Development of a Poly-ε-Lysine Contact Lens as a Drug Delivery Device for the Treatment of Fungal Keratitis

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Accepted: August 4, 2017
Submitted: May 26, 2017
Citation: Gallagher AG, McLean K, Stewart RMK, Wellings DA, Allison HE, Williams RL. Development of a poly-ε-lysine contact lens as a drug delivery device for the treatment of fungal keratitis. Invest Ophthalmol Vis Sci. 2017;58:4499–4505. DOI:10.1167/iovs.17-22301

PURPOSE. The purpose of this study was to develop a more efficient drug delivery device to overcome the limitations of current drop therapy for the treatment of fungal keratitis.

METHODS. Amphotericin B (AmpB), 0 to 30 μg/mL, was associated with a poly-ε-lysine (pεK) hydrogel. Fungicidal effect against Candida albicans was assessed at 18 and 42 hours by optical density (OD600) and growth on agar. Tear film dilution effect was mimicked by storage of AmpB pεK gels in 3.4 mL sterile PBS for 24 hours prior to fungal incubation. Drug elution over 96 hours was evaluated by HPLC, and drug stability was tested while associated with the gel by OD600 up to 48 hours. Lack of cytotoxicity toward the HCE-T corneal epithelial cell line was assessed over 7 days.

RESULTS. AmpB pεK gels show fungicidal activity in normal conditions (0.057 OD600, SD 0.003, P < 0.005) and in the presence of horse serum (0.048 OD600, SD 0.028 P < 0.005) at 18 hours. The drug release profile was above therapeutic levels (0.188 μg/mL) for up to 72 hours. Tear dilution had no significant effect at higher concentrations of AmpB (3 to 10 μg/mL). AmpB pεK gels were not cytotoxic to the HCE-T cell line.

CONCLUSIONS. We demonstrated that AmpB pεK gels confer sustained therapeutic antifungal activity for at least 48 hours without corneal epithelial cell line cytotoxicity, suggesting their potential for in vivo use as an antifungal bandage contact lens. This could avoid the need for intensive topical medication in the treatment of fungal keratitis.

Keywords: hydrogel, drug delivery, antimicrobial, amphotericin, bandage lens, cornea, keratitis
components pεK and bis-carboxylic fatty acids (Fig. 1b). The high water content ensures excellent oxygen transmission to the avascular cornea reducing the risk of corneal hypoxia and vascularisation.17 The mechanical properties and optical transparency of these gels can be easily optimized by altering the cross-link density, the polymer density, and the molecular length of the bis-carboxylic fatty acid cross-linker. We demonstrated that pεK gels are noncytotoxic to a human corneal epithelial cell line (HCE-T).18 Furthermore, the amine functional groups in the polymer backbone can be used for the chemical attachment of a range of biomolecules to aid drug delivery. Amphoterin B (AmpB) is one of the first-line therapies against C. albicans keratitis.22 AmpB contains a 38-member lactone ring with amino and carboxyl moieties.20,21 These carboxyl moieties have the potential to interact with amine groups on pεK within the polymer matrix.

This study aimed to modify a pεK hydrogel (Su 60 15; octanedioic acid cross-linker with a density of 0.066 g/mL and 60 mol% cross-linked) by associating AmpB with the amine functional groups to provide an effective drug delivery mechanism against C. albicans under different growth conditions. An investigation of AmpB stability after associating it with the hydrogel and the subsequent toxicity of the AmpB hydrogels toward the HCE-T cell line was also investigated.

Materials and Methods

Poly-ε-lysine, octanedioic acid, n-methylmorpholine (NMM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), and n-hydroxysuccinimide (NHS) were supplied by SpheriTech Ltd. (Runcorn, UK). HCE-T cells were donated by Kaoru Araki-Sasaki (Itami City, Japan).22 All other reagents including AmpB (A2942, 250 µg/mL stock were supplied by Sigma Aldrich (Gillingham, UK) unless otherwise stated.

