Effectiveness of Antimicrobial Peptide Immobilization for Preventing Perioperative Cornea Implants Associated Bacteria Infection

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Abstract

Titanium (Ti) is a promising candidate biomaterial for an artificial corneal skirt. Antimicrobial peptide (AMP) immobilization could improve the bactericidal effect of the Ti substrate. In this study, we tested the bactericidal efficacy of a functionalized Ti surface in a rabbit keratitis model. A corneal stromal pocket was created by a femtosecond laser. The Ti films were then inserted into the pocket and *staphylococcus aureus* (*S. aureus*) or *pseudomonas aeruginosa* (*P. aeruginosa*) were inoculated into the pocket above the implant films. The corneas with Ti-AMP implants were compared with corneas implanted with unprotected Ti by slit lamp observation and anterior segment optical coherence tomography (AS-OCT). Inflammatory response was evaluated by bacteria counting, haematoxylin and eosin staining and immunostaining. There was a lower incidence and less extent of infection on rabbit corneas implanted with Ti-AMP than those corneas with unprotected Ti implants. The bactericidal effect of AMP against *S. aureus* was comparable to post-operative prophylactic antibiotic treatment hence SESB2V AMP bound to the Ti implant provided functional activity *in vivo* but its efficacy was superior against *S. aureus* compared to *P. aeruginosa*. This work suggests that SESB2V AMP can be successfully functionalized in a rabbit keratitis model to prevent perioperative corneal infection.
INTRODUCTION

Infection or biofilm formation on the surface of implants following implantation of surgical devices is a clinically devastating complication that accounts for about 60% of all hospital-associated infections (1). Devices that penetrate the skin or those that are within tissues e.g. the cornea, present the highest risk of infection (2,3). Endophthalmitis, a severe pan-ocular infection, remains one of the most devastating complications after implantation of any artificial corneal device e.g. intra-corneal stromal rings to correct refractive errors (4,5), Boston keratoprosthesis (6,7,8) or osteo-odonto keratoprosthesis (OOKP) (9,10) used to restore vision in patients with end-stage corneal blindness. Patients with endophthalmitis present with discharge, lid swelling, pain, reduced vision acuity, implant erosion or exposure (11). Infected patients normally receive systematic or local antibiotic/antifungal treatment once their infection has been diagnosed and sometimes implant removal is required. Implant related infections, are generally difficult to manage, result in an adverse effect on patient’s quality of life, require prolonged hospital admission and culminate in a higher final cost both to the patient and the healthcare provider (12,13). Currently, there are limited strategies in preventing infections associated with surgical implants. Hence, clinically applicable and effective strategies are urgently needed to address this issue.

Both systemic and locally applied antibiotic agents have been used to prevent infections associated with surgical implants. Local application of antimicrobial agents usually provide high drug levels on the surface of devices and the immediate vicinity compared to systemic administration (1). Various approaches of local prophylaxis have
been tried to prevent bacterial colonization, these include antimicrobial irrigation of the surgical field, such as sterilizing of the ocular surface with povidone-iodine before ophthalmic surgery (14), placement of antimicrobial carriers, e.g. intraoperative insertion of an antibiotic–treated prosthetic devices in orthopaedics (15), dipping of implants into antimicrobial solutions, e.g. immersion of heart valve prostheses into antibiotic solution before implantation (16), and antimicrobial coating of surgical implants (17). Among these methods, antimicrobial coating of implants possesses the unique advantage of potential delivering the antimicrobial drug in a controllable manner via conditioned biodegradation of chemical bonds that cross-link the implant surface with the antimicrobial drugs. Clinical trials with the use of Silzone-coated St. Jude Medical valves (18) or the use of silver coated pins in orthopedics have proved that antimicrobial coatings are an effective approach (19). In previous studies, we have shown that Titanium (Ti) is a prospective biomaterial for a synthetic Osteo-Odonto Keratoprosthesis (OOKP) skirt as it shows excellent biocompatibility (20) and Ti is stable in inhospitable environments often seen in ocular microbial infections (21,22). We (23) and others (24,25) have previously shown the effectiveness of functionalized Ti with immobilized antimicrobial coatings in vitro. Compared with other antimicrobial agents, such as antibiotics, antimicrobial peptides (AMPs) have a broad-spectrum of activity and possess a low propensity for developing pathogen resistance (26). Antimicrobial peptides can be effectively immobilized on Ti substrates by the use of a phosphate lipid spray or crosslinking agent e.g. sinalization or polyethylene glycol (PEG) (27,28). Despite several publications on the in vitro efficacy
of AMP’s (23,24,25), there are few studies demonstrating their efficacy in vivo (29). In the current study we examined the effectiveness of a functionalized implant in a rabbit keratitis model for its potential application as an artificial cornea skirt to prevent perioperative corneal infection. The AMP coating of a Ti synthetic OOKP could improve the biocompatibility and bactericidal effect of an artificial corneal device for this group of patients with end-stage corneal diseases.

