A Novel Peptide Hydrogel for an Antimicrobial Bandage Contact Lens

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Corneal ulcers are one of the most common conditions to affect the cornea, accounting for ≈5% of total cases of blindness worldwide.[1–4] The causes of corneal ulcers range from noninfectious autoimmune disorders and chemical burns to infectious causes such as the herpes simplex virus and fungal or bacterial keratitis.[5,6] Of the infectious cases there is a global divide with fungal keratitis most prevalent in the developing world due to manual laborers working in optimal environments for fungal pathogens. In the developed world, bacterial keratitis is the most common cause of corneal ulcers and is most often contracted by improper use of contact lenses.[7] Current treatment focuses on the administration of broad spectrum antibiotic drops sometimes followed by the application of a bandage lens to protect the wound.[8] However, less than 7% of the drug actually reaches the point of injury due to the method of administration. This results in a requirement for multiple administrations sometimes at 3–4 intervals per hour over a 24 h period often with an initial dose every 5 min for the first 30–60 min.[9,10] There could be major advantages in the design and use of slow release antimicrobials from a bandage contact lens material as a more efficient delivery method.[11,12] Corneal bandage materials include collagen, amniotic membrane-derived materials, and advanced hydrogel polymers. A synthetic corneal bandage has many advantages over biologically derived products in terms of reproducibility of its properties and overcoming the need to use animal derived materials or an allogeneic graft.[13–15] An ideal bandage contact lens should be transparent, have a high water content, have sufficient mechanical properties for handling and be antimicrobial. This study developed poly-ε-lysine (peK)-based hydrogels as bandage contact lenses with optimized mechanical and antimicrobial properties.

The peptide-based polymer in this study has previously been developed in macroporous form as a wound dressing and as 3D scaffolds for tissue engineering applications. The polymer is based on peK cross-linked with bis-carboxy fatty acids. A feature which makes this hydrogel unique is the incorporation of two naturally occurring components; peK is an edible, non-toxic material currently used as an emulsifier and preservative in foodstuffs and classified as “generally regarded as safe” (GRAS) by the US Food and Drug Administration since 2004.[16–18] Short chain bis-carboxy fatty acids are found in both plants and animals. In the latter they are products of the oxidative degradation of longer chain fatty acids.[19,20] The hydrogels produced have a high water content, excellent transparency, and their mechanical properties can be tailored by altering the density of the polymer, the molecular length of the cross-linker and the cross-linking density. Further advantages of these hydrogels are that peK is naturally antimicrobial and the surface chemistry may be used to attach a variety of biomolecules.

peK (Zhengzhou Bainao Bioengineering Ltd, China) was cross-linked with bis-carboxy fatty acids ranging from C6 to C10 (Sigma-Aldrich, St. Louis, MO, USA) (Figure 1a) using an N-hydroxysuccinimide (NHS)/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI)-mediated cross-linking technique.[21] This cross-linking reaction occurs randomly and it is noted that it may also include amide bonds between the bis-carboxy acid and a single peK molecule. The density of the polymer ranged from 0.066 to 0.1 g cm\(^{-3}\) and the cross-linking density was varied between 60 mol% and 80 mol%, (Table S1, Supporting Information). The optimum gelation time was shown to be 5 h (Figure S2, Supporting Information). Appropriate lens shaped structures can be cast from these materials (Figure 1b). Post-polymerization penicillin G was attached via ionic interactions and free peK was covalently attached to remaining free surface amine functional groups (Figure S1 and Table S2, Supporting Information).

Details of the hydrogels synthesis and surface molecule attachment are available in Supporting Information. The ultimate tensile strength (Linkam TST350 tensile tester, Linkam, Surrey, UK; 20 N load cell, strain rate of 100 μm s\(^{-1}\)) increased as the molecular length of the bis-carboxy fatty acid increased from C6 to C9 before a statistically significant decrease for C10 (Figure 1c). The cross-link density increase from 60 mol% to 80 mol% generally caused an increase in strength again except for C10 where a significant decrease was observed. The factor that had the greatest effect on the ultimate tensile strength was the density of the polymer. In each case the increase in density from 0.066 to 0.1 g cm\(^{-3}\) more than doubled the ultimate tensile strength. The transparency of these gels assessed qualitatively (Figure S3,
Supporting Information) demonstrated a reduction as the chain length of the fatty acid increased. The reduction in transparency and tensile strength for C10 bis-carboxy fatty acid could be due to the hydrophobicity of the longer alkyl chain disrupting the structure of the hydrogel or the influence of intramolecular bonding or a combination of both.

