Antimicrobial Activity of Poly-epsilon-lysine Peptide Hydrogels Against *Pseudomonas aeruginosa*

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**PURPOSE.** To determine the antimicrobial activity of poly-epsilon-lysine (pεK) functionalization of hydrogels against *Pseudomonas aeruginosa*.

**METHODS.** Antimicrobial activities of pεK and pεK+ hydrogels were tested against both keratitis and a laboratory strain of *P aeruginosa* at a range of inocula sizes, over 4 and 24 hours. The number of viable CFU on pεK and pεK+ hydrogels or commercial contact lenses (CL) was investigated. Ex vivo porcine corneas were inoculated with *P aeruginosa* PAO1 (10³ CFU) and incubated with pεK+ hydrogels or commercial hydrogel CL for 24 hours and the effects of infection determined.

**RESULTS.** PrK+ hydrogels showed log reductions in viable CFU compared with pεK hydrogels for all *P aeruginosa* strains, depending on inocula sizes and incubation time. After 24 hours pεK+ hydrogels showed >5 and >7.5 log reduction in CFU compared with commercial hydrogel CL at 10³ and 10⁶ CFU, respectively. In an ex vivo porcine corneal infection model, pεK+ hydrogels led to a significant decrease in viable PAO1 CFU and histologic analysis indicated a decreased infiltration of PAO1 into the stroma.

**CONCLUSIONS.** PrK+ hydrogels demonstrated enhanced antimicrobial activity versus nonfunctionalized pεK hydrogels against clinically relevant *P aeruginosa* strains. PrK+ hydrogels have the potential to be used as a bandage CL with innate antimicrobial characteristics to minimize the risk of microbial keratitis.

Keywords: hydrogel, pseudomonas keratitis, contact lenses, antimicrobial, bandage lens
Poly-epsilon-lysine (pEK) is a cationic peptide with intrinsic antimicrobial properties and broad-spectrum antimicrobial activity against gram-positive and -negative bacteria, yeasts, and fungi.\textsuperscript{24,25} We have previously demonstrated that pEK hydrogels decreased the growth of laboratory strains of \textit{Staphylococcus aureus} and \textit{Escherichia coli}.\textsuperscript{26} The mechanism of action of pEK previously described involves disruption of the cell membrane and the cell wall.\textsuperscript{27,28} Cross-linking pEK with a dicarboxylic acid using carbodiimide chemistry results in a transparent hydrogel with excellent mechanical properties that can be cast into a CL and is nontoxic to corneal epithelial cells.\textsuperscript{26} Pek hydrogels, therefore, have the potential to be used as a bandage CL. The antimicrobial activity of pEK hydrogels can be enhanced by covalently binding additional pEK to the hydrogels free amine groups (pEK+), as previously described by Gallagher et al.,\textsuperscript{26} which showed approximately a 10-fold increase in amine functionality after functionalization owing to the additional pEK molecules, compared with the nonfunctionalized hydrogel. In this study, we compared the relative antimicrobial properties of pEK+ hydrogels with nonfunctionalized pEK hydrogels and commercial hydrogel CL using \textit{P aeruginosa} PAO1 and two different clinical isolates, \textit{exoU}\textsuperscript{+} and \textit{exoS}\textsuperscript{+}, in both in vitro assays and an ex vivo corneal infection model.

**Methods**

**pEK Hydrogel Synthesis and Functionalization**

The fabrication of pEK hydrogels (cross-linked to 60 mol\% with octanedioic acid to a polymer density of 0.071 g mL\textsuperscript{-1}), pEK functionalization, sterilization, and wash steps were performed as previously described.\textsuperscript{26,29}

