Collagen Tethering of Synthetic Human Antimicrobial Peptides Cathelicidin LL37 and its Effects on Antimicrobial Activity and Cytotoxicity

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ABSTRACT

Wound infections, particularly of chronic wounds, pose a substantial challenge for designing antimicrobial dressings that are both effective against pathogens, and do not interfere with wound healing. Due to their broad-spectrum antimicrobial and immunomodulatory activities, naturally-occurring antimicrobial peptides (AMPs) are promising alternative treatments. However, their cytotoxicity at high concentrations and poor stability hinders their clinical use. To mitigate these undesirable properties, we investigated the effects of tethering human AMP cathelicidin LL37 to collagen, one of the main extracellular matrix proteins in wound sites, secreted by fibroblasts, and in commercially-available wound dressings. The active domain of human AMP cathelicidin, LL37, and two chimeric peptides containing LL37 fused to collagen binding domains (derived from collagenase – *c*CBD-LL37 or fibronectin - *f*CBD-LL37) were synthesized and adsorbed to PURACOL[®] type I collagen scaffolds. After 14 days, 73%, 81% and 99% of LL37, *c*CBD-LL37 and *f*CBD-LL37, respectively, was retained on the scaffolds and demonstrated undiminished antimicrobial activity when challenged with both Gram-positive and Gram-negative bacterial strains. Loaded scaffolds were not cytotoxic to fibroblasts despite retaining peptides at concentrations 24 times higher than the reported cytotoxic concentrations in solution. These findings indicate that biopolymer-tethered AMPs may represent a viable alternative for preventing and treating wound infection while also supporting tissue repair.

KEYWORDS

Antimicrobial peptides; LL37; collagen; collagen-binding domain; tether; wound healing

1. INTRODUCTION

Wound infections, particularly of chronic wounds, pose a substantial challenge to patient health and quality of life, and cost billions of dollars each year to manage in the United States alone [1, 2]. Current approaches to combatting wound infections are insufficient and expensive due to bacterial resistance to conventional antibiotics [2-4]. Alternatively, silver- and chemical-based compounds can prevent and treat infections, but are toxic to mammalian cells and may impede tissue regeneration [4, 5]. Thus, there is a critical need for alternative approaches to wound care and infection control that eliminate pathogenic microbes and support functional tissue repair.

Antimicrobial peptides (AMPs) have demonstrated promise as a class of broad-spectrum antimicrobial agents that may also promote wound healing. AMPs are short, naturally-occurring peptides that act by the general mechanism of membrane disruption, allowing antimicrobial activity against a wide variety of microbes, including antibiotic-resistant organisms [6, 7]. Further, AMPs are important components of host innate immunity, serving additional *in vivo* functions such as modulation of the inflammatory response [8], lipopolysaccharide (LPS) binding and neutralization [9], and chemotaxis [6, 7]. With over 2,600 natural AMPs discovered to date and thousands more being synthesized [10], this technology could provide a platform for the development of new antimicrobial agents and address the critical problem of antimicrobial resistance. Current AMP-based therapies have been limited to topical applications, despite their high potential and promise as alternative systemic antimicrobials [11].

Several challenges have limited the use of AMPs in the clinic to date, primarily their toxicity to mammalian cells at high concentrations, *in vivo* instability, and their high cost of production [6, 7, 11-14]. Low stability of AMPs *in vivo* leads to higher required dosages, which not only

increases costs but also lowers their therapeutic index [14]. In some cases, slow release or covalent tethering of AMPs to various substrates such as resins [15, 16], titanium [17], silicon dioxide [18-20], gold [21, 22], polymer brushes [23], polystyrene [24-26], contact lenses [27] or synthetic polymer multilayers [28, 29] have been studied. However, few have focused on tethering or release from biopolymer scaffold materials [30]. Targeted delivery of AMPs using collagen scaffolds, including tethering to wound dressing materials, may improve AMP technology for broader clinical application.

Collagen is a component of the extracellular matrix (ECM) and a prevalent biopolymer in both regenerating tissues and commercially available wound dressings. Several previous studies have described the collagen-tethering of growth factors and other proteins via various collagen-binding domains (CBDs), short sequences derived from molecules such as fibromodulin [31-33], von Willebrand's factor [34, 35], collagenase [35, 36] and fibronectin [37, 38]. However, tethering and interactions of antimicrobial peptides with biopolymer materials such as collagen remains predominantly unexplored.

