ACS OMEGA

Article

Cite This: ACS Omega 2019, 4, 2584−2589

http://pubs.acs.org/journal/acsodf

Investigating the In Vivo Antimicrobial Activity of a Self-Assembling Peptide Hydrogel Using a *Galleria mellonella* Infection Model

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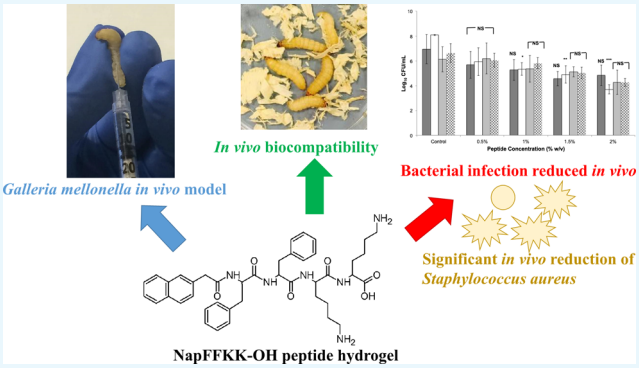
Supporting Information

ABSTRACT: Technological advances in protein biochemistry now enable researchers to modify the structure of peptides to enable them to possess self-assembling properties, forming hydrogels at low concentrations. Peptides can be altered further to provide multifunctional characteristics, for example, to demonstrate antimicrobial properties. The aim of this article is to investigate the in vivo toxicity and antimicrobial properties of a low molecular weight (naphthalene-2-ly)-acetyl-diphenylalanine-dilysine-OH (NapFFKK-OH) peptide hydrogel using an innovative waxworm (*Galleria mellonella*) model, as an alternative to mammalian/vertebrate testing. NapFFKK-OH hydrogels did not demonstrate any observable in vivo toxicity or death in *G. mellonella* larvae over 5 days at concentrations studied (≤2% w/v). A dose-dependent $\log_{10}$ reduction in viable (CFU/mL) Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria implicated in nosocomial infections was observed over 72 h. NapFFKK-OH was especially effective against in vivo infection models of *S. aureus* with a significant 4.4 $\log_{10}$ CFU/mL reduction in viable bacteria at 2% w/v after 72 h. Our results show *G. mellonella* to be a useful model for preliminary determination of in vivo toxicity and antimicrobial efficacy profiles of novel nanomaterials, including peptide-based hydrogels. This contributes to the 3R principles of animal testing, reduction, refinement, and replacement. The results also show NapFFKK-OH to be a promising alternative to standardly employed antimicrobials with the potential to be utilized as a novel therapeutic in the treatment and prevention of hospital infections.



INTRODUCTION

The rise of healthcare-associated infections and their increasing resistance to current therapeutic strategies are major concerns of the modern society. This is problematic at global and cross-sectoral levels, i.e., affecting humans, agriculture, and the environment. It is acknowledged that the challenge faced is best resolved by a multidisciplinary approach as highlighted by the One Health approach to antimicrobial resistance, which connects the human health and resistance concerns with that of animals.[1] It is predicted that if the current threat of antimicrobial resistance is not resolved and innovative solutions are not provided, 10 million lives will be at risk by 2050.[2] A number of therapeutic alternatives to standard antimicrobials are being explored, some of which hold promise in the treatment and prevention of hospital infections, including the use of antimicrobial peptides.

Unlike most conventional antibiotics, antimicrobial peptides target multiple sites (e.g., cell membrane and intracellular processes) on bacterial cells rather than individual metabolic processes; hence, the likelihood of resistance developing is reduced.[3] Research has also focused on the use of antimicrobial peptides against biofilm phenotypes that demonstrate increased tolerance to antimicrobial strategies and provide optimal conditions for resistance to spread.[4] Antimicrobial peptides are able to interact with polysaccharide components of the biofilm architecture, resulting in breakdown of their polymeric matrix.[5] Their promising activity has allowed peptides to become marketed antimicrobials (e.g., daptomycin) with several in the advanced stages of clinical trials for mainly topical applications.[6] However, concerns remain relating to the cost of large-scale manufacturing, their potential for systemic delivery due to reports of toxicity, and limited stability of L-enantiomeric natural variants. To overcome these issues, many groups including our own are focusing on ultrashort peptides. These comprise up to seven amino acids and are thus increasingly attractive peptide therapeutics due to reduction in associated cost, ease of synthesis, and simple modifications allowing improved resistance to proteolysis (e.g., use of D-enantiomers).[7] Amino acids can also be rationally selected in line with a