**Bacterial Strains and Culture Conditions**

\textit{P aeruginosa} (PAO1) (ATCC 47085) and clinical keratitis isolates (cytotoxic strain \textit{exoU}\textsuperscript{−} PA39016 and invasive strain \textit{exoS}\textsuperscript{+} PA50817) (kindly donated by Prof. C. Winstanley, University of Liverpool, Liverpool, UK),\textsuperscript{30} were cultured overnight on Luria–Bertani (LB) agar (Sigma-Aldrich, Dorset, UK) and vortexed for 30 seconds to remove adherent bacteria. Bacteria from the original overnight culture and suspended bacteria were serially diluted and 10 μL of each dilution plated onto LB agar plates using the Miles and Misra method\textsuperscript{31} and incubated at 37°C for 24 hours for enumeration. After vortexing in PBS buffer, pEK and pEK+ hydrogels, and LB agar discs were placed directly onto LB agar plates and incubated overnight at 37°C to determine bacterial regrowth. A minimum of four independent experiments were performed for each condition, with a minimum of three repeats for each condition per experiment.

**Ex Vivo Corneal Culture**

Fresh porcine eyes were obtained from 6-month-old pigs within 6 hours of slaughter from a local abattoir and corneas were excised as previously described.\textsuperscript{35} Eyes containing visible lacerations identified using 2% (w/v) Fluorescein sodium (Bausch & Lomb, Kingston-upon-Thames, UK) were excluded from the study. Corneas were washed for 2 minutes in sterile PBS containing 1% (v/v) penicillin, streptomycin, and amphotericin B (Sigma Aldrich), followed by a 2-minute wash in 3% (v/v) iodinated povidone (Ecolab Ltd, Leeds, UK) and washed thoroughly in antibiotic-free PBS. Corneas were placed epithelial side down into sterile bijou tube lids. UltraPure Agarose (Thermo Fisher Scientific, Loughborough, UK) (0.5% [w/v]) dissolved in Dulbecco's modified eagles medium (approximately 65.5°C), cooled to approximately 37°C and pipetted onto the endothelial side of corneas to fill the cavity and solidified at room temperature (approximately 25°C). Corneas and agarose supports were transferred into 6-well plates, epithelial side up, containing 3 mL Dulbecco's modified eagles medium (antibiotic free, containing 10% [v/v] fetal bovine serum [Labtech, Heathfield, UK]) and incubated at 37°C in 5% CO\textsubscript{2} for 24 hours before PAO1 infection, to ensure they were antibiotic and infection free before the start of the assay.\textsuperscript{35}

**Ex Vivo Corneal Infection**

Corneal epithelia were debrided using sterile 6-mm filter paper discs (Grade AA Discs, Whatman, Maidstone, UK) soaked in 70% (v/v) EtOH placed onto cornea for 5 seconds, followed by removal of epithelium with a surgical blade. Corneas were rinsed in Dulbecco's modified eagles medium and air-dried in a laminar flow cabinet for 10 minutes before inoculation. An inoculum of 10 μL (10\textsuperscript{5} CFU PAO1) was seeded onto the central cornea and incubated at room temperature for 15 minutes, enabling attachment of the PAO1 into the corneal surface, with minimal movement of the cornea. Sterile 8.5-mm diameter pEK+ hydrogels or commercial hydrogel CLs were placed onto the corneal surface. Control infected and noninfected, de-epithelialized corneas were run in parallel with 10 μL PBS added onto cornea as a mock inoculum, plus control corneas with epithelium intact. Corneas were incubated at 37°C in 5% CO\textsubscript{2} for 24 hours.

After overnight PAO1 infection, the central infected area of the cornea, or the cornea area under pEK+ hydrogels or commercial hydrogel CLs, was trephined using a sterile...
8.5-mm diameter CORONET long-handled corneal trephine (Network Medical Products Ltd, Ripon, UK), transferred into 500 μL PBS and homogenized using a Qiagen Tissue ruptor (Qiagen, Manchester, UK). Suspected bacteria were serially diluted in PBS and plated onto LB agar plates as previously described and quantified as CFU/cornea. Four independent experiments were performed, with a minimum of three corneas for each condition. A separate set of corneas from each independent experiment were fixed in 10% (v/v) neutral buffered formalin overnight for histology.

**Histology**

Corneas were processed using a Leica ASP300 tissue processor. Paraffin-embedded tissue was sectioned at a thickness of 5 μm and stained with a Gram stain (Tissue) kit (Thermofisher, Loughborough, UK) following the manufacturer's protocol and imaged using a Nikon CI upright microscope using a 60× objective.