MATERIALS AND METHODS

Minimum inhibitory concentration (MIC) of soluble SESB2V. MIC was performed in Muller-Hinton Broth (MHB) using broth macro-dilution method following CLSI guidelines (30). SESB2V peptide was dissolved in water to make 1000µg/mL stock solutions. Serial 2-fold dilutions of the peptide were prepared in cation-adjusted MHB in test tubes. The bacterial suspension was added in each tube to maintain a final concentration of approximately 5X10⁵ Colony Forming Units (CFU)/ml. The tubes were then incubated at 35 °C for 20-22 hours. The bacterial growth was checked in all the test tubes and tabulated for each concentration as “+” for growth and “-” for no growth. The MIC of the peptide was defined as the lowest concentration that prevented the visible growth of the bacteria.

Preparation of substrates and SESB2V peptide coating. The SESB2V and truncated peptide were purchased from EZbiolabs Inc., (Carmel, IN, USA). The Ti substrate and AMP coating were prepared as previously described (23). Briefly, Ti substrates were
cleaned ultrasonically for 10 minutes each in dichloromethane, acetone, water, and placed in HNO3 (40%) for 40 minutes for surface passivation. Acid-treated titanium substrates were washed with ultrapure water before further treatment. For surface polydopamine (PDOP) coating, the titanium substrates were immersed in dopamine hydrochloride solution (2 mg/mL) that was dissolved in tris buffer (10 mmol, pH 8.5) for 12 hours in darkness. The PDOP-coated substrates were then coated with AMP, SESB2V solution (500 μg/mL) on both surfaces. After being briefly washed with phosphate buffer saline (PBS), modified substrates were stored at 4°C prior to further analysis.

Thin (0.005 mm thickness) Ti films (Goodfellow, Cambridge, UK) were used for in vitro and in vivo studies. For in vitro peptide stability testing, FITC conjugated SESB2V peptide was immobilized onto the Ti surface. The functionalized substrate films were then soaked in phosphate buffer saline (PBS) and kept in dark. The fluorescence images were captured by a camera attached to microscope (Axioplan 4.4, Carl Zeiss, Gottingen, Germany).

**Bactericidal activities of SESB2V peptide.** Bactericidal function of SESB2V was analyzed with Live/dead baclight™ bacterial viability kit (Molecular probes, Carlsbad, CA). *Staphylococcus aureus* (*S. aureus*, ATCC 29213) and *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 9027) was used for the bactericidal assays. Yeast-dextrose broth containing peptone (10 g/l), beef extract (8 g/l), sodium chloride (5 g/l), glucose (5 g/l) and yeast extract (3 g/l) was used as the growth medium. The bacteria were incubated overnight at 37°C with agitation then washed and re-suspended in phosphate buffer saline (PBS) at a concentration of optical density (OD) 600=1.5. Bacterial suspension (500 μl)
was then added to each substrate in a 24-well plate and incubated for 30 minutes at 37°C. Substrates (n=3 for each) were then rinsed with PBS to remove non-adherent bacteria before analyzing with the protocol provided by the merchandiser. Live/dead bacteria images were taken with a fluorescent microscope (Carl Zeiss) and images of six areas were randomly captured. Numbers of live/dead bacteria in each image were counted from three repeats.

**Animal surgery.** The animal protocol of the study adhered to the Statement for Use of Animals in Ophthalmic Vision and Research by the Association for Research in Vision and Ophthalmology. The protocol was approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee at Singapore Eye Research Institute.

Thirty-six New Zealand white rabbits, aged 1 to 2 months and weighing 2 to 2.5 kg were used in the study. Animals were anaesthetised using an intramuscular injection of ketamine hydrochloride (35 mg/kg; Parnell Laboratories, Alexandria, Australia) and xylazine hydrochloride (5 mg/kg; Troy Laboratories, Smithfield, Australia). Right eye of each rabbit was chosen for surgery. The contralateral eye of the implanted rabbit served as the control. Following anaesthesia, a 7 mm diameter and 75% deep corneal stromal pocket was created using a Visumax™ femtosecond laser machine (Carl Zeiss Meditec, Jena, Germany). A 5 mm wide circumferential incision was made by a guarded diamond knife (Storz, Bausch and Lomb, USA). With forceps gently holding the anterior flap in place, a Siebel spatula was inserted to separate the cornea along the laser created pocket.
A 4 mm diameter implant film was then inserted into the pocket. Following insertion, the incision was then closed with interrupted 10/0 nylon sutures (Supple. Fig. 1A-C).

