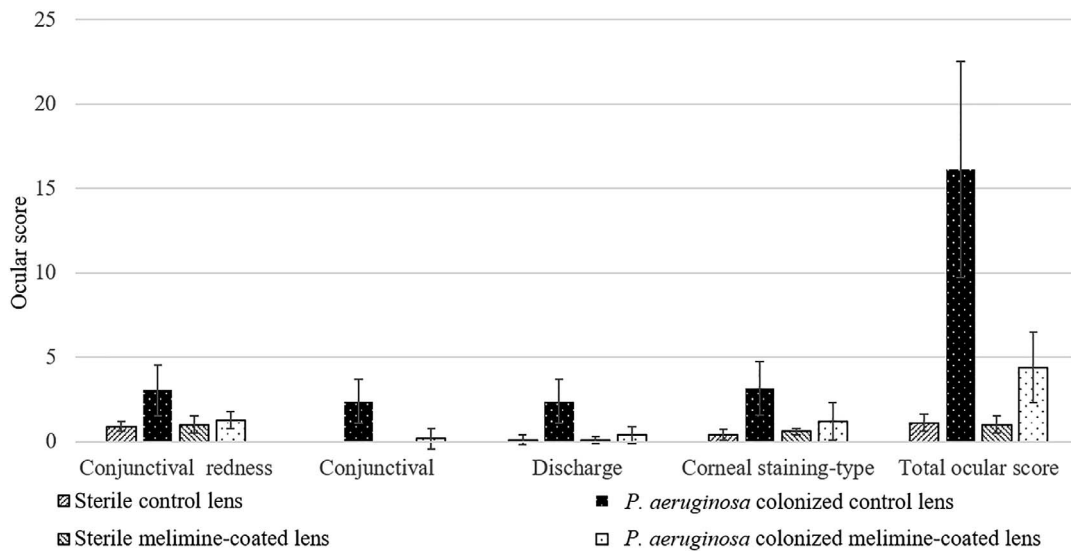


FIGURE 3. Ocular responses of rabbit eyes that retained contact lenses for 24 hours.

Our rabbit model is a more acute model of contact lens-induced MK. We previously reported that contact lens-induced MK in an acute Guinea pig model was dependent on prior scratching of the cornea,<sup>50</sup> much like is the case in a nonlens-wearing mouse model.<sup>51</sup> The reason why rabbits can produce an acute MK in the absence of corneal damage during

lens wear, and why not all rabbits are affected, requires further investigation, but there was no evidence of differences in corneal and conjunctival staining or conjunctival redness for those animals that developed MK before lens wear.

Melimine-coated contact lenses significantly reduced adhesion of viable bacteria in agreement with our earlier results.<sup>33,35</sup> However, when the number of total bacterial cells adherent to lenses was assessed using uptake and incorporation of radiolabeled uridine, there was no apparent reduction in total bacterial adhesion. Bacterial growth and uptake of <sup>3</sup>H-uridine during bacterial growth and incorporation into bacterial RNA is an established method of tracking adhesion.<sup>52</sup> An earlier report using confocal microscopy and LIVE/DEAD staining<sup>35</sup> showed significantly reduced numbers of dead bacteria on melimine-coated lenses. Interaction of melimine causes rapid disruption of bacterial cytoplasmic membranes resulting in cell death.<sup>53</sup> The lack of visible intact dead bacterial cells<sup>35</sup> (or at least to levels comparable to the amount of radioactive RNA present on lenses in the current study) might indicate that the bacteria are totally destroyed on adhesion to bound-melimine, such that no cell form can be seen with microscopy but cellular debris (i.e., RNA) is associated with the lens surface. RNA is a negatively charged molecule (polyanion)<sup>54</sup> that may bind to the positively charged melimine surface. The presence of endotoxin on the surfaces of lenses is further evidence of bacterial debris on the lens surface. Endotoxins are released when gram-negative bacteria are killed.<sup>55</sup>

Given the presence of bacterial debris (endotoxin and RNA, perhaps other components) on the melimine-coated lens surfaces, it is intriguing that the melimine-coated lenses after interaction with *P. aeruginosa* did not result in the production of a large ocular inflammatory response. Lipopolysaccharide or bacterial RNA and DNA on melimine-coated contact lenses could be recognized by Toll-like receptor (TLR)4 or TLR9, which are expressed by polarized corneal epithelial cells, bulbar conjunctiva, and stromal fibroblasts.<sup>56</sup> 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 nonpathogenic microbiota.<sup>57</sup> Ocular surface epithelial cells are regularly exposed to these bacteria and thus may have a low propensity for TLR-mediated inflammation.<sup>57</sup> Also, the polarity of ocular surface cells,<sup>56</sup> and consequent sequestering of TLRs away