Preparation of Hydrogel Su 60 15

Hydrogel Su 60 15 (30 mL) with 60% cross-linking and a polymer density of 0.07 g/mL was prepared. pεK (2.077 g) was dissolved in sterile distilled water (7.5 mL) in a 15-mL Falcon tube (Starlab Ltd., Blakelands, UK). An aliquot of 5% Tween 80 (0.25 mL) was added. Octanedioic acid (0.65 g) was dissolved in sterile distilled water (6.125 mL) with NMM (1.325 mL). This was sonicated until dissolved and added to the pεK solution. NHS and EDCI were measured into two separate 15-mL Falcon tubes and dissolved in PBS (7.5 mL PBS; Oxoid Ltd., Hampshire, UK). All solutions were filtered in a class II microbiology hood using a 0.2-µm syringe filter. EDCI (3.471 g) and NHS (0.695 g) were mixed together and immediately added to the pεK/octanedioic acid solution. This was inverted five times. An aliquot of the polymer solution (5 mL) was pipetted into 63-cm² nontreated tissue culture dishes (Greiner Bio-One GmbH, Kremsmünster, Austria). These were incubated for at least 5 hours at room temperature until the gel had polymerized, followed by addition of PBS to prevent dehydration of the gel. Sheets of gel were washed five times with 10% NMM for 5 minutes, followed by thorough washing in deionized water (dH2O, 10 × 5 minutes) to remove excess NMM. Gels were subsequently sterilized in 70% ethanol overnight before washing five times in sterile PBS and using them for experiments. Prior to commencing experiments, gels were cut into circular discs (0.36 cm²) using a metal trephine.

Culture of C. albicans

C. albicans strain SC5314 was revived from −80°C storage on potato dextrose agar (PD-agar; Formedium Ltd., Norwich, UK) streak plates incubated at 37°C overnight.23 C. albicans was subcultured by inoculating a PD agar streak plate with one colony taken from a previous PD agar plate and incubated overnight at 37°C. Colonies were maintained on PD agar stored at 5°C. Broth cultures were initiated by inoculating PD media (10 mL) with one colony of C. albicans taken from a PD agar plate. Cultures were incubated overnight at 37°C with constant shaking at 100 rpm. These were subcultured by taking an aliquot of C. albicans broth culture (0.5 mL) and diluting it in sterile PD broth (10 mL). For experiments, overnight broth culture (0.5 mL) was diluted with PD broth (10 mL), and optical density readings at 600 nm (OD600) were measured hourly with a Jenway 6705 UV/Vis Spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK). An initial OD600 measured between 0.1 and 0.2 and was monitored until an OD600 of 0.6 was obtained. The culture was diluted 1:1 with sterile PD prior to experiments. Candida cultures were dispersed in an ultrasonic bath for 15 minutes prior to experiments to prevent aggregation.

Fungicidal Effects of AmpB-Loaded Gel

AmpB (250 µg/mL) was diluted in sterile PBS to concentrations of 30, 25, 20, 15, 10, 5, 3, and 0 µg/mL. Gels were incubated in the various concentrations of AmpB for 2 hours before being transferred to 96-well microtiter plates. Control wells containing PD only and PD with C. albicans were used throughout all these experiments. Wells containing PD (0.1 mL) were...
inoculated with *C. albicans* (0.0125 mL). Microtiter plates were incubated overnight at 37°C, and OD_{600} readings were taken after 18 hours (Fig. 2, step 1).

This protocol was repeated to include *C. albicans* with the addition of horse serum (10% vol/vol) to PD broth to promote differentiation and upregulate the expression of virulence genes.24,25 After the gels were removed from the microtiter plate, they were individually washed in sterile PBS (5 mL) to remove any loosely attached fungi, and the wash solution was collected (Fig. 2, step 2). The gels were extracted using sterile forceps and placed on PD agar plates to identify the presence of viable fungi. An aliquot of each PBS wash solution (0.02 mL) was also plated (Fig. 2, step 2). The agar plates were incubated at 37°C overnight before imaging and counting the colony-forming units (Fig. 2, step 3). After the gels were removed, the plates were incubated at 37°C for a second night to establish whether there was any further growth of *C. albicans*. OD_{600} readings were taken after 24 hours (Fig. 2, step 3).