We chose LL37, the 37 amino acid active antimicrobial peptide domain of human cathelicidin, as the model AMP for collagen tethering. Cathelicidins are a class of AMPs typically produced by neutrophils and consist of a precursor region, a highly conserved cathelin-like domain and the broadly-active, species-variable C-terminus domain [39]. The precursor molecule to human LL37, hCAP-18 [40], is cleaved extracellularly by serine proteinase-3 to yield the active C-terminus domain, LL37 [41]. Human cathelicidin has been widely studied and has demonstrated potent antimicrobial and healing activities [39, 40, 42-44], although its tethering and release from ECM scaffolds has only been studied very recently [30].

The overall goal of the study was to generate and evaluate a new strategy for delivering AMPs via collagen-based scaffolds. For this, we designed two modified chimeric LL37 peptides with collagen binding domains (CBDs). Each has differing charges, amino acid compositions, lengths, and dissociation constants (K_D) make them interesting models to study LL37 binding ability, structure, and antimicrobial activity as a function of binding domain sequence and structure. We hypothesized that the modification of LL37 with a CBD will enable its tethering to collagen substrates while reducing its cytotoxicity and preserving its antimicrobial activity. Further, the addition of a CBD would provide improved retention of LL37 to the collagen scaffold, allowing for prolonged local bioactivities. To test this, we studied adsorption, antimicrobial activity and cytotoxicity of modified (cCBD-LL37 and fCBD-LL37) and unmodified LL37 while in solution and when applied to commercially-available 3D collagen scaffolds. These studies allowed us to observe how biopolymer tethering altered AMP bioactivity, compare the use of two different CBDs to tether LL37, and provide a foundation for future study of biopolymer-tethered AMPs for wound healing applications.

MATERIALS and METHODS

2.1. Synthetic Peptides

Synthetic LL37 (LLGDDFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES) was purchased from Anaspec, Inc. (Fremont, CA) at >95% purity, confirmed by high-performance liquid chromatography (HPLC). The modified LL37 peptides with a collagenase-derived CBD (cCBD-LL37) (LLGDDFRKSKEKIGKEFKRIVQRIKDFLRNLPRTESDYKDDDDKTKKTLRT) and fibronectin-derived CBD (fCBD-LL37)

(LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTESDYKDDDDKCQDSERTFY) were synthesized by New England Peptide, Inc. (Gardner, MA) at >90% purity, also confirmed by HPLC. A FLAG™ domain (DYKDDDDK) linker sequence was included between the LL37 and CBD sequences in each peptide to preserve flexibility [12, 15, 18, 19], and as an epitope tag [45]. The peptides were received at greater than 90% purity confirmed by high performance liquid chromatography. Stock solutions (0.67 mM) of each AMP were prepared according to manufacturer recommendations (LL37 in sterile PBS (pH 7.2) and cCBD- and fCBD-LL37 in sterile water (pH 3.5), each supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA)) and stored at -20°C.

2.2. Bacterial Strains and Culturing

Escherichia coli (B78) [46] was originally isolated from a woman with cystitis, provided by Dr. James Johnson (VA Medical Center, Minneapolis, MN), and *Bacillus subtilis* (R01792) was provided by Robert Stote (NSRDEC, Natick, MA). *E. coli* (ATCC 33694), *Staphylococcus aureus* (ATCC 48366), *Staphylococcus epidermidis* (ATCC 49461), Methicillin-resistant *S. aureus* (ATCC 33592), Gentamycin- and Methicillin-resistant *S. aureus* (ATCC 43300), and *Pseudomonas aeruginosa* (ATCC 29260) were purchased from the American Type Culture Collection, ATCC (Manassas, VA). Bacteria were cultured in 9.8 g/L Mueller Hinton broth (MHB; SigmaAldrich, St. Louis, MO). For all experiments, fresh cultures were prepared by choosing colonies on a Mueller Hinton agar (MHA; SigmaAldrich) stock plate (4-6 hours at 37°C). Cell turbidity was corrected to a McFarland Standard 0.5 to make 1×10^8 colony-forming units per mL (CFU/mL) and then diluted 100-fold in MHB to make 1×10^6 CFU/mL prior to inoculation.