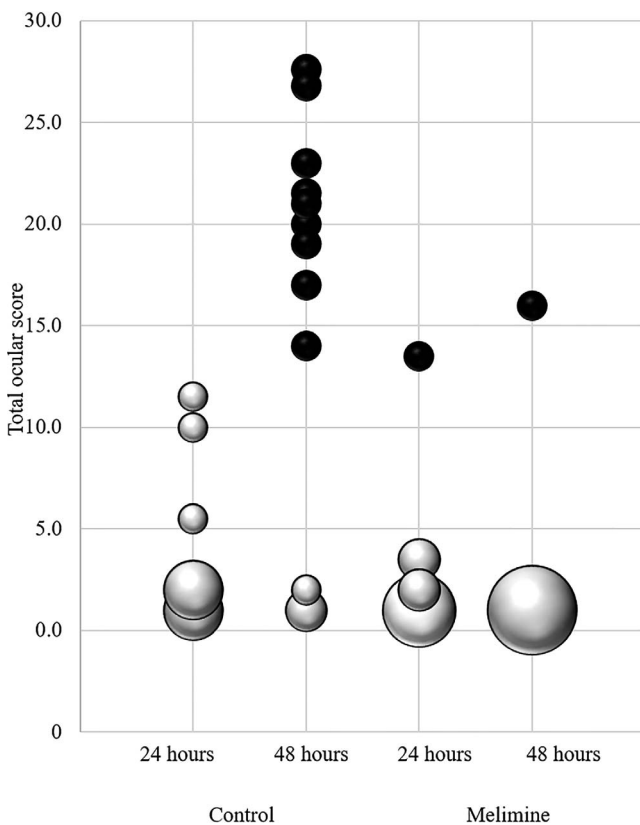


FIGURE 4. Total ocular scores following wear of *P. aeruginosa* adherent to contact lenses after 24 and 48 hours. Total ocular scores of 10 or more were regarded as a significant adverse event. Bubble size indicates the number of animals with same ocular score, and the smallest bubbles seen represent data from one rabbit. Black bubbles indicate incidence of microbial keratitis.

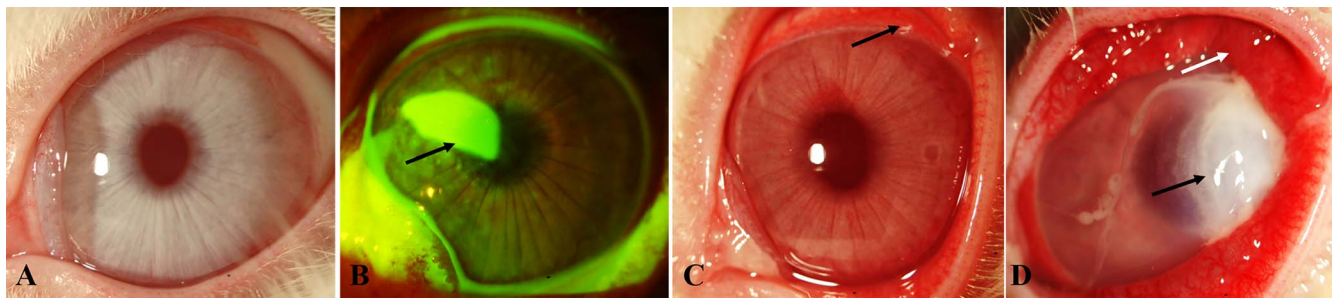


FIGURE 5. Representative images of slit-lamp findings of rabbit eyes captured at  $\times 7.5$  magnification. (A) Healthy rabbit eye; (B) fluorescein staining of full-thickness loss of epithelium; (C) conjunctival hyperemia with mild discharge; (D) microbial keratitis.

from the surface of the eye, also may prevent a healthy nonwounded ocular surface from interacting with the cellular debris. We have previously shown in a rabbit model of noninfectious keratitis for the human condition known as contact lens-induced peripheral ulcer, that dead staphylococci are not able to provoke an ocular surface inflammatory response during lens wear for 24 hours.<sup>58</sup> It may be that a time longer than 24 hours of exposure to melimine-coated lenses with associated bacterial debris is required for an ocular surface inflammatory response to occur, and we are currently investigating this possibility.