All rabbits received the following topical medications immediately after implantation: tobramycin 0.3% (Tobrex; Alcon Laboratory, Inc., Fort Worth, TX, USA) and prednisolone acetate 1% (Pred Forte, Allergan; Irvine, CA, USA). At 1 week post-operation, 50 µl bacterial solution (2X10^4 CFU/ml) was inoculated into the corneal pocket above the titanium discs with a syringe attached to a 29 G needle (Fig. 1D). For corneas with pristine Ti implants (n=21), one group of rabbits was sacrificed at Day 2 after inoculation of *S. aureus* (n=9) or *P. aeruginosa* (n=6). A second group of rabbits (n=6) received antibiotic eye drops immediately after *S. aureus* (n=3) or *P. aeruginosa* (n=3) inoculation. Antibiotics used were 0.3% gatifloxacin eye drops (Zymar®, one drop at every 2 hours) and subconjunctival injection of antibiotics solution once a day (cefazoline 330 mg/ml, 100 µl and gentamycin 40 mg/ml, 100 µl). The latter group of rabbits (n=6) were sacrificed after treatment with antibiotics continuously for two days. For corneas with Ti-AMP implants (n=15), rabbits were also divided into 2 groups. One group of rabbits were followed up till Day 2 after bacteria inoculation (n=6 for *S. aureus* and n=3 for *P. aeruginosa*) and another group of rabbits (n=3 for *S. aureus* and n=3 for *P. aeruginosa*) were followed up till Day 7 post-inoculation. The study design is pictured in Figure 1.

**Slit lamp imaging and anterior segment optical coherence tomograph (AS-OCT).** Slit lamp photographs and AS-OCT were taken before/after implantation, before bacterial...
inoculation, and at day 1/2/7 after bacterial injection. Slit lamp photographs were taken with a Zoom Slit Lamp NS-2D (Righton, Tokyo, Japan). Post-infection corneal slit lamp images were graded by a scoring method reported by Johnson et al (31). Briefly, the infection grade (0 to 4) is based on the evaluation of following infection symptoms: none to severe conjunctival infection; none to severe chemosis; none to severe iritis (cell & flare); none to severe fibrin formation; area of hypopyon formation; area of stromal infiltration (% of stroma with white cell infiltrate); area of stromal edema (% of stroma which is swollen). Corneal cross-sectional visualization was performed by the Visante AS-OCT (Carl Zeiss). Corneal thickness was measured by the software provided by the manufacturer.

Quantification of viable bacteria from infected rabbit cornea. At the determined time points, the infected eyes (both controls and treated) were enucleated from the rabbits and used for bacterial quantification. The corneas were dissected from rest of the eye ball and individually homogenized in sterile phosphate buffered saline (PBS) using plastic pestles followed by fine homogenization with bead beating using sterile glass beads (2 mm). The homogenate then underwent serial dilution plating using TSA plates (Beckman, USA). The plates were incubated at 35°C for 48 hours. The numbers of colonies were counted and results were expressed as log_{10} number of CFU/ cornea.

Tissue fixation and sectioning. After euthanization, the rabbit corneas were removed, post fixed in 4% paraformaldehyde followed by dehydration with a serial concentration of ethanol. After dehydration, tissue blocks were embedded into paraffin for Hematoxylin
& Eosin (H&E) staining. For immunostaining, following fixation, tissues blocks were embedded in optimum cutting temperature (OCT) cryo-compound (Leica Microsystems, Nussloch, Germany). Frozen tissue blocks were stored at −80°C until sectioning. Serial sagittal corneal 10 μm sections were cut using a cryostat (Microm HM550; Microm, Walldorf, Germany). Sections were placed on polylysine-coated glass slides and air dried for 15 minutes.

Hematoxylin & Eosin (H&E) and immunofluorescent staining. For H&E staining, tissue sections were immersed in hematoxylin (Sigma-Aldrich, Oakville, Canada) and eosin (Sigma) solutions for 10–20 s before cleaning with pure xylene. For immunostaining, tissue sections were post-fixed with 4% paraformaldehyde for 15 minutes, washed with 1X PBS, blocked with 10% normal goat serum in 1X PBS and 0.15% Triton X-100 for 1 hour. The sections were incubated with either rat monoclonal antibody against CD11b (Abcam, SanFrancisco CA) diluted 1:100, rat monoclonal antibody against F4/80 (Abcam) diluted 1:50, or mouse monoclonal antibody against MMP9 (Sigma) diluted 1:200 at 4°C overnight. After washing with 1X PBS, the sections were incubated with goat anti-mouse or goat anti-rat Alexa Fluor 488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) at room temperature for 1 hour. Slides were then mounted with UltraCruz Mounting Medium containing 4’, 6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA). For negative controls, non-immune serum was used in place of the specific primary antibody. Sections were observed and imaged with a fluorescence microscope (Carl Zeiss).
Statistical analysis. Data were represented as mean ± SE. The P value was determined using the one way ANOVA with the Microsoft Excel 2007 software (Microsoft, Redmond, WA). Student t test was used for bacteria live/dead ratio counting. Data was considered to be statistically significant when P value was less than 0.05.

