In Vivo Performance of Melimine as an Antimicrobial Coating for Contact Lenses in Models of CLARE and CLPU

Nerida Cole,1,2,3 Emma B. H. Hume,1,3,4 Ajay K. Vijay,1,2 Padmaja Sankaridurg,1,2,5 Naresh Kumar,5 and Mark D. P. Willcox1,2,3

PURPOSE. One strategy to minimize bacteria-associated adverse responses such as microbial keratitis, contact lens-induced acute red eye (CLARE), and contact lens-induced peripheral ulcers (CLPUs) that occur with contact lens wear is the development of an antimicrobial or antiadhesive contact lens. Cationic peptides represent a novel approach for the development of antimicrobial lenses.

METHODS. A novel cationic peptide, melimine, was covalently incorporated into silicone hydrogel lenses. Confirmation tests to determine the presence of peptide and anti-microbial activity were performed. Cationic lenses were then tested for their ability to prevent CLPU in the Staphylococcus aureus rabbit model and CLARE in the Pseudomonas aeruginosa guinea pig model.

RESULTS. In the rabbit model of CLPU, melimine-coated lenses resulted in significant reductions in ocular symptom scores and in the extent of corneal infiltration (P < 0.05). Evaluation of the performance of melimine lenses in the CLARE model showed significant improvement in all ocular response parameters measured, including the percentage of eyes with corneal infiltrates, compared with those observed in the eyes fitted with the control lens (P ≤ 0.05).

CONCLUSIONS. Cationic coating of contact lenses with the peptide melimine may represent a novel method of prevention of bacterial growth on contact lenses and consequently result in reduction of the incidence and severity of adverse responses due to Gram-positive and -negative bacteria during lens wear.

From the 1Institute for Eye Research, the 2School of Optometry and Vision Science, and the 3School of Chemistry, The University of New South Wales, Sydney, NSW, Australia; the 4Vision Cooperative Research Centre, Sydney, NSW, Australia; and the 5School of Optometry and Institute of Health and Biomedical Innovation, Queensland University of Technology, QLD, Australia.

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Corresponding author: Mark D. P. Willcox, Institute for Eye Research, Level 4, North Wing, Rupert Myers Building, Gate 14, Barker Street, The University of New South Wales, Sydney, NSW 2052, Australia; m.willcox@ier.org.au.

Adhesion and colonization of bacteria on contact lenses has been shown to play a role in several adverse responses, such as contact lens acute red eye (CLARE) and contact lens-induced peripheral ulcer (CLPU). CLARE and CLPU are relatively common adverse responses to contact lens wear, with CLARE developing in as many as 34% of patients in a study of continuously worn hydrogel lenses, whereas the incidence of CLPU has been reported to be as high as 13% per year in some studies. CLARE occurs only during extended wear and is associated with Gram-negative bacterial contamination of the lenses. Organisms implicated in CLARE events include Pseudomonas aeruginosa and Haemophilus influenzae. Conversely, CLPU is associated with both daily wear and extended wear of contact lenses and is an acute inflammatory response characterized by a small circular full-thickness epithelial erosion for which an association with microbial contamination of contact lenses particularly by Staphylococcus aureus has been established. Development of contact lens surfaces that are antimicrobial or antiadhesive is a strategy that is likely to be effective in reduction of contact lens-associated adverse responses.

There have been several antimicrobial surfaces described in the literature, including antibiotic coatings such as cefazolin, minocycline-rifampin, teicoplanin, and vancomycin which have been tested on biomaterials, particularly catheters. Alternative antimicrobial coatings such as salicylic acid, quaternary ammonium compounds, and chlorhexidine have also been trialed. However, in many cases development of microbial resistance makes these forms of therapy short-lived. Silver has also been used as an antimicrobial for biomaterials. However, in randomized controlled trials, silver coated material show no clinical advantage over uncoated material, and the coating can be cytotoxic. Other common problems with antimicrobial coatings are the loss of activity after covalent attachment to devices and the inability to sterilize the coatings once attached. There have been recent reports of biocompatibility testing of novel coatings for contact lenses, selenium and fibrinolides, which have been found to be antimicrobial in vitro. These coatings showed good compatibility with the cornea, but remain to be tested for retention of antimicrobial efficacy in vivo. In other studies the use of silver-impregnated lens case has been trialed, to reduce bacterial contamination of contact lenses in a clinical setting. The results have shown that fewer lens cases were contaminated compared with standard polypropylene alone only when used in conjunction with a multipurpose disinfection system.