Received: December 20, 2018
Accepted: January 18, 2019
Published: February 4, 2019

DOI: 10.1021/acsomega.8b03578
ACS Omega 2019, 4, 2584−2589

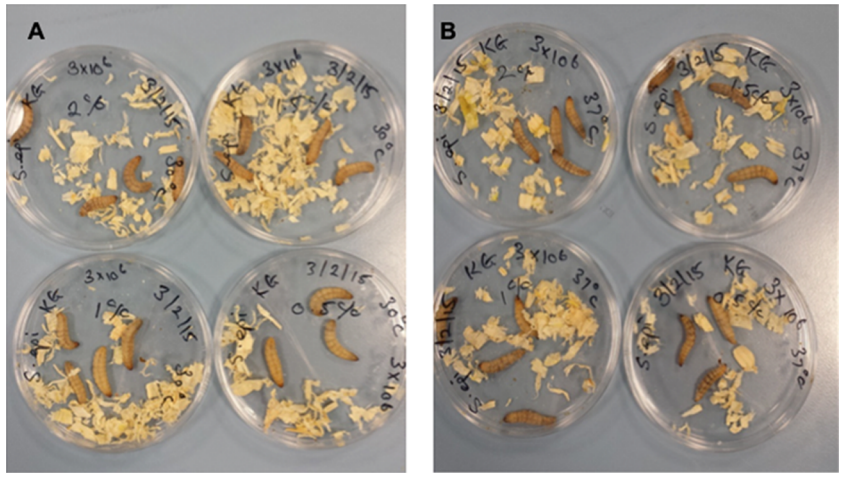


Figure 1. *G. mellonella* demonstrated full survival after injection with varying concentrations (0.5−2% w/v) of the NapFFKK-OH hydrogel: (A) 30 °C and (B) 37 °C, 120 h after administration.

minimum pharmacophore required to permit antimicrobial activity.[8]

Ultrashort peptides also have the ability to self-assemble to higher architectures, for example, hydrogels, in response to external, physiological stimuli (e.g., pH and specific enzymes). This provides the peptide with a range of desirable, multifunctional properties for biomedical use. Their potential applications include as localized, topical treatments for infected wounds owing to their antimicrobial activity and as controlled-release drug delivery systems. The chemical, physical, and structural properties that govern peptide self-assembly are closely associated with factors that determine the antimicrobial potency and spectrum of activity of synthetic antimicrobial peptides.[9] Previous work by our group created ultrashort self-assembling peptide hydrogels capable of killing biofilm forms of infection.[10] (Naphthalene-2-ly)-acetyl-diphenylalanine-dilysine-OH (NapFFKK-OH) was particularly promising, demonstrating broad-spectrum activity against a range of biofilms implicated in hospital-acquired infections. Although in vitro investigations provide a useful indication of preliminary activity, in vivo studies must be performed to facilitate translation of novel medical products to a clinical setting. This provides several challenges relating to ethics and cost.

Standard animal models include mainly vertebrae species, including rabbits, mice, guinea pigs, and monkeys.[11,12] Concerns exist relating to the use of such animals as a representation of human health and disease; however, there are limited viable alternatives.[13] There has recently been increased emphasis on the "Three Rs" (3Rs) concept and the necessity for novel or alternative methods to in vivo testing.[14] In vivo testing should be used in cases where it will provide beneficial advances to human or animal health or the environment. The 3Rs aim to reduce the adverse effects caused to animals, improve animal welfare, and enhance the quality of science in which animals are used so that: the minimum possible number of animals is used (reduction); they experience negligible pain or pain is avoided entirely (refinement); and, if possible, nonanimal models are used for experimentation (replacement). Refinement takes precedence over reduction and replacement in that a method that uses a greater number of animals but with milder harm is judged preferable to one that uses fewer animals but causes greater suffering to each individual animal.[15]