**Fungicidal Effect of the AmpB-Loaded Gel After 24-Hour Storage in PBS**

Discs (0.36 cm²) of gel were incubated in various concentrations of AmpB (0.3, 3, 5, and 10 µg/mL) for 2 hours. Subsequently, the gel samples were individually incubated in 3.4 mL sterile PBS for 24 hours to mimic and assess the dilution effect of the tear film in vivo. The gels were then transferred to a 96-well microtiter plate containing PD (0.1 mL), and the PBS tear film solution was collected. The gels were inoculated with *C. albicans* (0.0125 mL) and incubated at 37°C overnight. OD_{600} readings were taken after 18 hours with and without gels in the microtiter plate. After removal from the microtiter plate, the gels were individually washed as described previously. The gels and an aliquot of the tear film wash solutions (0.02 mL) were plated onto PD agar. The agar plates were incubated at 37°C overnight before imaging, and the number of colony-forming units was determined.

An aliquot of each PBS tear film solution (0.1 mL) was diluted with PD (0.1 mL) and added to a 96-well microtiter plate. The microtiter plate was then inoculated with *C. albicans* (0.0125 mL) and incubated overnight at 37°C. OD_{600} readings were taken after 18 hours.

**AmpB Elution From the Hydrogel**

Discs of the gel (0.36 cm²) were incubated in a 30-µg/mL AmpB solution for 2 hours. The gels were removed and incubated in PBS at one gel per 0.1 mL for 24, 48, 72, and 96 hours. At each time point, the supernatant was removed and used to monitor AmpB elution via HPLC. Fresh PBS was added to the same gel samples prior to reincubation.

Reversed-phase HPLC (RP-HPLC) was performed on a SpectraSYSTEM AS3000 (Thermo Scientific, Waltham, MA, USA) with a C18 Vydac column (4.6 × 250 mm, 5 µm). The column temperature was set at room temperature, and 0.2 mL was the injection volume at a flow rate of 1 mL/min. The gradient was set to 40% to 100% B over 15 minutes with A: 0.1% TFA in H₂O, B: 0.1% trifluoroacetic acid (TFA) in MeCN and a detection wavelength of 380 nm.

**AmpB Stability While Associated With the Hydrogel**

Discs of the gel (0.36 cm²) were incubated in a 30-µg/mL AmpB solution for 2 hours. The gel was removed and incubated without media at 25°C for 1, 24, or 48 hours. The AmpB incubation solution was also stored for the same period. PD (0.1 mL) was added to each test well in a 96-well microtiter plate, and then a test gel or the corresponding AmpB incubation solution was added (0.0125 mL) and inoculated with *C. albicans* (0.0125 mL). Microtiter plates were incubated

![Figure 2](https://arvojournals.org/figure2.png)
overnight at 37°C, and OD
600 readings were taken after 18
hours.

**Cytotoxicity of AmpB-Loaded Gels**

A cell counting kit-8 (CCK-8) assay kit (Dojindo Laboratories, Kumamoto, Japan) was used to monitor AmpB gel cytotoxicity toward the HCE-T cell line. Discs of the gel (0.36 cm²) were incubated in a 30-μg/mL AmpB solution for 2 hours prior to incubating one gel per 0.1 mL in DMEM/F12 cell culture media at 37°C for 3 days. Media without gels was also incubated for the same period as a control. HCE-T cells were then seeded at a density of 2.5 × 10⁴ in a 48-well microtiter plate and incubated with media from the preincubation with the gels or the control media (0.25 mL). At time points 4, 24, 48, 96, and 168 hours, CCK-8 (0.025 mL) was added to each well and incubated at 37°C for 2 hours. Aliquots (2 × 0.1 mL) of media were removed and transferred to a 96-well microtiter plate, and the absorbance was read at 485 nm on a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany).

**Statistical Analysis**

GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA) was used to model 2-way univariate ANOVA with Tukey post hoc analysis, as well as model 1-way univariate ANOVAs to analyze data at the same concentrations or time points where appropriate. All the statistical data were presented as means ± SD.

**RESULTS**

**Fungicidal Effects of AmpB-Loaded Gels**

The loading of AmpB and its release from the gels significantly reduced the growth of *C. albicans* compared with no AmpB, with no obvious changes in transparency (Fig. 3). However, there was no statistically significant difference between concentrations of AmpB or between the different culture conditions (-NH₂, -NH₂ gel cultured in PD + 10% horse serum) compared across all concentrations.