RESULTS

Previous studies have indicated that incubation of the full length peptide with trypsin generated a number of proteolytically processed fragments and a partial loss of antimicrobial activities in the presence of tear fluid (32). Since the microenvironment in the stroma encompass a number of proteases, we sought to investigate the efficacy of full length SESB2V and its various sequentially truncated variants. First, we determined the MIC value of full length and truncated peptides against S. aureus and P. aeruginosa strains. As shown in Figure 2A, the MIC of truncated peptides to S. aureus, except the shortest peptide [(R)2K]2KK, varied between 3.125 µg/ml to 25 µg/ml. The MIC value of full length peptide [(RGRKVVRR)2K]2KK] was 6.125 µg/ml. For P. aeruginosa, except [(VvRR)2K]2KK and [(R)2K]2KK, the MIC values of truncated peptide varied between 6.25 µg/ml to 25 µg/ml. The MIC value of the full length peptide was 12.5 µg/ml. These results indicated that SESB2V peptide retained high bactericidal effect to both S. aureus and P. aeruginosa even after truncation.

We then studied the in vitro bactericidal effect of immobilized AMP with a live/dead bacteria imaging assay. Clusters of live bacteria (stained green) were seen on pristine Ti
substrates (Fig. 2B) while on SESB2V functionalized Ti substrates most of the bacteria were dead (stained red). Quantitative live/dead ratio of bacteria was calculated to compare the antimicrobial properties of the pristine Ti and Ti-AMP substrates (Fig. 2B). The mean live/dead ratio of *S. aureus* and *P. aeruginosa* on pristine Ti substrates (15.8:1, for *S. aureus* and 1.66:1 for *P. aeruginosa* respectively) were significantly higher than those on Ti-AMP substrates (2.5:1 for *S. aureus*, *p*=0.01 and 0.48:1 for *P. aeruginosa*, *p*=0.03, between Ti and Ti-AMP group).

We also studied the stability of AMP coating on Ti metal substrates *in vitro*. The fluorescence signal of FITC conjugated SESB2V peptide (SESB2V-FITC) lasted at least 60 days after covalent binding onto Ti substrates (Fig. 2C). Pristine Ti showed no fluorescence after laser excitation by the same microscope.

Bactericidal effects of AMP were tested in a rabbit keratitis model. No clinically signs of toxicity were observed in any rabbit eyes after Ti or Ti-AMP implantation. After the *S. aureus* bacterial injection, signs of inflammation and infection were visible clinically in the pristine Ti implanted rabbits by Day 1 post-inoculation (n=9/9) and there was worsening in seven of the nine eyes by Day 2. Clinical symptoms included conjunctival chemosis, discharge, hypopyon in anterior chamber, severe corneal edema, and iritis. Most corneas (n=5/9) melted due to severe edema, inflammatory cellular infiltration, and cell sloughing. However, for the corneas with Ti-AMP implants, most of the corneas (n=7/9) developed no obvious signs of inflammation and the corneas remained clear after bacteria inoculation. Three eyes were followed up to 7 days. Two of
the three eyes still had minimal signs of infection/inflammation up to Day 7 post-inoculation (Fig. 3A). For corneas with the *P. aeruginosa* inoculation, inflammatory and infection signs occurred in corneas with both pristine Ti (n=6/6) and AMP coated Ti (n=4/6) on Day 1 post-inoculation and worsened on Day 2 post-inoculation (n=6/6 for pristine Ti group and n=4/6 for Ti-AMP group). However, for corneas with Ti-AMP implants, the infection was localized to the central area of the cornea and not as extensive compared with the pristine Ti implants. On Day 7 post-inoculation, there were still signs of localized inflammation and infection in the Ti-AMP group (n=2/3, Supple. Fig. 2A). Due to the severe melting of cornea, rabbits with pristine Ti implantation group were sacrificed at day 2 post-inoculation hence there was no data collected at day 7 from this group.