Alternative lower model organisms to traditional vertebrae models, such as the invertebrate *Galleria mellonella* (greater wax moth larvae), can serve as effective preliminary in vivo models and a possible alternative to species protected by Schedule 3 of the Animals (Scientific Procedures) Act 1986. Typically, there are fewer ethical concerns regarding their use within in vivo testing. *G. mellonella* serves as a useful tool of pathogenicity and infection for human bacterial and fungal species.[16] It is widely available, economically advantageous, small in size (15−25 mm), and possesses a short life cycle, meaning that it can be handled easily and injected with smaller quantities (20 μL) of test compounds. *G. mellonella* serves as an ideal model for clinical infections as it can be maintained at physiological temperatures (37 °C) for up to 5 days.[17−19]

A number of studies have been conducted using *Galleria* as infection models including those outlined in the review by Tsai and colleagues.[20] *G. mellonella*'s innate response to infections is structurally and functionally similar to that of mammals. Humoral responses trigger release of reactive oxygen species and antimicrobial peptides into the hemolymph (blood system). Hemolymph clotting, equivalent to mammalian blood clotting, occurs followed by melanization triggered by an upstream proteolytic cascade involving serine proteases. The cellular response results in encapsulation of the infecting pathogen and phagocytosis.[17,21,22] Previously conducted antimicrobial investigations have demonstrated *Galleria* and mammalian immune systems to share some important similarities, highlighting the promise of *Galleria* as an alternative model to determine antimicrobial efficacy and immune response.[23−25] Validation of antimicrobial efficacy and determination of pharmacokinetic and pharmacodynamic properties have also been investigated using *G. mellonella* models.[26,27]

In this study, we performed preliminary in vivo toxicity studies via inoculation of *G. mellonella* larvae with self-assembling antimicrobial peptide hydrogels (NapFFKK-OH: 0.5−2% w/v) and found that the peptide was less toxic than in vitro studies had indicated. We determined a suitable inoculum concentration threshold to represent in vivo bacterial infection and evaluated the ability of NapFFKK-OH to reduce infection in *G. mellonella*. A reduction in both Gram-positive and Gram-negative infections was observed over 24 and 72 h treatment windows.

DOI: 10.1021/acsomega.8b03578
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RESULTS

NapFFKK-OH In Vivo Toxicity. The NapFFKK-OH peptide hydrogel proved to be biocompatible at concentrations studied (0.5−2% w/v). No death and minimal melanization were observed for all treatment groups after 120 h (Figure 1).

Verifying Maximum Nonlethal Dose of Bacteria. *G. mellonella* larvae were treated with a range of inoculating doses (1×10^8−1×10^2 CFU/mL) and observed for death alongside phosphate-buffered saline (PBS) controls. The maximum nonlethal dose was determined and used as the inoculating dose for infection model studies, as displayed in Tables S1−S4, highlighted in yellow. Of the four pathogens investigated, *Staphylococcus epidermidis* proved to be the least lethal with 100% survival observed at concentrations as high as 1×10^7 CFU/mL bacteria. *Staphylococcus aureus* and *Escherichia coli* displayed moderate killing activity (1×10^5 CFU/mL). *Pseudomonas aeruginosa* proved to be the most lethal, with limited survival above concentrations of 1×10^2 CFU/mL.

Infection Models. Infection models were performed over the shorter time frame of 24 and 72 h to avoid cocooning of waxworms that began to be observed over longer incubation times at 37 °C. A range of reduction in bacterial numbers was observed for each pathogen between 2.5 and 4.5 $\log_{10}$ CFU/mL (Figures 2 and 3). For 24 and 72 h time points, the greatest reduction was seen for *S. aureus* 2% w/v NapFFKK-OH treated larvae with significant reduction observed at concentrations of ≥1% w/v NapFFKK-OH after 72 h.