Following these initial results, further tailoring of the hydrogel properties was investigated using octanedioic acid as the cross-linker. As expected the ultimate tensile strength increased with an increase in polymer density from 0.067 to 0.077 g cm\(^{-3}\) (Figure 1d). The elastic modulus (Figure 1e) for hydrogel Su 60 13 (octanedioic acid cross-linker with a density of 0.077 g cm\(^{-3}\) and 60 mol% cross-linked) was significantly greater than the other hydrogels tested. However, optical clarity of this hydrogel was compromised significantly due to the increased polymer density, resulting in a refractive index (Figure 1f) of 1.395 (AR200 digital refractometer (Reichert, New York, USA)). The hydrogel with the next greatest elastic modulus was hydrogel Su 60 14 (octanedioic acid cross-linker with a density of 0.071 g cm\(^{-3}\) and 60 mol% cross-linked) which, despite no significant difference in ultimate tensile strength compared to Su 60 13, was superior to both Su 60 15 (octanedioic acid cross-linker with a density of 0.066 g cm\(^{-3}\) and 80 mol% cross-linked) and Su 65 15 (octanedioic acid cross-linker with a density of 0.066 g cm\(^{-3}\) and 65 mol% cross-linked). Hydrogel Su 60 14 possessed a refractive index of 1.390 making it closely associated with the 1.380 of the human cornea.\(^{[22]}\) All of the hydrogels tested had a significantly smaller refractive index than the cornea, which is why this hydrogel was chosen for further investigation.

Figure 1. a) A schematic of the hydrogel backbone highlighting the proposed amide formation between short chain bis-carboxy fatty acids and the amine groups of p\(\varepsilon\)K. – amide bond. b) The hydrogel cast using a bandage contact lens mold. c) A comparison of tensile strength of hydrogels cast with differing bis-carboxy fatty acid cross-linkers and polymer densities -60 mol% cross-linked, 0.1 g cm\(^{-3}\) -60 mol% cross-linked, 0.066 g cm\(^{-3}\) -80 mol% cross-linked, 0.1 g cm\(^{-3}\) -80 mol% cross-linked, 0.066 g cm\(^{-3}\). d) A comparison of stress between all octanedioic hydrogel variants. e) A comparison of the elastic modulus between all octanedioic hydrogel variants. f) Refractive index measurements of all octanedioic hydrogel variants. Error bars ±SD, \(*p < 0.05, **p < 0.01, ***p < 0.005, n = 9.\)
lower refractive index than the commercial contact lens Etafilcon A at 1.412. Consequently, Su 60 14 was chosen as the hydrogel with superior mechanical properties while maintaining an acceptable optical clarity. The elastic moduli for all the hydrogels were in the range of 0.31–0.73 MPa, similar to commercial lens materials whilst maintaining a relatively high water content, measured gravimetrically, of between 67 and 73 w/w% regardless of the polymer composition (Table S3, Supporting Information). This may be extremely beneficial in that the mechanical properties may be optimized without significantly reducing the water content of the lens. This characteristic is important for both oxygen permeability and comfort of the lens to the wearer.\(^{[23]}\) The addition of penicillin G and free \(\varepsilon-K\) to Su 60 14 did not influence the ultimate tensile strength (Figure 1d) of the gels, but the addition of free \(\varepsilon-K\) did significantly increase the elastic modulus (Figure 1e) and resulted in a greater water content and lower refractive index than the penicillin G modified hydrogel (Figure 1f). The added \(\varepsilon-K\) to hydrogel Su \(\varepsilon-K\) results in an increase of hydrophilic amino groups and is directly associated with the increased water absorption capacity of the hydrogel.\(^{[24]}\)