We have recently reported a novel antimicrobial peptide, melimine, which incorporates active regions of protamine (from salmon sperm) and melittin (from bee venom), which is effective against both Gram-positive and -negative bacteria in solution, and retains its activity when covalently attached to contact lenses in vitro. Furthermore, this peptide is not cyto-
toxic at active concentrations, and bacteria do not appear to readily gain resistance. Melimine is also sterilizable and active in the presence of tears. Consequently, melimine as a surface coating for contact lenses represents a promising strategy for the reduction of adverse events resulting from contamination with Gram-negative or -positive bacteria. In this study, the performance of contact lenses with covalently linked melimine has been examined in vivo models of CLARE associated with *P. aeruginosa* and CLPU associated with *Staphylococcus aureus*.

**Materials and Methods**

**Animals**

Adult IMVS (Institute of Medical and Veterinary Science) male and female guinea pigs aged 10 to 14 weeks and weighing between 600 and 900 g were used for the CLARE model. New Zealand White rabbits (female) were used for the CLPU model. All procedures were approved by the institutional Animal Care and Ethics Committee and all procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Contact Lenses**

Silicone hydrogel lenses containing covalently incorporated melimine were produced as previously described. Antimicrobial lenses for guinea pigs and rabbits had a target of 100 μg of melimine per lens incorporated during the process. Melimine was omitted for control lenses. Final concentrations of melimine in the lenses were estimated by using a previously published method. In addition, the stability of melimine incorporation was tested by re-estimation of melimine levels after removal, cleaning, and sterilization of lenses on completion of the experiment. Briefly, the contact lenses were stained for 24 hours with Coomassie blue R250 (Sigma-Aldrich, Castle Hill, NSW, Australia) 0.025% wt/vol in 10% acetic acid and 10% iso-propanol (vol/vol) at 37°C and then destained in 10% acetic acid and 10% iso-propanol at 37°C. The lenses were then extracted in 25% (vol/vol) pyridine overnight. The extracted solutions were analyzed spectrophotometrically at 600 nm. Melimine quantities were determined by correlating the extracts to a standard curve.

**CLARE Model**

**Preparation of Bacteria and Contact Lenses.** *P. aeruginosa* (Paer 1), originally isolated from a CLARE event in humans, was grown overnight in tryptone soya broth (TSB; Oxoid, Basingstoke, UK), washed in phosphate-buffered saline (PBS; NaCl 8 g L⁻¹, KCl 0.2 g L⁻¹, Na₂HPO₄ 1.15 g L⁻¹, and KH₂PO₄ 0.2 g L⁻¹) and resuspended in PBS to an OD₆₆₀nm of 2.0. The suspension was examined by light microscopy to ensure clumping did not occur. Sterile guinea pig contact lenses prepared as described herein, with or without melimine, were removed from vials and washed three times in 1 mL PBS before use and then immersed in 1 mL of bacterial suspension for approximately 30 minutes before insertion.

The contact lenses were removed from the bacterial suspension with sterile forceps, washed once in 1 mL sterile PBS, and placed on the eyes. The lenses were evaluated for the number of both total and viable adherent bacteria before insertion and on removal at the completion of the experiment. The viable bacteria were quantitated by homogenizing the lenses (DIAX 500 homogenizer; Heidolph, Berladingen, Germany). A 100-μL aliquot of the resulting homogenate was serially diluted 1:10 in sterile PBS. Triplicate aliquots (20 μL) of each dilution, including the original homogenate, were plated onto nutrient agar (Oxoid). The plates were incubated for 24 hours at 37°C before colony-forming units (cfu) were enumerated and the units per lens calculated. The total number of bacteria on the lens surface (colony-forming units per square millimeter) was estimated by using light microscopy according to the method of Williams et al. Five high-power fields (×1000) were counted per lens sample by a masked observer.

**CLPU Model**

**Preparation of Bacteria and Contact Lenses.** *S. aureus* (Saur 31) a strain isolated from a case of CLPU was grown overnight in TSB and then washed three times in PBS. The bacteria were resuspended in PBS to an OD₆₆₀nm of 2.0. The suspension was examined by light microscopy to ensure the excessive clumping did not occur. Contact lenses were washed three times in 1 mL PBS before use and immersed in 1 mL of the bacterial suspension for approximately 30 minutes before insertion into the rabbit eyes.