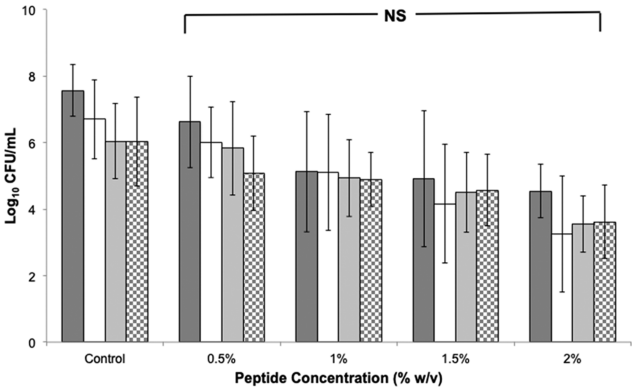

Figure 2. Bacterial viable counts ($\log_{10}$ CFU/mL) after 24 h treatment of infected *G. mellonella* larvae (n = 5) with NapFFKK-OH hydrogels (0.5−2% w/v). Key: dark gray, *S. epidermidis* ATCC 35984; white, *S. aureus* ATCC 6538; light gray, *E. coli* NCTC 11303; black and white checked, *P. aeruginosa* PAO1; and NS, no significant difference ($p \geq 0.05$) between negative untreated control and peptide treatment.

DISCUSSION

NapFFKK-OH hydrogel toxicity studies using in vivo models of *G. mellonella* expand on previously conducted in vitro studies by our group whereby a monolayer of murine fibroblast NCTC 929 cells was exposed to varying concentrations of solubilized and hydrogel forms of NapFFKK-OH. NapFFKK-OH was nontoxic in the solubilized micromolar range (≤500 μM: >80% cell metabolic activity),[28] according to the International Standard classification for biomaterial toxicity,[29] but less than 60% NCTC 929 cell metabolic activity was observed at concentrations of ≥1.5% w/v in the hydrogel form.[10] By comparison, the in vivo studies presented (Figure 1) indicate that in more complex cell environments, for example, at an organism level, toxicity is less

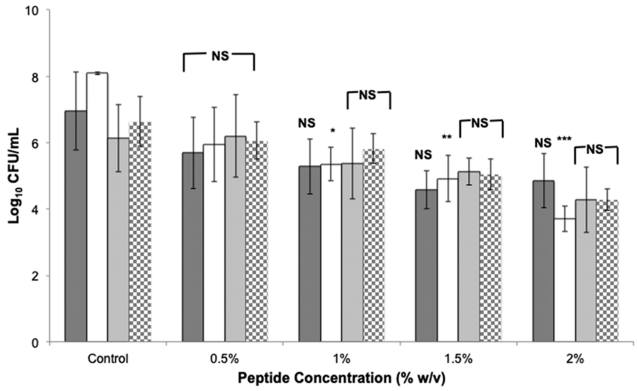

Figure 3. Bacterial viable counts ($\log_{10}$ CFU/mL) after 72 h treatment of infected *G. mellonella* larvae (n = 5) with NapFFKK-OH hydrogels (0.5−2% w/v). Key: dark gray, *S. epidermidis* ATCC 35984; white, *S. aureus* ATCC 6538; light gray, *E. coli* NCTC 11303; black and white checked, *P. aeruginosa* PAO1; NS, no significant difference ($p \geq 0.05$); *$p < 0.05$ significant difference; **$p < 0.01$ significant difference; ***$p < 0.001$ significant difference between negative untreated control and peptide treatment.

pronounced. There was no evidence of *Galleria* death (100% survival) following inoculation with test concentrations of NapFFKK-OH (0.5−2% w/v). Placebo inoculum (PBS controls) served as evidence that no death or injury resulted as part of the handling or injection process.[30] Inoculation of *Galleria* larvae via the proleg, directly into the hemolymph, reduced the likelihood of precipitating release of stress factors, which results in melanization. There was, however, evidence of melanization in most treatment groups by 120 h, which could also be attributable to incubation time and possible cocooning rather than stress factor release.[17]