Table 1 summarized the mean ocular infection score for each bacterial inoculum group by slit lamp examination. For corneas with pristine Ti implants, all eyes (n=9) developed signs of *S. aureus* bacterial infection within 48 hours post inoculation while for corneas with Ti-AMP implant, two out of nine eyes developed the signs of infection. The mean ocular inflammation score for Ti group was 2.78 ± 0.36 on Day 1 post-inoculation and 3.56 ± 0.18 on Day 2 post-inoculation, while the mean ocular inflammation score for Ti-AMP group was 0.22 ± 0.15 on Day 1 post-inoculation, 0.56 ± 0.38 on Day 2 post-inoculation (p=0.01 between Ti and Ti-AMP groups at both day 1 and day 2 time points) and 0.34 ± 0.31 on Day 7 post-inoculation. As for *P. aeruginosa* inoculation group, all six eyes with Ti implants developed severe signs of infection, and four of the six eyes with Ti-AMP implants developed mild to moderate signs of infection.
The mean ocular inflammation score for Ti group was 3.17 ± 0.33 on Day 1 post-inoculation and 3.67 ± 0.22 on Day 2 post-inoculation, while the mean ocular inflammation score for Ti-AMP group was 1 ± 0.39 on Day 1 post-inoculation, 1.67 ± 0.78 on Day 2 post-inoculation (p=0.01 between Ti and Ti-AMP groups at both day 1 and day 2 time points) and 1.33 ± 0.67 on Day 7 post-inoculation.

On AS-OCT examination, the Ti film appeared as a hyper-reflective line on the scanned image, confirming the position of the implant (Fig. 3B). Within 48 hours of inoculation with *S. aureus*, corneal thickness anterior to pristine Ti implant increased to 757 ± 76.7 µm while corneal thickness anterior to Ti-AMP implant increased to 408 ± 37 µm (p=0.02 between Ti and Ti-AMP group). At Day 7 post-inoculation, the corneal thickness was 360 ± 12.3 µm in the Ti-AMP group (Fig. 3C). The infection in the AMP coated Ti corneas was worse after *P. aeruginosa* inoculation than those after *S. aureus* inoculation. Within 48 hours of *P. aeruginosa* inoculation, corneal thickness anterior to pristine Ti implant increased to 1010.7 ± 225.4 µm while mean corneal thickness anterior to Ti-AMP implant increased to 460.3 ± 100.1 µm (p=0.01 between Ti and Ti-AMP group). At Day 7 post-inoculation, the corneal thickness of Ti-AMP group was 409.2 ± 31.4 µm (Supple. Fig. 2C).

We counted bacteria clones from the rabbit’s corneal tissue to analyze the bacterial load. By 48 hours post-inoculation, the number of *S. aureus* organisms increased from the initial inoculums of 1000 CFU per cornea to approximately 1.3×10^7 CFU per cornea in Ti group and increased to 6.6×10^3 CFU per cornea in Ti-AMP group (p=0.01 between Ti and Ti-AMP groups). At Day 7 post-inoculation, there was no bacterial growth in Ti-
AMP group in culture plate. Similarly, within 48 hours of bacteria inoculation, the number of *P. aeruginosa* organisms increased from the initial inoculum of 1000 CFU per cornea to approximately $1.9 \times 10^6$ CFU per cornea in Ti group and $9.4 \times 10^3$ CFU per cornea in Ti-AMP group ($p = 0.01$ between Ti and Ti-AMP group). At Day 7 post-inoculation, there was $1.74 \times 10^2$ CFU per cornea in Ti-AMP group (Fig. 4).

Tissue sections were analyzed to further evaluate the inflammatory response following bacterial inoculation. At 48 hours post-inoculation, H&E staining of *S. aureus* infected corneas with pristine Ti implant revealed loss of corneal integrity as a result of “melting” of the epithelial layer in the central cornea (Fig. 5B). Blood vessels grew into the cornea stroma (Fig. 5C) and inflammatory cells infiltrated into the central cornea surrounding the area of the implant pocket (Fig. 5C). However, *S. aureus* infected corneas with Ti-AMP implant revealed very mild cellular infiltration (Fig. 5E and F) and the keratocyte nuclei were aligned as in normal cornea (Fig. 5G).

In corneas with pristine Ti implant, inflammatory markers, CD11b, F4/80 and MMP9 were all highly expressed in the anterior cornea stroma area while there was reduced expression in the corneas of Ti-AMP implant group (Fig. 6). For *P. aeruginosa* infection group, similar different patterns of inflammatory cell infiltration were observed in corneas with pristine Ti or AMP coated Ti implants (Supple. Fig. 3 and Supple. Fig. 4).

We compared the bactericidal effect of immobilized AMP with post-infective antibiotics. At Day 1 of antibiotics treatment, all three rabbits still had severe clinical
signs of *S. aureus* infection with an average infection score of 3.67 ± 0.33. At Day 2 of treatment, the signs improved with an average score of 2.67 ± 0.33 (Fig. 7A). For corneas with antibiotics treatment, the number of *S. aureus* organisms increased from the initial inoculums of 1000 CFU to 8.3×10⁴ CFU per cornea (Fig. 7B) which was significantly higher (p=0.02) than Ti-AMP group at post inoculation day 2 (6.6×10³ CFU per cornea).

