Multifunctional Ultrashort Peptide Hydrogels for Chronic Wound Healing

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MULTIFUNCTIONAL ULTRASHORT PEPTIDE HYDROGELS FOR CHRONIC WOUND HEALING

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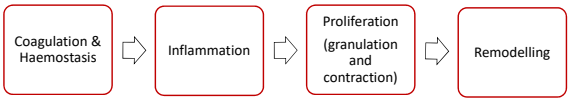
INTRODUCTION

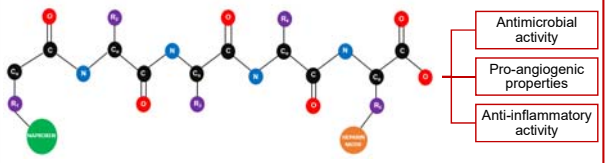
Wound healing is a complex and dynamic process consisting of four main steps:



In chronic wounds the wound remains in the proliferation stage with excess inflammation occurring and fails to progress to remodeling [1].

The use of peptides represents a novel approach to enhance wound healing. Inherent antimicrobial activity, increased biocompatibility and tunable biodegradability render peptides more suitable for application to the chronic wound environment than currently administered synthetic materials [2]. Uncontrolled local inflammation and the presence of infectious pathogens can result in the development of chronic non-healing wounds, leading to increased patient morbidity and the failure of standard therapies such as antibiotics [3].

The chemical versatility of the peptide motif enables three main qualities to be incorporated into a structure which is capable of self-assembly:



Self-assembly can occur in response to a number of different infective stimuli including pH, temperature and specific enzymes to enable targeted action at the required site. The incorporation of multifunctional properties overcome limitations with existing topical therapies, which fail to address a profile of increased inflammation and microbial load, with reduced angiogenesis that combine to prevent healing.

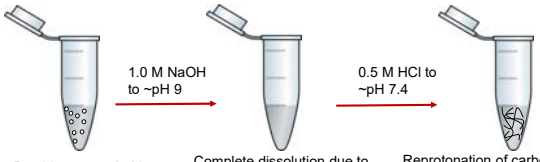
AIM

To develop a therapeutically active hydrogel network with the potential to be employed as a dressing, where the hydrogel is applied to the wound and conforms to its shape, whilst providing a moist environment and enabling gas exchange at the wound site.

METHODS

The peptide was synthesized according to standard Fmoc-based solid phase peptide synthesis. The structure was based on a diphenylalanine-dilysine sequence (FFKK), which was previously shown to gelate and display antimicrobial properties [4]. The non-steroidal anti-inflammatory drug naproxen was conjugated to the end of the amine terminal of the peptide sequence to confer the desired anti-inflammatory properties and a heparin motif was incorporated to provide pro-angiogenic qualities.

Peptide gelation ability at various concentrations was examined via pH modulation.



Hydrogels form at concentrations above the minimum gelation concentration (MGC) (% w/v). Vial inversion assays and Scanning Electron Microscopy were employed to examine gelation.

Bacterial susceptibility assays were performed against Gram-negative *Pseudomonas aeruginosa* (PA01) and Gram-positive *Staphylococcus aureus* (NCTC 10788) using the Miles and Misra drop count method.

Haemolytic activity was measured using equine erythrocytes and biocompatibility was determined via *in vitro* cell culture assays to determine cell viability using the CellTiter 96® AQueous One Solution Cell Proliferation Assay with NCTC clone 929 (ATCC CCL 1) murine fibroblast cells with 6, 24 and 72 hour incubation times.

RESULTS AND DISCUSSION

The self-assembly process is dependent on the amino acids incorporated in the primary sequence of the peptide structure and the formation of intermolecular interactions including Van der Waals interactions and π – π stacking. Vial inversion assays demonstrated the formation of peptide hydrogel at a concentration of 1.5 % w/v and above and the fibrous network was confirmed via SEM imaging with Figure 1 below showing the dense fibrous network at the highest concentration employed.

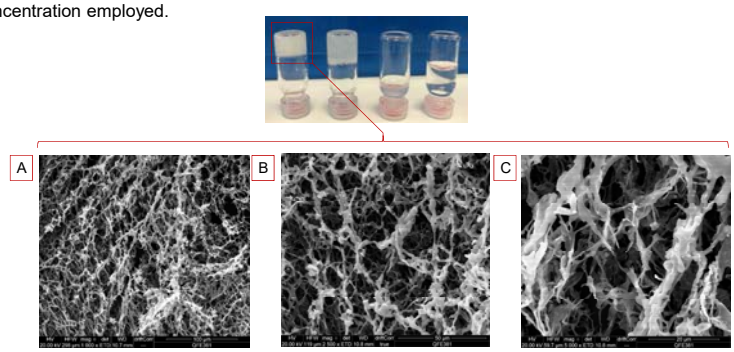


Figure 1. Scanning electron microscope images of freeze-dried, gold-sputtered samples of naproxen-conjugated peptide at a concentration of 2.0 % w/v at: [A] a magnification of x 1,000, [B] a magnification of x 2, 500 and [C] a magnification of x 5, 000.