An indirect cytotoxicity assay (CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan)) was carried out to determine if any leachables from the hydrogel material had an effect on a human corneal epithelial cell line (HCE-T cells donated by Kaoru Araki-Sasaki, Japan\(^{[25]}\) in vitro. Over the period of an 8 d culture (Supporting Information) no significant effects upon cell viability were observed when compared with the control (Figure 2a). This suggests that leachables from the hydrogel were noncytotoxic to the HCE-T cell line and provided the initiative to investigate how direct contact of the gel influenced the HCE-T cell behavior. A scratch assay was used to determine the rate of re-epithelialization of a HCE-T cell monolayer when in direct contact with the hydrogel material. The majority of scratch wounds with the hydrogel present closed by 23 h. This was also true of the control and no significant difference was observed in scratch wound closure rates when quantified with TScratch software.\(^{[26]}\) This suggests the Su 60 14 hydrogel does not inhibit HCE-T cell re-epithelialization when in direct contact (Figure 2b). The rate of wound healing is of great importance when considering a bandage contact lens material ensuring a faster healing process and less discomfort for the patient.\(^{[27]}\) Specific investigation of the closed cell monolayers was undertaken by staining for zonal occludens-1 (ZO-1). The ZO-1 protein functions in the formation of tight junctions which are expressed around...
the cell membrane when a cell monolayer is intact and the epithelial barrier functioning. Microbial infection may result in a compromised corneal epithelial barrier and repair of this is essential. Specifically, staining for ZO-1 (Primary antibody ZO-1 (Invitrogen 402200, California, USA) 1:100; Secondary antibody Alexa Fluor 594 Goat anti-Rabbit (Invitrogen A11008, California, USA) in 1% BSA 1:500) identifies whether the epithelial barrier function of the reformed monolayers is compromised in any way. A strong ZO-1 fluorescence signal was obtained for each of the different samples of closed scratch wounds (Figure 2c). This suggested that the presence of the hydrogel had no adverse effect on the reformation of a functioning HCE-T cell monolayer. These data coupled with data for scratch wound closure and cytotoxicity suggest that the hydrogel has no adverse effects on HCE-T cells in vitro, indicating it is cytotcompatible with this cell type. To be a commercially viable alternative to conventional bandage lens materials, however, a drug delivery capability is beneficial. A repeat of the above cytocompatibility tests on the antimicrobial hydrogels highlighted several novel insights. Minor toxicity arising from the antimicrobial hydrogels was observed after 24 h in culture possibly due to pH changes in the media from biomolecule elution or insufficient neutralization of salts associated with the hydrogels (Figure 2a). To highlight this, hydrogel Su PeK PO₄ with its associated phosphate salt showed the most toxicity which was evident after only 2 h. This toxicity was due to the phosphate salt leaching from the hydrogel into cell culture media and highlights the need to consider the effect of hydrogel-associated molecules prior to cell culture. A repeat of the HCE-T scratch assay and ZO-1 staining found no difference in re-epithelialization with the antimicrobial hydrogels present when compared with the Su 60 14 hydrogel and control (Figure 2b). ZO-1 staining was evident for all the hydrogels, except with Su PeK PO₄ where no monolayer was present and almost all cells were dead after 23 h (Figure 2c). The toxicity observed at 24 h resulting from the antimicrobial hydrogels may not have a negative impact in vivo as the natural flushing mechanism associated with blinking would maintain the tear film turnover surrounding the cornea epithelium.