For corneas with *P. aeruginosa* inoculation and antibiotics treatment, the rabbit corneas developed mild clinical signs of keratitis with an averaged infection score of 1.67 ± 0.33 at Day 1 and there were no obvious clinical signs of corneal infection at Day 2 of treatment (Fig. 7C). No *P. aeruginosa* organisms were cultivated from the cornea tissue (Fig. 7D).

**DISCUSSION**

An artificial corneal device bridges the non-sterile ocular surface with the sterile anterior chamber. There is a high risk of rapid invasion of bacterial pathogens into the cornea stroma where the artificial cornea device is situated. Our objective in this study was to develop a local administrative antimicrobial approach to inhibit the bacterial colonization on an implant placed into a cornea. AMP (SESB2V) was immobilized onto the surface of Ti via a cross linker, polydopamine (33, 34). To mimic keratoprosthesis surgery, Ti films were then inserted into rabbit corneal stromal pockets. Ti implants whose surface was functionalized with AMP efficiently inhibited corneal bacterial infections with minimal adjacent tissue damage. Immobilized AMP, proved to be more efficient *in vivo* against *S. aureus* compared *P. aeruginosa*. We also demonstrated that Ti-AMP showed superior
bactericidal activity against *S. aureus* compared to the use of post inoculation topical antibiotics.

Current strategies to combat implant associated ocular infections include the continuous use of topical antibiotics (1). However, a serious concern regarding the continuous use of conventional antibiotics is the potential development of antibiotic–resistant pathogens such as the Methicillin-Resistant *Staphylococcus Aureus* (MRSA) (35-37) and fungi (7). Antibiotic-resistant infections can lead to devastating effects and uncontrolled bacterial infection. Therefore AMPs that have a wide-spectrum of bactericidal activity and are less likely to develop bacterial resistance (26, 37) would be an ideal substitute. There are few studies on the *in vivo* efficacy of covalently bound AMPs. Gao et al have demonstrated the efficacy of AMP coated Ti wire, used in orthopaedic surgery, in a dorsal skin pocket model of wound infection (29). Here we reported the successful surface functionalization of a metallic implant with immobilized AMP in an infectious keratitis model. Our model mimics precisely the clinical *in situ* placement of the synthetic artificial cornea devices as opposed to other reported studies (29). We have previously proved that SESB2V has a wide spectrum of bactericidal activity against both gram-positive and gram-negative bacteria (23). We have also determined the MIC of SESB2V against MRSA (DM21455) to be 6.25 µg/ml. In this study, we have shown a low MIC value (3.125 to 25 µg/ml) of soluble SESB2V and its truncated peptides against both Gram-positive *S. aureus* and Gram-negative *P. aeruginosa* bacterial strains. These results indicated that fragmented SESB2V peptide still maintained a bactericidal effect, which is important clinically following protease
digestion in vivo. These properties make SESB2V peptide a promising candidate for ocular therapeutic use. After immobilization onto Ti substrates, SESB2V peptide retained its bactericidal activities against both *S. aureus* and *P. aeruginosa* with a coating concentration of 500 µg/ml, this was about 80 times higher than the MIC values of soluble SESB2V. We used this concentration since the covalent binding of the AMP to the substrate surface is likely to alter the MIC value. Previous studies have shown higher MIC concentrations of immobilized AMPs compared to soluble peptides (39-41). The difference in dead-live ratio between *S. aureus* and *P. aeruginosa* on pristine Ti surface is possibly because *P. aeruginosa* contains much higher levels of dead/weakened bacteria. Another possibility is that the pristine Ti itself has different bactericidal effect to different bacteria strain. However, the results are still valid since we compared the live/dead ratio of bacteria before and after AMP immobilization.

