Bacteriophage Delivering Hydrogels Reduce Biofilm Formation *In Vitro* and Infection *In Vivo*

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Statement of Purpose: Biofilm formation is a common complication in bone grafting procedures, and accounts for over one million nosocomial infections per year[1]. Because biofilms reduce the already declining efficacy of traditional antibiotics[2], these infections typically result in removal of the infected implant and are associated with high patient morbidity[1]. This necessitates the development of biomaterials resistant to bacterial infection and colonization that do not rely on antibiotics. To this end, we have engineered a bone-regenerating poly(ethylene) glycol (PEG) hydrogel[3] with encapsulated bacteriophage, ϕPaer14, which replicates by infecting and killing *Pseudomonas aeruginosa*, the gram-negative bacteria most commonly implicated in implant infections. We then studied the ability of a strain of *P. aeruginosa*, PsAer-9, to colonize the gels *in vivo* and *in vitro*.

Methods: ***ϕPaer14-encapsulating hydrogel synthesis:*** PEG hydrogels were functionalized with RGD peptide and crosslinked with VPM in a solution of either pure 100 mM MES buffer or the same buffer with a suspension of approximately 10^7 plaque forming units (PFU) per µL.

In vitro *biofilm formation:* 25 µL PEG hydrogels were cast in acrylic channels mounted on glass slides. Each slide was capped with a silicone microfluidic device designed to produce a laminar flow over the surface of the gel, and sealed using a UV activated adhesive. The slides were then warmed to 37°C, and a liquid culture of PsAer-9 with optical density of 0.5 was flowed over the gel for one hour. Concentrated trypticase soy broth (TSB 25%) was then flowed over the gel for an additional 24 hours.

Bacteria viability count: PEG hydrogels were recovered following biofilm formation or implantation, weighed, and degraded in collagenase at 37°C for one hour. Serial dilutions of each degraded hydrogel were then plated on TSA and incubated for 24 hours. Colonies were counted and normalized to the mass of hydrogel recovered to determine the original CFU content in the hydrogel.

Mouse radial segmental defect infection model: 8-10 week old male C57/B6 mice were anesthetized through isoflurane inhalation and their right forelimb was injected with buprenorphine as an analgesic. A 1 cm incision was made over the radius, and a bone cutting tool was used to remove 2.5 mm of the radius. A 4 mm polyimide tube containing a PsAer-9-infected hydrogel was then fitted over the defect, and the wound was sutured shut. Mice were euthanized via CO_2 inhalation at 1 week, then implants were removed with surrounding tissue, homogenized and suspended for serial dilution in PBS and plating to enumerate live bacteria.

hMSC toxicity: 5000 human mesenchymal stem cells (hMSCs) per well were plated in a 96 well plate and incubated in 200 µL of hMSC media for 24 hours. Media were then replaced with either 200 µL of unaltered hMSC media or hMSC media containing $2*10^6$ PFU/µL, and cultured for an additional 24, 48, or 72 hours. Cell survival was then assessed by incubating the cells with a 10% solution of CCK-8 reagent for 45 minutes and measuring fluorescence intensity at 450 nm.

Statistics: Test groups were compared to controls using an unpaired Student's t-test, unless samples had significantly different variances (in which case Welch's t-test was used), or had non-normal distribution (in which case a Mann Whitney U-test was used). Outliers were detected using a ROUT test with a false discovery rate of 1%. Values of PFU or CFU per unit volume were transformed by $\log_{10}$ before analysis. All analyses were performed using Prism Graphpad.

Results: hMSCs cultured in the presence of ϕPaer14 showed no significant difference in survival compared to controls at 24 hours or 48 hours. At 72 hours, cells cultured in the presence of ϕPaer14 showed significantly higher survival ($p = 0.0047$) than controls. Biofilms grown *in vitro* on ϕPaer14-delivering hydrogels showed a significant reduction in the number of recovered viable bacteria (Fig. 1) compared to virus-free control gels.

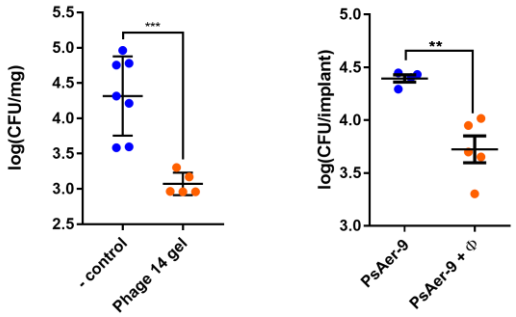

Figure 1. *CFU/mg recovered from PEG hydrogels after biofilm formation* in vitro *(left) and* in vivo *(right). Error bars show standard error of the mean* (** $p<0.01$,***$p<0.001$).

ϕPaer14-delivering hydrogels recovered from the radial defect infection model showed significantly fewer viable bacteria than control hydrogels at 1 week ($p=0.0296$).

Conclusions: The results demonstrate that ϕPaer14 has no negative impact on the viability of hMSCs, and that encapsulating ϕPaer14 in PEG hydrogels significantly reduces the ability of PsAer-9 to colonize the surface of the gel. Furthermore, ϕPaer14-delivering hydrogels significantly reduce infection in bone defects. This shows bacteriophage, such as ϕPaer14, are a viable candidate for further investigation as potential antimicrobial agents in biomaterials implants.

References: [1] Darouiche et al. *N Engl J Med*, 2004, 350, 1422-1429. [2] Knapp et al. *Environmental Science & Technology*, 2010, *44*(2), 580-587. [3] Shekaran et al. *Biomaterials*, 2014, 35, 5453-5461. [4] Brouqui et al. *Antimicrobial Agents and Chemotherapy*. 1995, 39(11), 2423-2425.

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