**Statistical Analysis**

Bacterial counts were log_{10} transformed before data analysis. Data are presented as standard deviation of the mean and statistical analyses was carried out using GraphPad Software Prism version 8.4.1 (La Jolla, CA) using a two-way ANOVA statistical analyses was carried out using GraphPad Software Data are presented as standard deviation of the mean and nonfunctionalized pK+ hydrogels were determined at 4 hours with nonfunctionalized pK hydrogels was reduced, compared with nonfunctionalized pK hydrogels with inocula from 10^3, 10^4, 10^5, 10^6, and 10^7 CFU at 4 hours with corresponding 3.58, 3.90, 3.67, 2.50, and 2.28 log decreases, respectively (Fig. 2A; see Supplementary Table S2 for log reductions). Similar effects in viable count reductions of both strains PA39016 and PA58017 for pK+ hydrogels compared with nonfunctionalized pK hydrogels with all inocula sizes were determined.

Pronounced differences between the functionalized and nonfunctionalized pK hydrogels were determined at 24 hours. pK+ hydrogels decreased the number of associated PAO1 CFU compared with nonfunctionalized pK hydrogels by 4.75, 5.98, 7.85, 8.76, and 8.42 log, with inocula of 10^3, 10^4, 10^5, 10^6, and 10^7 CFU, respectively (Fig. 2B; see Supplementary Table S2 for log reductions). To a similar extent, pK+ decreased viable CFU compared with nonfunctionalized pK hydrogels at inocula of 10^3, 10^4, 10^5, and 10^6 CFU for both keratitis isolates PA39016 and PA58017 at 24 hours.

**RESULTS**

**Antimicrobial Activity of pK+ Hydrogels Against *P aeruginosa***

The innate antimicrobial activity of pK+ hydrogels compared with nonfunctionalized pK hydrogels was determined against *P aeruginosa* PAO1 and two clinically relevant strains (PA39016 [exoU] and PA58017 [exoS]) at varying inocula sizes (10^3, 10^4, 10^5, 10^6, and 10^7 CFU) cultured in PBS buffer. The number of viable CFU from the surrounding PBS buffer of the hydrogels was determined after 4 or 24 hours incubation. LB agar discs served as positive controls for *P aeruginosa* bacterial growth. pK+ hydrogels showed increased antimicrobial activity compared with nonfunctionalized pK hydrogels (Fig. 1; see Supplementary Table S1 for log reductions). Data showed significant decreases in viable CFU within the PBS buffer for all *P aeruginosa* strains and inocula after 4 and 24 hours incubation. PAO1 and PA58017 showed decreases between 2 and 4 log, whereas PA39016 showed decreases between 1.63 and 2.43 log, depending on inocula size, after 4 hours incubation. After 24 hours incubation, pK+ hydrogels decreased the number of viable CFU further by 4.53 to 9.27 log, depending on the strain and inocula size.

pK+ hydrogels decreased the number of viable PAO1 CFU in the PBS buffer after 4 hours incubation to below the starting inocula. PA39016 showed decreases in viable CFU below the starting inocula for 10^3 and 10^4 CFU, but no decreases at higher starting inocula (Fig. 1). PA58017 showed increases in viable CFU below the starting inocula for 10^3, 10^4, and 10^5 CFU, but not at the higher inocula. After 24 hours incubation, viable CFU were below the level of detection in the PBS buffer for starting inocula between 10^3 and 10^6 CFU for all *P aeruginosa* strains. At the higher starting inocula of 10^6 and 10^7 CFU, viable CFU were less than 10^3 CFU for PAO1 and PA39016 and less than 10^5 CFU for PA39016. Nonfunctionalized hydrogels showed the numbers of viable CFU greater than the starting inocula at 4 and 24 hours.