Titanium films with immobilized SESB2V were placed into the rabbit cornea stroma and there were no clinical signs of cornea inflammation within one week after implantation compared to those with pristine Ti. AMPs have been shown to have immunomodulatory activities that include angiogenesis, modulation of cytokine activity and reduction of lipopolysaccharide (LPS)-mediated pro-inflammatory responses (42, 43). The cytotoxicity associated with AMPs is usually related to the high concentrations used to compensate for the relatively short half-life of AMP due to the rapid tissue protease digestion (44). Covalent immobilization of SESB2V could decrease the long-term cytotoxicity to corneal tissue since covalent binding would prolong the lifespan and function of the peptides and reduce the necessity of using high concentrations of AMP.
We observed that SESB2V was stable in a humidified environment for at least 2 months after immobilization onto Ti surface. Additionally, the bactericidal effect of functionalized Ti discs could be achieved even after 2 weeks of implantation (1 week after bacteria inoculation). The results indicated that AMP activity was still present even though these peptides may face the risk of degradation by tissue protease. The degraded product of SESB2V may still possess the bactericidal effect in vivo as evidenced by the low MIC value of the truncated SESB2V peptides. However, we did observe a difference of bactericidal efficacy of the AMP coated implants against both *S. aureus* and *P. aeruginosa* at Day 7 post-inoculation (2 weeks after implantation). This was possibly due to the biodegradation or dysfunction of SESB2V peptide in the corneal stroma. Our results are encouraging in the early post-operative period but bacteria endophthalmitis may occur months or years after device placement (4-6). Hence, future work will examine the role of AMP combined with a controlled drug delivery system, e.g. nanoparticles or biodegradable films.

The clinical score of corneal inflammation on slit lamp images and the quantitative analysis of corneal thickness on AS-OCT images, as well as the histological studies demonstrated that immobilized SESB2V significantly reduced the corneal inflammation and stromal swelling caused by either *S. aureus* or *P. aeruginosa* pathogens. Moreover, immobilized SESB2V was more effective in preventing a post-implantation infection caused by *S. aureus* compared to *P. aeruginosa*. After inoculation of *S. aureus* in the AMP coated implants, the clinical analysis and histological images showed less obvious signs of infection/inflammation compared to those inoculated with *P. aeruginosa*. This
was possibly because *P. aeruginosa* was less sensitive to the bactericidal activities of SESB2V peptide compared with the same inoculum of *S. aureus*. Additionally, we observed that the inoculation of *S. aureus* or *P. aeruginosa* caused infiltration of different types of inflammatory cells in the absence of AMP. For example, an inoculation of *S. aureus* caused more F4/80 positive cells infiltration near the implant site while an inoculation of *P. aeruginosa* caused more CD11b positive cell infiltration. Hence, we cannot exclude the possibility that the difference of bactericidal effect was due to the difference in the virulence of the pathogens, (46, 47). As the rabbits developed a more severe keratitis after *P. aeruginosa* compared to *S. aureus* even without the presence of AMP. However, the superior efficacy against *S. aureus* is clinically advantageous since the latter is the leading cause of endophthalmitis and bacterial keratitis post ocular implantation (7).

Following a clinical diagnosis of an ocular implant infection, the initial treatment will involve the use of intensive topical antibiotics. We hence compared the effect of intensive topical antibiotic therapy similar to a clinical situation with that of immobilized SESB2V. Compared with topical antibiotics, there was significantly less clinical inflammation score and fewer bacterial clones with the AMP coating following *S. aureus* inoculation. However, the topical antibiotics were superior to SESB2V peptide to treat infections caused by *P. aeruginosa*, as the inflammatory symptoms resolved and there was no growth of bacteria clone after treatment by the current choice of antibiotics (Fig. 7C and D). In reality clinically the topical therapy would be used adjunctive to the AMP coating,
hence localized control of the infection by the AMP coating, as indicated by our results, may prevent further spread of the infection into a fulminant endophthalmitis.

Infections associated with the implantation of biomaterials remain one of the major barriers to the use of medical devices in patients. We have successfully immobilized a novel AMP to deliver a bactericidal effect in vivo. Hence, our study demonstrated the efficacy of a local antimicrobial prophylactic measurement to prevent implant associated cornea infections in an infectious keratitis model.

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REFERENCES


**Figure legends**

Table 1. Scoring of corneal infection from slit lamp examinations.

Figure 1. A diagram to interpret the design of rabbit study.

Figure 2. *In vitro* bactericidal effect and stability of SESB2V AMP.

A: a table to show the MIC value of the full length and truncated SESB2V peptide.

B: representative images of live/dead bactericidal assay. Green: live bacteria, Red: dead bacteria. The ratio of live to dead bacteria number was counted and graphed. *p <0.05, n=6, student t test.

C: the fluorescence images of Ti surface after immobilization with SESB2V-FITC peptide in phosphate buffer saline.

Figure 3. *In vivo* bactericidal effect of SESB2V AMP against *S. aureus*.

A: slit lamp examination of the rabbit eyes before and after implantation and bacteria inoculation.

B: AS-OCT scanning of the rabbit eyes before and after implantation and bacteria inoculation.

C: measurement of corneal thickness. Only cornea thickness anterior to implant was measured at pre and post-inoculation D1 and D2. **p <0.05 between pristine Ti and Ti-AMP group at post-inoculation day 1. ***p <0.05 between pristine Ti and Ti-AMP group at post-inoculation day 2. one way ANOVA.**
Figure 4: Bacteria counting of *S. aureus* and *P. aeruginosa*. Results are presented as log10 CFU/cornea ±SEM, “*” and “**” *p*<0.05, *n*=3 in each group. One way ANOVA.