Use of *Galleria* as an infection model was required to be validated to produce consistent, reproducible, and statistically relevant results.[31] The maximum nonlethal dose defined as one sufficient to kill a proportion of larvae during the validation time (120 h) was decided as the maximum concentration of bacteria at which there was at least 80% or greater survival.[18] A 10-fold serial dilution method of bacterial inoculum, similar to that of Olsen and colleagues, was adopted.[25] Survival data was linked to inoculum concentration (Tables S1−S4), whereas PBS controls ensured that viability was independent of injection and handling. Larvae death was confirmed by a change of color from golden to dark brown/black and lack of movement when manipulated with a pipette tip.[32] *S. epidermidis* proved the least fatal with survival even at concentrations as high as 1×10^7 CFU/mL (Table S2). Higher concentrations of *S. aureus* (1×10^8−1×10^6 CFU/mL) resulted in significant melanization (Table S1). To minimize the influence of innate larval self-defense mechanisms on survival and bacterial numbers, an inoculum of 1×10^5 CFU/mL was chosen for further NapFFKK-OH investigations. A similar assumption was adopted for *E. coli* samples (Table S3). *P. aeruginosa* proved particularly lethal as observed by other researchers. Inoculating doses employed previously by other papers include 1.4×10^3 CFU/mL,[33] and those as low as 1×10^2 CFU/mL have proven capable of inducing mortality.[34] However, within our investigation, a low dose of 1×10^2 CFU/mL provided sufficient viability within the 80% maximum nonlethal dose threshold to be used as the inoculating concentration for infection models (Table S4).

Infection models were performed using inoculating concentrations determined from the validation assays (highlighted in

DOI: 10.1021/acsomega.8b03578
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yellow in Tables S1−S4). At all treatment times (24 and 72 h), 100% survival of *Galleria* was observed. Reduction in bacterial numbers was concentration-dependent for the 24 h treatment group with 2% w/v NapFFKK-OH having the greatest antimicrobial activity against all pathogens; however, no results were statistically significant at the 24 h time point. The findings are biologically and clinically interesting, given that greater than 3 $\log_{10}$ CFU/mL bacterial reduction was obtained against *S. aureus* for 2% w/v NapFFKK-OH.[35,36] At least a 2 $\log_{10}$ CFU/mL reduction in bacterial numbers was observed for 2% w/v NapFFKK-OH against all pathogens after 72 h, the greatest and also most significant ($p < 0.001$) being 4.4 $\log_{10}$ CFU/mL against *S. aureus*. The authors recognize that again there is a lack of statistical significance against other pathogens at both 24 and 72 h time points. However, a reduction in bacterial load is demonstrated and the *G. mellonella* model serves as a useful tool in vivo.

The inoculating doses and techniques used in these investigations are similar to those conducted by other research groups. Gibreel and Upton,[22] for example, employed a higher dose of *S. aureus* (2.5×10^6 CFU/mL); however, the methods used reflect our own studies and determine peptide toxicity and larval viability over 120 h. Interestingly, they also determined in vivo stability and activity as concentration-dependent and determined that peptide degradation occurred at concentrations less than 50 mg/kg. Leuko and Raivio[37] crushed *G. mellonella* to determine bacterial counts, as opposed to hemolymph extraction employed in this work. Crushing was avoided in this work to prevent contamination of the test pathogen with commensal gut bacteria.[38] Prophylaxis with a standard antibiotic to reduce gut bacteria was not performed as only one formulation was investigated. In addition, prophylaxis may trigger upregulation of first-line inherent antimicrobial peptides; these may positively enhance the apparent antimicrobial activity of the test peptide.[17]

The antimicrobial mechanism of action of NapFFKK-OH is likely to follow that of other peptides whereby hydrophobicity and cationic charge enable the formation of ion channels in bacterial membranes and/or a surfactant-like action. Recent research has also shown a link between antimicrobial activity and molecular folding, structural conformation and assembly state.[39−41] Self-assembly has been increasingly linked to improved antimicrobial activity within peptides and proteins. β-amyloid, implicated as the main component of plaques linked to Alzheimer's and to which diphenyalanine (FF) forms a fundamental component of self-assembly, demonstrates significant antibacterial activity.[42,43] β-amyloid forms transmembranous pores in bacterial membranes, leading to membrane depolarization and cell death. Although this is a feasible mechanism for NapFFKK-OH, especially given the presence of the key β-amyloid motif FF, it is more likely that our hydrogel acts similarly to Schneider's β-hairpin peptide hydrogels (MAX, MARG, and PEP6R).[44−46] The combination of local cationic charge on the NpxFFKK-OH hydrogel surface and the porous network of cross-linked nanofibers allows increased interaction with anionic constituents of bacterial membranes, leading to membrane disruption and cell death.