**P aeruginosa Growth From pK+ Hydrogels Onto LB Agar Plates**

To determine whether we had successfully decreased the bacterial load on the pK+ hydrogels below the detection limit for viable counting, we assessed the growth of each strain directly from hydrogels and LB agar discs after incubation for 24 hours. There were no culturable cells of any of the *P aeruginosa* strains from pK+ hydrogels at inocula up to 10^6 CFU, whereas nonfunctionalized pK hydrogels and LB agar discs showed colony outgrowth at all inocula tested (Fig. 3).

**Antimicrobial Activity of pK+ Hydrogels Against *P aeruginosa* Compared With Commercial Hydrogel CL**

Having determined that pK+ hydrogels possess antimicrobial activity against *P aeruginosa*, we investigated their antimicrobial effects compared with a commercial hydrogel CL at 10^3 and 10^6 CFU, after 24 hours incubation with different *P aeruginosa* strains. At 10^3 CFU, pK+ hydrogels decreased the numbers of viable CFU from all strains of *P aeruginosa* in the PBS buffer or associated with pK+ hydrogels to below the level of detection, represented by more than 5 log and more than 4 log decreased, compared with the commercial hydrogel CL and nonfunctionalized pK hydrogel, respectively (Fig. 4). Using an inocula of 10^6 CFU, *P aeruginosa* in the PBS buffer and associated with pK+ hydrogels were still less than the detection limit. For each of...
the *P. aeruginosa* strains there were more than 7.5 and more than 6.0 log decreases in CFU in the PBS buffer and the PrK+ hydrogels, compared with the commercial hydrogel CL and non-functionalized PrK hydrogel, respectively. Non-functionalized PrK hydrogels showed no significant log decreases compared with the commercial hydrogel CL.

**Antimicrobial Effects of PrK+ Hydrogels Against *P. aeruginosa* on Ex Vivo Corneas**

The antimicrobial effects of PrK+ hydrogels and commercial hydrogel CL were investigated using an ex vivo porcine corneal infection model. Corneas infected with *P. aeruginosa*

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**Figure 1.** Antimicrobial activity of PrK+ hydrogel lenses, compared with PrK hydrogels and LB agar discs against *P. aeruginosa* isolates in PBS buffer. PrK+ hydrogel, PrK hydrogel lenses and LB agar discs were inoculated with *P. aeruginosa* (PAO1, PA39016, and PA58017) at $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ CFU for 4 and 24 hours. Viable bacterial counts were determined as CFU from PBS buffer. Values represent mean, error bars represent the standard deviation. *$P < 0.05$ using two-way ANOVA and post hoc Tukey’s analysis.*
FIGURE 2. Antimicrobial activity of PrK+ hydrogels, compared with PrK hydrogels and LB agar discs against *P. aeruginosa* isolates. PrK+ hydrogels, PrK hydrogel, and LB agar discs were inoculated with *P. aeruginosa* (PAO1, PA39016, and PA58017) at $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ CFU for 4 and 24 hours. Viable bacterial counts associated with PrK+ hydrogels, PrK hydrogels, or LB agar discs were determined as CFU. Values represent mean, error bars represent the standard deviation. $^*P < 0.05$ using two-way ANOVA and post hoc Tukey's analysis.

PAO1 at $10^3$ CFU with PrK+ hydrogels applied displayed decreased corneal haze and clouding at 24 hours, compared with infected corneas and corneas infected in the presence of the commercial hydrogel CL (Fig. 5A). Bacterial numbers of strain PAO1 associated with the corneas were quantified by CFUs; there was a $2.79 \pm 0.45$ log decrease in CFU in the presence of PrK+ hydrogels, compared with the infected cornea. The commercial hydrogel CL showed a negligible $0.01 \pm 0.41$ log decrease in CFU compared with the infected corneas (Fig. 5B).
### Histologic Analysis of *P. aeruginosa* on Ex Vivo Corneas

The effects of prK+ hydrogels on the localization of strain PAO1 within ex vivo corneas was investigated using histologic analysis and compared with infected corneas or those with the commercial hydrogel CL. Control nonwounded and wounded corneas, showed the presence and absence of an intact epithelium, respectively, with no evidence of bacteria within the corneal stroma (Fig. 6). In contrast, strain PAO1 wounded infected corneas showed the presence of the bacteria stained red using Gram staining, localized intensely at the corneal surface and gradual invasion into the stroma.