Figure 5: Hematoxylin & Eosin (H&E) pictures of rabbit cornea after inoculation with *S. aureus*.

A: normal rabbit cornea.

B-D: rabbit cornea with pristine Ti implantation and bacterial infection. (B) Corneal epithelium, (C) central cornea stroma, (D) corneal stroma surrounding the implant pocket.

E-G: rabbit cornea with Ti-AMP implantation and bacterial infection. (E) Corneal epithelium, (F) central cornea stroma, (G) corneal stroma surrounding the implant pocket.

Figure 6: Immunostaining pictures of rabbit cornea after infection with *S. aureus*. Green, inflammatory cell markers; Blue, DAPI cell nucleus counterstaining.

A-C: CD11b staining. (A) normal rabbit cornea, (B) cornea with pristine Ti implant, (C) cornea with Ti-AMP implant.

D-F: F4/80 staining. (D) normal rabbit cornea, (E) cornea with pristine Ti implant, (F) cornea with Ti-AMP implant.

G-I: MMP9 staining. (G) normal rabbit cornea, (H) cornea with pristine Ti implant, (I) cornea with Ti-AMP implant.

Figure 7. Antibiotics treatment of rabbit cornea after inoculation with *S. aureus* and *P. aeruginosa*. 
A and C: Scoring of slit lamp examinations with inoculation of *S. aureus* (A) and *P. aeruginosa* (C).

B and D: Bacteria counting of corneal tissue with inoculation of *S. aureus* (B) and *P. aeruginosa* (D). “*”*p*<*0.05, one way ANOVA.
Table 1. Scoring of slit lamp examinations.

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<th>P. aeruginosa</th>
<th>S. aureus</th>
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Figure 1: A diagram to interpret the design of the rabbit study.

Rabbits Cornea

1. Prisline Ti Implantation (21)
2. 7 days
3. S. aureus inoculation (6)
4. 2 days
5. Sacrifice (3)
6. Sacrifice (9)
7. Sacrifice (9)
8. S. aureus inoculation (6)
9. Prisline Ti Implantation
10. 7 days
11. P. aeruginosa inoculation (3)
12. 2 days
13. Antibiotics
14. 2 days
15. Sacrifice (3)
16. Ti AMP Implantation (16)
17. 7 days
18. P. aeruginosa inoculation (3)
19. 2 days
20. Antibiotics
21. 2 days
22. Sacrifice (3)
23. Sacrifice (3)
24. Sacrifice (3)
Figure 2

**A**

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>MIC S. aureus (μg/ml)</th>
<th>MIC P. aeruginosa (μg/ml)</th>
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**B**

S. aureus  P. aeruginosa

Ti  Ti-AMP

**C**

Ti  Ti-AMP-FITC

day 1  day 1  day 30

day 60

Figure 2. In vitro bactericidal effect and stability of SESB2V AMP.

A: A table to show the MIC value of the full length and truncated SESB2V peptide.

B: Representative images of live/dead bactericidal assay. Green: live bacteria, Red: dead bacteria. The ratio of live to dead bacteria number was counted and graphed. * p < 0.05, n=6, student t test.

C: The fluorescence images of Ti surface after immobilization with SESB2V-FITC peptide in phosphate buffer saline.
Figure 3. In vivo bactericidal effect of SESB2V AMP against S. aureus.

A: slit lamp examination of the rabbit eyes before and after implantation and bacteria inoculation

B: AS-OCT scanning of the rabbit eyes before and after implantation and bacteria inoculation

C: measurement of corneal thickness. Only cornea thickness anterior to implant was measured at pre and post-inoculation D1 and D2. *** p < 0.05 between pristine Ti and Ti-AMP group at post-inoculation day 1. **** p <0.05 between pristine Ti and Ti-AMP group at post-inoculation day 2. One way ANOVA.
Figure 4

A

Bacteria counting (S. aureus)

Bacteria counting (P. aeruginosa)

Log CFU/colonies

Figure 4. Bacteria counting of S. aureus and P. aeruginosa. Results are presented as log10 CFU/colonies ± SEM. **p<0.01, ***p<0.001 and ****p<0.0001, n=3 in each group. One way ANOVA.
Figure 6

A normal rabbit cornea
B cornea with positive T-implant
C cornea with positive T-implant
D normal rabbit cornea
E cornea with positive T-implant
F cornea with positive T-implant

Legend:
- Green: inflammation
- Blue: VCP-tail signals
- Red: DAPI staining