CONCLUSIONS

In this study, we have tested the in vivo antimicrobial properties of an ultrashort antimicrobial peptide hydrogel NapFFKK-OH using a unique *G. mellonella* infection model and a range of healthcare-associated Gram-positive and Gram-negative pathogens. *G. mellonella* is gaining increasing interest as an alternative model for mammalian in vivo testing and is particularly relevant for the testing of novel antimicrobial platforms, such as our NapFFKK-OH hydrogel. There is increasing need for the validation of techniques employed when using these models to confirm the reliability of the data produced. Previous investigations have indicated that there may be variations depending on the batch and/or the supplier. In addition, there is not any reference population yet (size, gene function, sequence, or phenotype); thus, standardization is required.

METHODS

Peptide Synthesis. Fmoc- and Boc-protected amino acids, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), and Wang resin (mesh size 100−200, 0.65 mmol/g), were obtained from Novabiochem, Merck KGaA (Darmstadt, Germany). The NapFFKK-OH peptide (Figure S1) was synthesized following standard Fmoc solid phase synthesis protocols, as previously outlined.[10] Synthesis was performed manually on lysine-conjugated Wang resin with elongation achieved using 4 molar equivalents (meq) of HBTU, 8 meq of DIEA, 4 meq of HOBt, and 4 meq of Fmoc-protected amino acid or naphthalene acetic acid. Peptides were cleaved from the resin using 95% trifluoro acetic acid (TFA), 2.5% triisopropylsilane, and 2.5% thioanisole.

Peptide identity and purity were determined by electrospray ionization mass spectroscopy, ^{1}H NMR (Varian Unity Inova 400 spectrometer) using DMSO-d_6, and reverse-phase HPLC (Agilent 1260 Infinity system fitted with a Gemini C18 250 mm × 4.6 mm column; 100 − 80% gradient over 20 min of 0.05% TFA−water/acetonitrile and a flow rate of 1 mL/min). Hydrogels were formulated using 0.5 M HCl, 1 M NaOH, and sterile deionized water as previously demonstrated by our group.[28]

NapFFKK-OH In Vivo Toxicity. A method similar to that used by Gibreel and Upton was used to assess peptide hydrogel toxicity.[22] *G. mellonella* larvae (Livefoods Direct Ltd., Yorkshire, U.K.) were stored at 15 °C until required for use. They were not fed as it is recommended to starve the larvae for at least 24 h prior to use.[16] Five larvae were inoculated with 20 μL of each peptide hydrogel (0.5−2% w/v) injected into the hemolymph via the bottom left proleg using 29G insulin syringes (0.3 mL) with needles (Figure 4). Percentage survival was monitored over 120 h by recording live/dead counts at 24, 48, 72, and 120 h. Toxicity was determined on the basis of percentage survival, where 100% survival during the experiment time represented minimal toxicity for that peptide and concentration (0.5−2% w/v). Toxicity was measured at two different temperatures 30 and 37 °C to see whether this would have an impact on *G. mellonella* survival. Controls were provided in the form of untreated larvae, and these were injected separately with PBS and the comprising elements of the peptide hydrogel (deionized water, 1 M NaOH, and 0.5 M HCl) in the relevant volumes for each hydrogel concentration (Figure S2).[18,28]

Verifying Maximum Nonlethal Dose of Bacteria. Methods similar to those of Hornsey and Wareham were used to conduct validation and antimicrobial studies.[12] Overnight bacterial cultures of the test bacteria (*S. epidermidis* ATCC 35984, *S. aureus* ATCC 6538, *E. coli* NCTC 11303, and *P. aeruginosa* PAO1) were grown in Mueller-Hinton broth (MHB) at 37 °C and adjusted using PBS to 1×10^8 CFU/mL and serially