Clinically significant Log_{10} reductions in the growth of Gram-negative *P. aeruginosa* and Gram-positive *S.aureus* was observed at higher concentrations employed, with significance denoted as at least a three Log_{10} reduction in viable counts since this is commonly employed to denote clinical significance [5]. *P. aeruginosa* and *S. aureus* are the most common ESKAPE pathogens isolated from chronic wounds and demonstrate increased resistance to topical antibiotics [6]. There is no necessity for a sterile environment for wound healing, however, a reduction in the bioburden can promote wound healing in the case of chronic wounds and this peptide demonstrates at least a three log reduction against each organism at a concentration of 1.5 and 2.0 % w/v.

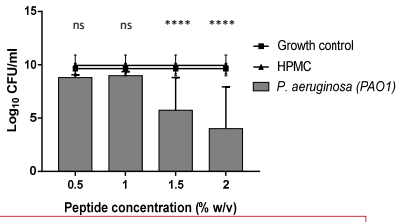


Figure 2. Logarithmic reduction in *P. aeruginosa* (PA01) viable count (Log_{10} CFU/mL) after 24 hour incubation with peptide (concentration 0.5 – 2.0 % w/v).
ns: no significant difference (P ≥ 0.05), ****: P < 0.0001 significant difference between Log_{10} CFU/mL of peptide and the growth control.

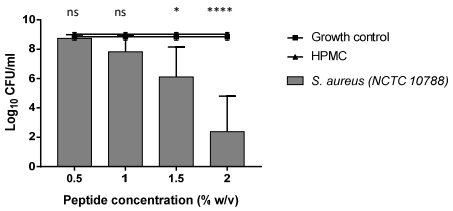


Figure 3. Logarithmic reduction in *S. aureus* (NCTC 10788) viable count (Log_{10} CFU/mL) after 24 hour incubation with peptide (concentration 0.5 – 2.0 % w/v).
ns: no significant difference (P ≥ 0.05), *: P < 0.01 and ****: P < 0.0001 significant difference between Log_{10} CFU/mL of peptide and the growth control.

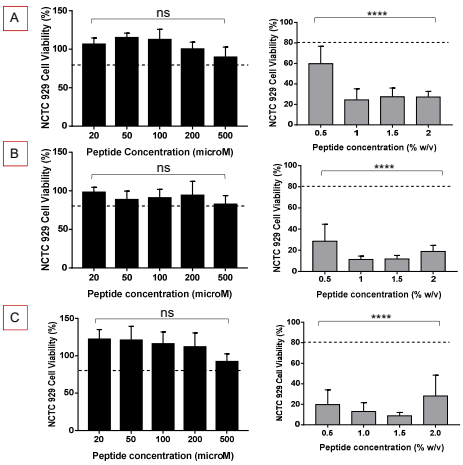


Figure 4. Percentage cytotoxicity of NCTC clone 929 (ATCC CCL 1) cells following a 6, 24 and 72 hour incubation time (A, B and C respectively) with varying concentrations of peptide. Black bars show micro molar concentrations and grey bars show % w/v concentrations. Dotted lines represent 80 % viability.
ns: no significant difference (P ≥ 0.05), ****: P < 0.0001 significant difference between peptide treated cells and the negative control (untreated cells).

The MTS assay was performed to assess the viability of cells after incubation with varying concentrations of the peptide as an initial assessment of biocompatibility. The micro molar concentrations employed had no significant effect on the cell viability whilst the higher concentrations (% w/v) showed a reduction in cell viability. The micro molar concentrations are more likely to be reflective of the concentrations of peptide which cells would be exposed to since the peptide will slowly diffuse from the gel matrix and into the surrounding environment to exert its effect. Further studies will be necessary to assess the biocompatibility of the peptide.

The peptide was shown to be relatively non-haemolytic at the lower concentrations employed, with the haemolysis observed at higher concentrations likely to be attributed to issues with tonicity or osmolarity due to osmotic variations in the test media resulting in over-estimation of the toxicity.

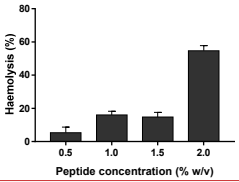


Figure 5. Percentage haemolysis of equine erythrocytes after one hour exposure to varying concentrations of peptide (0.5 – 2.0 % w/v).

CONCLUSIONS

Multifunctional ultrashort peptide hydrogels demonstrate potential as wound healing products. Peptide hydrogel dressing products may resolve issues in the case of chronic wounds which fail to heal, for example, diabetic ulcers. Future work will involve assessment of the mechanical properties of the gel structure via oscillatory rheology and wound healing properties using the *in vitro* wound scratch assay to assess cell migration using the human dermal endothelium cell line HMEC-1 and the human keratinocyte cell line HaCaT [7]. In addition to this it will be possible to modify the peptide sequence to tailor the properties to the exact desired requirements via modification of the primary sequence and incorporation of other non-steroidal anti-inflammatory drugs.

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ACKNOWLEDGEMENTS
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