2.3. Minimum Inhibitory Concentration (MIC) Assays

The MICs of each peptide (LL37, *c*CBD-LL37, *f*CBD-LL37) were determined using published methods [47], based on standards set by the Clinical and Laboratory Standards Institute [48] and the European Committee for the Antimicrobial Susceptibility Testing [49]. In short, two-fold serial dilutions were tested for each sample in MHB. Each well, including a no-peptide growth control (GC) was inoculated with 1×10^6 CFU/mL fresh inoculum prepared as described in **section 2.2**. A sterility control of MHB only was used as a negative control. After inoculation, plates were incubated at 37°C and absorbance at 590 nm, OD(590), was recorded using a Victor3 plate reader (Perkin Elmer, Waltham, MA) 12-16 hours after inoculation. GCs were diluted and plated on MHA plates and counted after overnight incubation to ensure that the original inoculum size was 1×10^6 CFU/mL. The MIC was determined as the concentration of peptide at which OD(590) was the same as the sterility control value, or where 100% visible growth was inhibited [47].

2.4. AlamarBlue[®] Cell Viability Assay

AlamarBlue[®] (Thermofisher, Waltham, MA) was used to determine the viability of CRL-2352 dermal fibroblasts (ATCC) or CT1005 dermal fibroblasts (tissue distribution program, University of Massachusetts Medical School, Worcester, MA) after exposure to the AMPs in solution. The linear ranges of signal and cell seeding densities for AlamarBlue[®] reduction were determined by creating a standard curve. Cells were cultured in medium (1:1 Dulbecco's modified eagle medium and Ham's F-12 media supplemented with 10% fetal bovine serum) in 96-well plates seeded at $12,500 \text{ cells/cm}^2$ for 12-16 hours. Medium was replaced with fresh medium supplemented with AMPs (1, 3, 5, 10, 15 and 20 μM) at pH 7.4 for 4 hours. AlamarBlue[®] solution (10% v/v) was then added to each well and incubated for another 4 hours. Plates were incubated at 37°C and 5% CO_2 . The fluorescent signal from AlamarBlue[®]

(excitation and emission at 540 nm and 590 nm) after 4 hours were read on a Victor3 plate reader (Perkin Elmer). According to the manufacturer, the AlamarBlue[®] signal directly correlates to the number of viable cells. Medium was refreshed until the second addition of AlamarBlue[®] at 24 hours.

2.5. Preparation, Handling and Loading of 3D Collagen Scaffolds

PURACOL[®] collagen type I dressings (Medline Industries, Mansfield, MA) were cut into 5 mm diameter disks using biopsy punches (Robbins Instruments, Chatham, NJ) under sterile conditions. Prior to additional testing, all scaffolds were sterilized with ethylene oxide. Each scaffold was loaded with peptides or sterile phosphate buffered saline (SigmaAldrich; PBS pH 7.4) as a negative control. Scaffolds were loaded by adsorbing 5 μ L of LL37 (1478 μ g/mL), cCBD-LL37 (2078 μ g/mL) or fCBD-LL37 (2178 μ g/mL) diluted with PBS, pH 7.4. This volume was selected because it is the minimum volume required to hydrate a 5 mm diameter scaffold. The peptide concentration was chosen as the maximum mass required to fully saturate each PURACOL[®] disk. This theoretical mass was calculated assuming a 1:1 ratio of collagenase binding sites to collagen triple helices in each disk composed of 100% native collagen type I, a mass of 280 kDa per triple helix, and approximately 10×10^{14} collagenase binding sites per helix [36, 50-52]. Scaffolds were hydrated with the peptide or PBS control solution on hydrophobic polydimethylsiloxane (PDMS) mats, placed in a 37°C incubator for 2 hours, and rinsed twice with either MHB, release medium, or mammalian cell medium and studied for antimicrobial activity (section 2.7), cumulative release over 14 days (section 2.8), or cytotoxicity (section 2.10), respectively.

2.6. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) images were collected of PURACOL[®] scaffold disks to visualize scaffold structure. Dry scaffolds were sputtered with an 8 nm coating of Pt/Pd (Cressington Scientific Instruments, Watford, England, UK). SEM images were taken at the University of Massachusetts Medical School Electron Microscopy Core (Worcester, MA) with an FEI Quanta 200 FEG MKII (FEI, Hillsboro, OR) SEM. Images were acquired at 300x magnification, 10 mm working distance, and 10kV under high vacuum condition.