The natural antimicrobial activity of the peK hydrogel towards *Staphylococcus (S.) aureus* and *Escherichia (E.) coli* was investigated by measuring cell metabolism using a resazurin assay adapted from Petit et al. The cross-link density of the hydrogels was reduced to leave a greater number of free surface amine functional groups to contribute toward antimicrobial activity. There was ≈40% less resazurin reduction for all hydrogels after 4 h and 18 h when compared with the growth medium control (Figure S4, Supporting Information). However, there was no significant difference in resazurin reduction when comparing hydrogels of different cross-link densities. This outcome may correlate with the 100 fold decrease in antimicrobial activity that peK, with <9 lysine residues, has when compared with longer chain residues. There may not be enough of a difference in the number of free amino groups side-by-side within the polymer backbone to make a significant difference to antimicrobial activity between the three cross-link densities. To test this hypothesis, peK was added post-polymerization to the hydrogel as longer chains. A similar trend was identified with both bacteria revealing little reduction of resazurin in the presence of the Su PeK hydrogel at the 2 and 4 h time points and only a minor resazurin reduction at the 18 h time point for both *S. aureus* (3%) and *E. coli* (5%) (Figure S5, Supporting Information). The unmodified hydrogel performed marginally better than the growth medium control at the 2 h and 4 h time points with both bacteria. After 18 h the resazurin reduction was approximately half of that of the growth medium control. A more detailed investigation into improving the antimicrobial activity of the hydrogel by either the ionic attachment of penicillin G or the covalent attachment of peK was undertaken with focus on *S. aureus* as the microbial model. A significant improvement of antimicrobial activity was observed against both planktonic and attached *S. aureus* on the hydrogel. The assay of planktonic *S. aureus* revealed the greatest log reduction of 2.8 was observed from Su Pen G, which may be explained from the fact that penicillin G was attached ionically and therefore becomes more bioavailable in the culture media when it releases from the hydrogel and has a greater effect than Su PeK on planktonic bacteria (Figure 3a). Activity toward *S. aureus* attached to hydrogel Su PeK revealed the greatest log reduction of 2.3 (Figure 3b). This could be due to the covalent attachment of cationic peK to the hydrogel promoting interaction with the net negative charge of *S. aureus* at the hydrogel surface. Propidium iodide staining of *S. aureus* attached to the surface of the hydrogels revealed that the bacteria were arranged as singular cocci where they were attached to Su PeK as opposed to the other hydrogel surfaces where their eponymous clustered growth conformation was observed (Figure 3c I-III). The expected electrostatic interaction between the negatively charged *S. aureus* surface and the highly cationic Su PeK hydrogel may be strong enough with this species to disrupt growth compared with other microbes. This interaction disrupts aggregation and biofilm formation leaving the microbes more susceptible to antimicrobial agents. Further staining with LIVE/DEAD BacLight of *S. aureus* retrieved from the hydrogel supports the conformational differences previously observed with the propidium iodide staining (Figure 3c IV-VI). There was also confirmation of significantly greater *S. aureus* death when in contact with both the Su PeK and Su Pen G hydrogels compared with the unmodified Su 60 14 hydrogel, underpinning an increased antimicrobial activity after biomolecule attachment. This study demonstrates the effectiveness of antimicrobials that can either be attached via covalent bonding or electrostatic attachment to the amine groups and thus should work for other more clinically relevant antimicrobials with these properties. Other agents may be incorporated and released via dissolution in the aqueous phase of the hydrogel. One such example being the attachment of antifungals to the hydrogel for the treatment of fungal keratitis.

In summary, peK hydrogel materials have been designed and characterized to show that they can be manufactured with properties similar to those of commercial contact lens materials. Their water content was determined to be high, which is a critical property for comfort to the wearer and oxygen permeability. Optimal monomer composition was established resulting in a highly reproducible final polymer material, hydrogel Su 60 14. This hydrogel was demonstrated to be non-cytotoxic to HCE-T cells in culture and does not inhibit re-epithelialization or indeed the re-formation of a fully functioning cell monolayer.
The natural antimicrobial activity of the hydrogel was improved with biomolecule attachment without inhibiting re-epithelialization or the integrity of the HCE-T cell monolayer. Interference with S. aureus biofilm formation was demonstrated by the pεK hydrogel modified with either a model antibiotic or the attachment of free pεK both for planktonic bacteria and those attached to the hydrogel. This study has demonstrated that pεK hydrogels are potential candidates for antimicrobial bandage contact lenses.

**Experimental Section**

The methods detailing hydrogel design, mechanical analysis, cytotoxic evaluation, and the investigation of antimicrobial activity are included in the Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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