**The Rabbit CLPU Model.** Twenty-two eyes of New Zealand White rabbits were trialed as has been published and received control and melimine lenses concomitantly. Briefly, an epithelial defect was introduced into the corneas of the rabbits by using a sterile 16-gauge needle after the topical application of 1% lignocaine. The defect was positioned 2 mm from the superior limbus. Evaluation of rabbit eyes was performed before and after the introduction of the defect at ×16 magnification under white light with a photo slit lamp biomicroscope (model FS3; Topcon Corp.). Lenses, prepared as described earlier, were inserted contralaterally. The guinea pigs were regularly monitored until lens removal 24 hours after insertion. At this time, the eyes of the guinea pigs were scored by a masked observer. Ocular responses such as bulbar, limbal, and conjunctival redness, chemosis and discharge were graded on a scale of 0 to 4, where 0 is no response and 4 is a severe response. Variables assessed and scores given for each are listed in Table 1. A CLARE-like response was considered to have developed when the eyes showed irritates and the sum of all scores was ≥10.

Viable bacteria adherent to the recovered lenses were enumerated as described elsewhere. Briefly, the lenses were washed once in sterile PBS (1 mL) and homogenized in 2 mL sterile PBS with a homogenizer (Heidolph 900; Drex, Schwabach, Germany). Viable total bacteria were estimated as described earlier. In addition, one lens from each group was retained for determination of the quantity of melimine present after wear. These lenses were washed three times in PBS, sonicated for 1 minute, and washed three times in PBS before being autoclaved. Melimine quantitation was performed as described herein.

**Conclusion**

In conclusion, melimine was incorporated into Hydrogel contact lenses as a surface coating for contact lenses represents a promising strategy for the reduction of adverse events resulting from contamination with Gram-negative or -positive bacteria. In this study, the performance of contact lenses with covalently linked melimine has been examined in vivo models of CLARE associated with *P. aeruginosa* and CLPU associated with *Staphylococcus aureus*.
Statistics

Student’s two-tailed unpaired t-test was used to determine significant differences between test (melimine coated lenses) and the appropriate control lenses in bacterial recovery experiments. Combined data from in vivo corneal evaluations in the CLARE model were examined for significance, with a paired t-test used for bulbar and limbal redness, chemosis, and discharge and the Fisher exact test or Mann-Whitney U analysis used as appropriate. For the CLPU studies, the Wilcoxon sign rank test was used for corneal score and the χ² test for epithelial defect.

RESULTS

Contact Lenses

The control uncoated guinea pig lens had a background staining equivalent to approximately 0.1 μg/lens of melime. Melimine lenses used to fit guinea pigs in the CLARE model were found to have a median quantity of melimine of 44 ± 14 μg/lens (~0.15 μg/mm²), and melimine distribution was found to be uniform in these lenses. Control uncoated contact lenses of the rabbit had background staining equivalent to approximately 1.5 μg/lens of melime, although the melamine-coated lenses, to fit the rabbit, had a concentration of approximately 94 μg/lens (~0.23 μg/mm²). Again, the topographic distribution of melimine in the lenses was uniform. The levels of melimine determined in lenses after removal, cleaning, and sterilization did not differ from those determined before use.

The CLARE Model

In Vitro Adhesion to Lenses. Control guinea pig lenses and melime guinea pig lenses immersed in P. aeruginosa strain Paer 1 were enumerated for the number of total and viable adherent bacteria before insertion into guinea pig eyes and after removal (Fig. 1A). Significant reductions in the counts of viable bacteria bound to the melimine lens compared with those in the control were found both before insertion into the guinea pig eyes and on removal at the end point of the experiment 24 hours later. Before insertion, control lenses had 4.6 ± 3.9 log cfu/mm² and melimine lenses had 3.2 ± 2.9 cfu/mm², a log reduction of approximately 1.4 (P = 0.05). On removal at the completion of the experiment, control lenses retained 4.7 ± 4.1 log cfu/mm² and melimine lenses retained 3.9 ± 3.4 cfu/mm², a log reduction of approximately 0.8 (P = 0.027). As opposed to the viable count, the total count of bacteria did not differ between lens type (control or melime coated) or between pre- and postwear determinations (Fig. 1A).