Corneas wounded and infected with strain PAO1 and incubated in the presence of the commercial hydrogel CL showed the presence of bacteria at the corneal surface and throughout the stroma, comparable with infected corneas. Corneas wounded and infected with strain PAO1 and incubated with prK+ hydrogel showed no evidence of bacteria directly under the hydrogel surface or throughout the stroma.

### DISCUSSION

CLs are important and popular medical devices helping with visual and ocular surface restoration. They are, however, associated with an increased risk of MK, particularly that...
Figure 4. Antimicrobial activity of pK+ hydrogels, compared with pK hydrogels and commercial hydrogel CL against \textit{P aeruginosa} isolates. Viable \textit{P aeruginosa} in PBS buffer and associated with pK+ hydrogel, compared with pK hydrogel and commercial hydrogel CL. PrK+ hydrogel, pK hydrogel, and commercial hydrogel CLs were inoculated with \textit{P aeruginosa} (PAO1, PA39016, and PA58017) at $10^3$ and $10^6$ CFU for 24 hours. Viable bacterial counts were determined as CFU. Values represent mean of four independent experiments, error bars represent the standard deviation. *$P < 0.05$ using two-way ANOVA and post hoc Tukey’s analysis.

Owing to \textit{P aeruginosa}, this risk becomes particularly problematic when the integrity of the ocular surface is disturbed. Provision, therefore, of a CL with inherent antimicrobial properties that can be used as a bandage CL would be of benefit in decreasing the risks of MK and in providing protection of the ocular surface. In this study, we demonstrated that hydrogel functionalization with pK increased its antimicrobial properties via enhanced activity against laboratory and clinically relevant \textit{exoU}+ and \textit{exoS}+ keratitis strains of \textit{P aeruginosa}, compared with the nonfunctionalized pK hydrogels and commercial hydrogel CL. This work supports the study from Gallagher et al., which demonstrated that pK functionalized hydrogels showed increased antimicrobial activity against laboratory strains of \textit{S aureus} and \textit{E coli}.  

PrK+ hydrogels showed increased antimicrobial activity compared with nonfunctionalized pK hydrogels within the PBS buffer for all inocula and \textit{P aeruginosa} strains; a scenario representing bacteria within the external environment of the CL and ocular surface. The effect of the inocula size upon pK and pK+ hydrogels was apparent at 4 hours, with higher inocula showing the lowest log decreases or no antimicrobial activity, possibly owing to the decreased ratio of molecules per bacteria. More important, we observed that pK+ hydrogels reduced viable CFU below the detection limit for all strains of \textit{P aeruginosa} within the surrounding PBS buffer environment compared with the commercial hydrogel CL. In contrast, both nonfunctionalized pK hydrogels and commercial hydrogels showed bacterial growth of less than 1.5 log above the starting inocula. CLs used as therapeutic bandages are in contact with the cornea for longer than 4 hours, so the extent of the reductions at 24 hours of \textit{P aeruginosa} with pK+ hydrogels is particularly encouraging for antimicrobial CL development.

CLs provide a potential surface for bacteria to colonize, proliferate, and infect the cornea, with adhesion being the initial step. Interestingly, \textit{P aeruginosa} reportedly adheres at greater numbers than \textit{S aureus} to CLs; however, different strains of \textit{P aeruginosa} do not adhere differently.
We obtained similar log decreases in CFU with prK+ hydrogels against all *P. aeruginosa* strains for each inocula size and time point tested. Importantly, we observed antimicrobial activity against the highly virulent *exoU* isolate PA39016, isolated from a patient with prolonged healing time and resistance to antibiotics. Decreases in bacteria associated with prK+ hydrogels may prevent and retard any further growth or spreading onto the cornea. Further studies are required to investigate if there is biofilm formation or if any resistance evolves to prK+ hydrogels over time.