**Viability of Cells Attached or Loosely Adhered to the Gel**

No loosely attached viable fungi were recovered from the gel wash. Minimal *Candida* growth was also observed when gels preincubated with AmpB (0.3 μg/mL) were placed directly on PD agar (Fig. 4).

**Viability of Fungi Remaining in the Wells**

No further growth of *C. albicans* was observed in wells that previously contained the gel across all AmpB preincubation concentrations (Fig. 5).

**Fungicidal Effect of AmpB-Loaded Gels After Storage in PBS**

A statistically significant decrease in the growth of *C. albicans* was measured in wells treated with 3 to 10 μg/mL AmpB compared with those treated with 0.3 μg/mL (Fig. 6).

**Figure 3.** Bar chart demonstrating the fungicidal effects of gels preincubated in various concentrations of AmpB under different growth conditions. Error bars denote ±SD, ***P < 0.005, N = 9.

**Figure 4.** *C. albicans* viability assays on gels and from PBS washes with enhanced images of representative wells for each sample. (I) Gels plated on PD agar for 0 + 3 μg/mL AmpB gel cultured in PD broth and PD broth supplemented with 10% horse serum. (II) Washes plated on PD agar for 0 + 3 μg/mL AmpB gel cultured in PD broth and PD broth supplemented with 10% horse serum.
AmpB Elution From the Gels

A therapeutic dose of AmpB (see Supplementary Material S1) was eluted from the gel for a period of 72 hours with a smaller dose after 96 hours (Fig. 7). The dose at 96 hours is still relevant compared with minimum inhibitory concentration data from the literature.26,27

AmpB Viability After Attachment to the Hydrogels

AmpB associated with the hydrogel remained stable and produced a killing effect against *C. albicans* for at least 48 hours (Fig. 8). There seemed to be less *C. albicans* killed at 48 hours compared with the earlier time points; however, this was not statistically significant. There was no difference between the efficacy of the AmpB gels and the 30-μg/mL AmpB incubation solution at each time point in terms of the eradication of *C. albicans*.

Cytotoxicity of AmpB Gels

No cytotoxicity to the HCE-T cell line was observed when comparing gels incubated in AmpB (30 μg/mL) with standard cell culture media (Fig. 9). The positive control (Su Pt:K PO₄), a Su 60 14 gel loaded with phosphate, was cytotoxic toward the HCE-T cell line as already determined for this cell line.18

**DISCUSSION**

Development of a bandage contact lens as a drug delivery device for antifungal drugs overcomes the limitations of topical antifungal eye drops, with great potential benefit in the management of fungal keratitis. Ideally a bandage contact lens may be worn for extended periods of time, aiding ocular surface healing and comfort with less frequent need for medical review and lens change. The lens material in this situation must have excellent oxygen permeability to preserve the integrity of the avascular cornea. High-water-content hydrogels would allow significant oxygen permeability through the water phase of the gel. The advantage of hydrogels for drug delivery is the potential to incorporate biomolecules through-
and is the organism that is most common in keratitis infection as it is the most well-studied strain of temperate climates. AmpB was used as the antifungal agent, isolates from the literature of 0.06 to 10% horse serum and correlates with MIC data for ocular report (see Supplementary Material S1) is between 0.094 and 1.7% horse serum (Su 60 15). This hydrogel has a positive charge due to the remaining uncross-linked amine groups within the polymer matrix.

C. albicans strain SC5314 was used to model a fungal keratitis infection as it is the most well-studied strain of Candida and is the organism that is most common in temperate climates. AmpB was used as the antifungal agent, given that it is one of the first-line choices against C. albicans and has a carboxyl group that can promote attachment of the drug molecule to the free amine groups on hydrogel Su 60 15 and aid its uptake into the hydrogel. The MIC found in this report (see Supplementary Material S1) is between 0.094 and 0.188 μg/mL for C. albicans cultured in both PD and PD with 10% horse serum and correlates with MIC data for ocular isolates from the literature of 0.06 to 1 μg/mL. A concentration of AmpB, well above the MIC, is necessary to ensure adequate dosing to the cornea considering that ≤7% reaches the target tissues. Using serial dilutions of the stock AmpB solution, we demonstrated that AmpB incorporated into the pK hydrogels significantly reduced the growth of C. albicans compared with no AmpB, independent of the solution concentration over 18 hours. Generally, the gels preincubated in AmpB and cultured under both normal growth conditions (and when the PD was supplemented with 10% horse serum) had a similar efficacy on C. albicans. When the gels themselves were evaluated, it was clear that there was minimal fungi growth on the gels or loosely attached to the gels.