In Vivo Evaluation of Lens Performance in a CLARE Model. Four of the six animals fitted with sterile contralateral control and melimine lenses completed five days of lens wear. All eyes were within normal limits, with no signs of increased redness or corneal staining on completion of the study. Sixteen guinea pigs were fitted with contact lenses exposed to P. aeruginosa cultures. The combined data for the ocular responses from replicates are summarized in Table 1 and typical ocular responses are shown in Figure 2.

Eyes fitted with lens melimine lenses had significantly lower scores for the individual parameters of ocular inflammation measured (e.g., bulbar and limbal redness), chemosis and discharge fitted compared with the control lens, with significantly fewer animals attaining a total score of 10 or greater in the melimine lens group compared with the control lens group.

![Figure 1.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933452/ on 10/17/2017)

**Figure 1.** (A) P. aeruginosa adhesion to control and melime-coated guinea pig contact lenses before insertion and after removal at the experimental end point 24 hours after insertion. (B) S. aureus adhesion to control and melime-coated rabbit contact lenses before insertion and after removal at the experimental end point 24 hours after insertion. Data are the enumeration of total and viable bacteria expressed as the mean ± SE. *P < 0.05.

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<tr>
<th>Table 1. Ocular Response Scores at 24 Hours in Guinea Pigs in the CLARE Model</th>
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<td><strong>Characteristic</strong></td>
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<td>Bulbar redness (0–4)</td>
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<td>Limbal redness (0–4)</td>
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<td>Chemosis (0–4)</td>
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<td>Discharge (0–4)</td>
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<td>Infiltrates</td>
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<td>Score (≥10)</td>
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Data are the mean score ± SD. 95% confidence intervals are given in parentheses.

* Paired t-test for bulbar and limbal redness, chemosis and discharge and the Fisher exact test for infiltrates and score ≥ 10.
The CLPU Model

In Vitro Adhesion to Lenses. Control and melimine rabbit lenses immersed in Saur 31 before insertion into rabbit eyes, were enumerated both before insertion and after removal for the number of total and viable adherent bacteria. No significant differences were found in bacterial counts between the control and melimine lenses after removal at the end of the experiment. Before insertion, control lenses had viable counts of $3.5 \pm 3.0 \log \text{cfu/mm}^2$ and melimine lenses $2.6 \pm 2.6 \log \text{cfu/mm}^2$ ($P = 0.058$; Fig. 1B). Postwear viable and total counts were not different between control and melimine lenses (Fig. 1B); levels of viable bacterial contamination were less than $0.7 \log \text{cfu/mm}^2$ for both lens types.

In Vivo Evaluation of Lens Performance in a Rabbit CLPU Model. Rabbits were fitted with contact lenses exposed to Saur 31. A total of 22 rabbit eyes fulfilled the inclusion criteria for this study (i.e., the eye retained the contact lens for the duration of the experiment). A score for the response of the rabbit eyes was generated from the measurement of parameters including edema, flare, hypopyon, hyphema, and conjunctival hyperemia and chemosis (summarized as “corneal score”). Other parameters measured were corneal infiltration and the presence of an epithelial defect. Findings are summarized in Table 2.

Eyes of rabbits wearing the melimine lens showed significantly lower scores for ocular symptoms of inflammation (corneal score) than did eyes wearing the control lens. Eyes wearing the melimine lens showed a threefold reduction in the presence of an epithelial defect at 24 hours after insertion ($P = 0.033$). Photomicrographs of eyes typical of rabbits wearing lenses are shown in Figure 3.

DISCUSSION

This study, which to our knowledge is the first report of the antibacterial performance of an antimicrobial contact lens surface in vivo, indicates that contact lenses with covalently incorporated melimine are well tolerated by the eye and able to reduce the clinical manifestations of two adverse responses to contact lenses associated with bacterial contamination of contact lenses.