We assessed antimicrobial activity at 4 and 24 hours, representing logarithmic and stationary phases of bacterial growth, respectively, and this assessment was undertaken in a nutrient-limited PBS buffer to provide an environment representative of the ocular surface. Our data demonstrate that prK+ hydrogels have an effect on a range of inocula sizes. At 4 hours we observed that the largest inocula sizes were associated with the greatest adhesion to prK+ and nonfunctionalized prK hydrogels, consistent with other studies using alternative CLs. After 24 hours incubation, however, bacterial CFUs were decreased for all inocula sizes, indicating that the prK+ hydrogels are effective at providing antimicrobial activity against larger inocula sizes and preventing any further growth on the prK+ hydrogel for up to 24 hours. The infectious dose of pathogenic bacteria before colonization of the eye or CLs is unknown. Sweeney et al. reported approximately 10 to 30 CFU in a noninfectious environment after 13 nights wear of a soft, high water content ionic lens. We suspect that CLs and corneas are in contact with lower inocula sizes compared with those tested in our experiments. Our data would suggest prK+ hydrogels could prevent colonization and growth of bacteria on both the cornea and surrounding environment in the presence of a low inocula.

In this study, we demonstrated that prK+ hydrogels decreased the number of viable CFU in ex vivo porcine corneas, compared with infected corneas with and without a commercial hydrogel CL. We observed lower log reductions in bacterial numbers from prK+ hydrogels in ex vivo corneas, compared with in vitro experiments. prK+ hydrogel activity acts by contact and high bacterial numbers arising from growth on the cornea are likely to invade deeper into the stroma, limiting the antimicrobial effect of prK+ hydrogels. Ex vivo cornea models have limitations compared with in vivo models, such as a lack of tear fluid and the immune response. They do, however, provide an understanding of cellular and structural changes that occur in the cornea following an external insult such as a microbial infection and CL.

Other studies have used different strategies to modify existing commercial CLs, which include both chemical and passive modifications using silver, free radicals, antimicrobial peptides, or nitric oxide–releasing polymers to either...
Antimicrobial $\text{pK}^+\text{hydrogels}$ Against $P.\text{aeruginosa}$

**FIGURE 6.** $\text{pK}^+$ hydrogels prevent PAO1 infection in ex vivo porcine corneas. Histologic analysis of gram-stained corneal tissue sections from an ex vivo cornea infection model incubated with $P.\text{aeruginosa}$ strain PAO1 for 24 hours, with or without $\text{pK}^+$ hydrogel or commercial hydrogel CL. Images show nonwounded healthy cornea showing intact epithelium (E) and stroma (S) and wounded cornea in the absence of infection with no epithelium present (tissue stained yellow). Wounded infected corneas with or without $\text{pK}^+$ hydrogel or commercial hydrogel CL, bacteria stained red (labelled with arrow heads) throughout stromal tissue. Scale bar = 50 μm, insert scale bar = 10 μm.

Although these strategies have their own advantages, a critically important advantage of $\text{pK}$ hydrogels are their capacity to be functionalized owing to the free amine groups to exert increased antimicrobial activity. Although $\text{pK}$ was used in this study, alternative agents can be attached similarly to engender additional antimicrobial activity or to improve healing of the ocular surface. Additional benefits of covalently binding additional $\text{pK}$ to the free amine groups on the hydrogels is that the antimicrobial activity does not rely on the release of any antimicrobial agents via diffusion onto the ocular surface. Therapeutic effects of the $\text{pK}$ hydrogel are therefore not decreased owing to the presence of corneal barriers such as increased tear fluid and blinking reflex.

Previous studies have demonstrated the antimicrobial activity of $\text{pK}^+$ hydrogels against laboratory strains of $S.\text{aureus}$ and $E.\text{coli}$, and this study demonstrates antimicrobial activity against clinically relevant $P.\text{aeruginosa}$, expanding its application as an antimicrobial CL. In summary, this study demonstrates the development of the $\text{pK}^+$ hydrogels as antimicrobial CL that are effective against clinically relevant $P.\text{aeruginosa}$ strains could potentially lessen the risk of CL associated MK.
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