DOI: 10.1021/acsomega.8b03578
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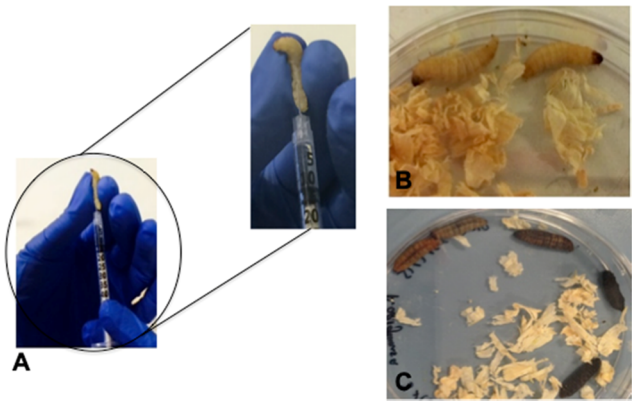

Figure 4. (A) Inoculation via the proleg using a 29G insulin syringe with needle. (B) Healthy larvae (golden larvae) following inoculation with NapFFKK-OH 2% w/v. (C) Evidence of varying degrees of melanization (brown larvae) and death (black larvae).

diluted. As per the toxicity study, sets of five larvae for each dilution (1×10^8–1×10^2) were inoculated the bottom left proleg with 20 μL of bacteria. Live/dead counts were recorded at 24, 48, 72, and 120 h and are presented in Tables S1–S4. We defined the maximum nonlethal dose as the one at which there was 80% or greater larvae survival.[18] Following determination, a suitable inoculum concentration was selected (the one at which there was >80% survival after 120 h) and prepared using PBS from an overnight culture (grown, as detailed, in MHB at 37 °C in a gyrorotary incubator). Larvae were inoculated with the maximum nonlethal dose of bacteria via the bottom left proleg. NapFFKK-OH was selected as the model antimicrobial hydrogel due to its broad-spectrum antibiofilm activity demonstrated previously by our group against in vitro biofilm models.[10]

Infection Models. Triplicate sets of five *Galleria* larvae were infected with 20 μL of the maximum nonlethal bacterial dose with antimicrobial treatment in the form of NapFFKK-OH administered via injection into the bottom right proleg 2 h after inoculation with the test microorganism, as utilized by Aperis and colleagues.[47] An equivalent volume of peptide hydrogel to bacterial inoculum was used (20 μL), and 0.5–2% w/v concentrations of peptide were used. The larvae were incubated at 37 °C for 24 or 72 h after which hemolymph was extracted. In each case, controls were provided in the form of larvae inoculated with bacteria only (maximum nonlethal dose) and larvae injected with PBS only. These represented untreated (negative control) and uninfected control larvae, respectively.

Hemolymph Extraction and Bacterial Enumeration. Following the appropriate incubation time (24 or 72 h), larvae were placed in a falcon tube on ice for 10 min to aid handling. Anesthetized larvae were placed in a sterile Petri dish, and a sterile scalpel was used to make an incision between the bottom two prolegs where inoculation had occurred. Hemolymph was collected from the five larvae per treatment group in a sterile 1.5 mL microcentrifuge tube. Hemolymph was serially diluted in PBS (10^{-1} – 10^{-6}). Miles and Misra bacterial viability counts were performed using MHA of dilutions 10^{-3} – 10^{-6} and incubated overnight at 37 °C. Hemolymph was diluted and plated within 10 min to prevent it turning brown and coagulating. The Miles and Misra plates were counted 24 h following incubation, and $\log_{10}$ CFU/mL bacterial reduction was determined.[47]

Statistical Analysis. Results were analyzed using GraphPad Prism, 6.0. Reduction in $\log_{10}$ CFU/mL bacterial numbers was analyzed following inoculation, and treatment with NapFFKK-OH included employment of one-way analysis of variance and Tukey's posthoc test to identify individual differences. This enabled comparisons to be made between changes in bacterial counts in the hemolymph of treated *Galleria* larvae compared to those of the positive untreated (maximum nonlethal dose) controls.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03578.

NapFFKK-OH chemical structure (Figure S1); validation of maximum nonlethal dose of bacteria data (Tables S1–S4); photos of untreated controls 24 and 120 h postinjection (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to acknowledge the contribution of Patrick Catterson, Catherine Cassidy, Jack McCallion, and Karl Gartland, who all helped to validate the *G. mellonella* assays for use with peptide hydrogels as part of their final year research projects.

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DOI: 10.1021/acsomega.8b03578
ACS Omega 2019, 4, 2584−2589