Guinea pigs have been reported to provide a useful model for human keratoconjunctivitis, and the CLARE model in guinea pigs has been previously shown to give ocular responses similar to those reported in humans. The ocular responses in the guinea pig to a challenge with contact lenses contaminated with \textit{P. aeruginosa} were characterized by moderate to severe redness and chemosis, with associated discharge and corneal infiltration. These responses were similar to CLARE/IK (infiltrative keratitis) responses previously reported in humans with the exception of conjunctival chemosis, which is not typically seen in humans with this condition. Evaluation of the performance of the melimine-coated lens in the CLARE model showed significant reductions in the number

| Table 2. Summary of the Significant Rabbit Ocular Responses to \textit{S. aureus} Challenge during Wear of Melimine and Control Contact Lenses |
|-----------------|-----------------|-----------------|---|
| Ocular Characteristics | Melimine Lens | Control Lens | $P$ |
| Corneal score | $4.5 \pm 1.7$ (3.8–5.1) | $7.0 \pm 4.5$ (5.3–8.9) | 0.05 |
| Presence of epithelial defect, % eyes | 9 | 27 | 0.033 |

Results are expressed as the average score or infiltrate depth ± SD. The presence of an epithelial defect is expressed as the percentage of eyes with a defect present 24 hours after lens insertion. The Wilcoxon sign rank test was used for corneal score and the $\chi^2$ test for epithelial defect. 95% confidence intervals are given in parentheses.
of eyes demonstrating a red eye response compared with eyes fitted with the control lens. Of importance, these findings correlate with the reduction of adhesion of viable bacteria to the melimine-coated lenses. Relatively high numbers of dead bacteria were observed only on the melimine lenses in the CLARE model. It has been demonstrated that there is a dose response for the number of viable bacteria adherent to a lens and the number of eyes showing CLARE in this model, indicating a requirement for metabolically active bacteria, a finding that is mirrored in the human condition. No differences were observed in the total number of adherent P. aeruginosa to either lens type at either time point, suggesting that with this method of incorporation of melimine into the lens matrix, the lens is not antiadhesive. The effectiveness of the melimine contact lens against CLARE reflects the requirement for viable bacteria to be associated with the contact lens after the adverse event. Indeed studies that recovered contact lenses from eyes experiencing CLARE show that the number of colony-forming units isolated from lenses at the time of the event are usually large and significantly higher in comparison to bacteria recovered during normal lens wear. It has also been demonstrated previously that a critical viable bacterial load is necessary, below which CLARE does not develop, and the use of melimine in contact lenses is able to sufficiently reduce the levels of viable bacteria, even when a large inoculum is used. It is possible that these lenses are more effective in ameliorating a CLARE event in a situation in which it seems likely that the bacteria associated with the lens would be smaller initially and would build up over time, a situation that is likely to be prevented by using melimine-coated lenses.

In the rabbit model of CLPU, using Saur 51 isolated from a human case of this adverse response, eyes wearing melimine-coated lenses showed significant reductions in the scores of ocular symptoms and in depth of corneal infiltration. Similar to observations with the Gram-negative P. aeruginosa, these data correlate with a reduction in the adhesion of viable bacteria to the lens surface before insertion. Differences were not observed in the number of viable bacteria at lens removal, as the ocular defenses are able to largely eliminate the Gram-positive staphylococcal load. This result is consistent with the very low number of bacteria that have previously been recovered from corneas of rabbits in a CLPU model. However, it has also been shown that in the CLPU model there is no ocular response to challenge with lenses exposed to 10^12 cells/mL of killed S. aureus. The numbers of bacteria recovered from the model reflects the findings in lenses of patients with this condition and is likely to result from tears containing a range of molecules with potent activity against Gram-positive bacteria such as S. aureus, including secretory phospholipase A2 which is present in rabbit tears. Whereas certain of the anti-Gram-negative bacteria substances in tears including defensins are not active, and Gram-negative bacteria, including P. aeruginosa, can grow in the presence of many potentially antibacterial substances in tears. Similar to P. aeruginosa, significant differences were not observed in the total number of adherent S. aureus on the lenses.

Extending our findings in vitro, in which we demonstrated that melimine is stable to heat sterilization, is efficacious against several Gram-negative and -positive organisms, and remains active in the presence of tear fluid, we demonstrated here that covalent incorporation of the cationic peptide melimine in contact lenses may represent a novel method of prevention of adhesion of viable bacterial to contact lenses and consequently may result in a reduction of the incidence and severity of corneal adverse responses due to Gram-positive and -negative microorganisms during lens wear.

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