Once the gels had been removed from the wells, it was important to evaluate whether any fungi remaining could proliferate even if growth was low when the gels were present. This could model the situation where the medicated lens was removed from the eye without knowing if all the infection had been eliminated. Over the subsequent 24 hours, minimal fungal growth was observed in the wells that contained the gels. These data suggest that the concentration of AmpB attached to, and released from, the gels is fungicidal.

The approximate volume of tear fluid produced over 24 hours is 1.7 mL in a normal eye. When an eye is infected, however, there is often a considerable increase in tear volume. To mimic the dilution effect of the tear film, gels preincubated in AmpB were stored in 3.4 mL sterile PBS for 24 hours. Over this period, it is assumed that AmpB will diffuse out of the gel into the media. After incubation, both the gels and the tear fluid were evaluated for their antifungal activity. For the gels, the growth of C. albicans in any of the wells (3 to 10 μg/mL) after storage was significantly reduced compared with the lowest concentration of AmpB (0.3 μg/mL). Similarly, aliquots of the tear fluid from around these gels caused a significant reduction in fungi growth. Evaluation of the tear fluid from these gels demonstrated that only gels incubated in the greater concentrations of AmpB (3 to 10 μg/mL) possessed a sufficient concentration of AmpB to cause a significant reduction in fungal growth.

It is important to evaluate how much AmpB diffuses out of the gels over time to help optimize the drug loading regime. We demonstrated that after storage for all time points, the gel released a clinically relevant dose of AmpB for 72 hours, only falling below the MIC after 96 hours. These data demonstrate that the gels are effective at delivering a clinically relevant dose of AmpB. Furthermore, we could demonstrate that the dose of AmpB delivered by the gel was stable enough after 48-hour storage to kill C. albicans with a similar efficacy as a gel used immediately after preincubation in AmpB. Effective treatment of fungal keratitis will require the bandage contact lens to maintain the level of AmpB against the cornea at a therapeutic level to promote penetration of the drug into the corneal stroma. These data suggest that the gel may be an effective AmpB delivery device. It also suggests that the loading dose could be tailored to release the appropriate dose at different stages of the healing process.

The AmpB gel was also investigated for any cytotoxicity toward the HCE-T cell line. The gels, with AmpB, as expected and corroborated by previous work, were not cytotoxic toward the HCE-T cell line. An appropriate next step will be to investigate the AmpB gel in a diseased animal model or an ex vivo model of bacterial keratitis.

**CONCLUSIONS**

This study demonstrated the potential of pK gels to act as a drug delivery bandage contact lens. The gels have a proven antifungal activity against C. albicans under normal growth conditions and in the presence of horse serum after preincubation with AmpB. A therapeutic amount of AmpB was released from the gel for a period of at least 48 hours, retaining its therapeutic fungicidal activity for at least 48 hours. The AmpB gels also proved noncytotoxic to the HCE-T cell line, further outlining its potential clinical use as a bandage.
Antifungal Bandage Contact Lens

contact lens to replace the laborious AmpB administration by eye drops in the fight against fungal keratitis.

Acknowledgments

Supported by bursaries awarded from The Wolfson Foundation and the Institute of Ageing and Chronic Disease, University of Liverpool, is grateful to funding KM, as well as the Engineering and Physical Sciences Research Council (EPSRC) for Grant EP/M002209/1, which contributed to this study and Spheritech Ltd. for funding KM, as well as supported by bursaries awarded from The Wolfson Foundation.

Disclosure: A.G. Gallagher, SpheriTech Ltd. (E); K. McLean, Richard Martin Optics Ltd. (E); D.A. Wellings, SpheriTech Ltd. (I), P. H.E. Allison, None, R.L. Williams, None.

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