As the aim of developing melimine-coated contact lenses is to use them to reduce contact lens-associated keratitis in humans; it is worthwhile considering the possible effect of the bacterial debris on the human ocular surface. During the study, we found that commensal ocular microbiota of the rabbits was similar to earlier reports<sup>59</sup> and healthy human ocular microbiota,<sup>60</sup> which is predominantly composed of gram-positive bacteria, especially *Staphylococcus* sp. There was no evidence of cultivable *P. aeruginosa* as part of the normal ocular microbiota of rabbit eyes, and so we assume that all these bacteria cultured after contact lens wear were from the colonized contact lenses. Although the number of pathogenic bacteria that initially colonize a contact lens before the onset of MK is not known, it is unlikely that it is at the level used in the current study ( $1.0 \times 10^8$  CFU mL<sup>-1</sup>). It is perhaps more likely that much lower numbers of bacteria initially colonize a contact lens, and then grow to levels that initiate the infection. Over the course of 13 nights of wear of etafilcon A contact lenses, the numbers of bacteria that could be cultured from lenses usually ranged between 10 and 30 CFU lens<sup>-1</sup>.<sup>61</sup> During daily wear of contact lenses, lenses are stored in contact lens cases, but reports on the number of viable bacteria in a lens case rarely exceeds  $1 \times 10^5$  CFUs per lens case well.<sup>62</sup> Therefore, if lower numbers of bacteria adhere initially, melimine coatings would likely greatly reduce the likelihood of them growing to large numbers on the lens surface. This would mean that it might take several days for debris to accumulate on lenses. Reusable contact lenses are commonly disposed of on a monthly or 2-weekly basis.<sup>63</sup> We are currently running a clinical trial of melimine-coated contact lenses worn on a 2-weekly disposable extended wear schedule to examine the production of noninfectious keratitis compared with noncoated lenses, and this will definitively examine whether melimine coatings are associated with reductions, no change, or even increases in adverse events.

The finding that melimine coatings can reduce the adhesion of *P. aeruginosa* to contact lenses is confirmation of earlier studies that showed that melimine coatings can reduce microbial adhesion to a variety of biomaterials, including contact lenses in vitro.<sup>35,64,65</sup> The reduction of the viable numbers of *P. aeruginosa* after wear is similar to previous findings with a Guinea pig model of noninfectious keratitis.<sup>38</sup> This reduction in

viable numbers of adherent bacteria is likely the reason for the reduced incidence of MK and severity of ocular responses in the absence of MK. When MK did occur in the presence of melimine-coated lenses, the numbers of bacteria that were cultured from the cornea were the same as the numbers in non-melimine-coated eyes, indicating that if viable bacteria are released from melimine-coated lenses they still have the capacity to cause disease. Thus, it is possible, given the data described herein and elsewhere,<sup>38</sup> that melimine-coated lenses will reduce the incidence of both MK and noninfectious keratitis, but will not completely eliminate the disease.

In this study, some rabbits lost lenses during the 24 hours of wear of lenses with adherent *P. aeruginosa*, and this rate of lens loss was higher than when rabbits wore lenses in the absence of adherent *P. aeruginosa* (Table 3).<sup>39</sup> This may indicate that the rabbits experienced increased discomfort in the presence of the adherent bacteria and so actively removed the lenses from their eyes. Melimine-coated lenses also have high activity against bacteria other than *P. aeruginosa* that are implicated in MK.<sup>35</sup> Although it is likely, further work is needed to confirm its capacity in reducing such MK in vivo. This study was a proof-of-principal test of the effectiveness of antimicrobial contact lenses in a model of MK; as such, we did not consider the feasibility to manufacture melimine-coated lenses as a commercial product or its potential cost for large-scale production. It may be that melimine or other antimicrobial coatings are eventually brought to market, and the likelihood of this happening will depend on their antimicrobial efficacy as well as ease and cost of manufacture.

In summary, this study for the first time has shown that melimine-coated antimicrobial contact lenses have the capacity to significantly reduce the incidence of MK associated with *P. aeruginosa* contamination. Development of MK requires viable bacteria present at the ocular surface, and bacterial debris adherent to the lens, such as endotoxin, does not cause MK and results in only minor ocular changes.

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The corresponding author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and there is no discrepancy from the study as planned.

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