2.7. Antimicrobial Activity of Peptide-Loaded 3D Scaffolds

To test the antimicrobial activity of peptides applied to 3D collagen scaffolds, a bacterial inoculation and growth assay based on medium turbidity was adapted from a prior study [53]. Under sterile conditions, scaffolds were loaded with peptides as described in **section 2.5**, placed in a 96-well plate and rinsed twice with MHB. Then, scaffolds were transferred with sterile forceps into fresh MHB. Each well was inoculated with 1×10^6 CFU/mL bacteria prepared as described in **section 2.2**. A growth control, with no scaffolds or peptide, was also inoculated and sterile MHB only was used as a negative sterility control. The inoculated scaffolds and control samples were incubated 1 hour at 37°C.

After 1 hour, bacterial solution was aspirated and scaffolds were rinsed twice with sterile MHB. Samples were then transferred into new wells with fresh MHB and incubated for 12 hours at 37°C. After 12 hours, scaffolds were removed from the wells and solution turbidity (indicating bacterial growth) in the form of OD(590) was recorded using a Victor3 plate reader (Perkin Elmer). All values were normalized to the average sterility control values; turbidities from peptide-loaded samples were compared with untreated, PBS-loaded samples. Antimicrobial activity was determined by the significant reduction of turbidity of peptide-loaded versus unloaded scaffolds.

2.8. Peptide Adsorption and Release

After loading and incubating the scaffolds with peptide or PBS control solutions, scaffolds were rinsed twice with release medium (sterile PBS pH 7.4 supplemented with 1% wt/v bovine serum albumin (BSA)) and rotated on a Labquake Tube Rotator (Thermofisher) at 37°C. At specific time points (1 hour, 4 hours, 1 day, 4 days, 7 days and 14 days), release medium was collected from each tube and replaced with fresh medium under sterile conditions. This time period of 14 days provides a peptide release period that matches the majority of the duration of the two critical phases of wound healing – inflammatory and proliferative – where LL37 may have the most impact on infection prevention and wound healing [54]. ELISA (section 2.9) was used to measure peptide concentrations in the collected aliquots, as well as in the initial scaffold rinses to determine cumulative release and initial peptide adsorption, respectively. Scaffold replicates from day 14 of the release study were also used for the antimicrobial assay (section 2.7) versus *E. coli* followed by IHC (section 2.11) and additional replicates were collected for IHC to visualize LL37 on the scaffolds without bacterial exposure.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

A direct ELISA for determining AMP release was designed and validated. Aliquots from the release study were placed into ELISA plates (BrandTech Scientific, Essex, CT) and incubated 12 hours at 4°C. Wells were then rinsed with Tween-20 in PBS (0.05% v/v), twice, followed by PBS pH 7.4, twice. All subsequent “rinses” were carried out the same way. BSA in PBS (1% wt/v), was added for 2 hours at 37°C, followed by rinsing. Then, 0.4 µg/mL, primary rabbit polyclonal anti(human)-LL37 antibody (Anaspec; cat. no. AS-55927) was added for 1 hour at 37°C. After another rinse, 0.3 µg/mL horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, CA; cat. no. 1706515) was added for 1 hour at 25°C. Another rinse

was followed by the addition of TMB-ELISA developer (ThermoFisher). The reaction was stopped at 30 min with 2M H₂SO₄, and absorbance at 450 nm, OD(450), was recorded using a Victor3 spectrophotometer (Perkin Elmer).

To analyze these data, the OD(450) for each sample was compared to standard curves, created by constructing OD(450) versus concentration curves of serial dilutions for each peptide, LL37, *c*CBD-LL37 and *f*CBD-LL37. The concentration of peptide in each aliquot was calculated using these standard curves, total protein released at each time point was calculated, and the cumulative release profiles were constructed.

2.10. Cell Viability on Peptide-Loaded 3D Scaffolds

AlamarBlue[®] was also used to measure the cytotoxicity of peptide-loaded scaffolds (prepared as in section 2.5) when seeded with human dermal fibroblasts, cultured as described in section 2.4. Non-tissue treated, low binding Corning 96-well plates (Corning, NY) were used. The chosen cell density to seed over the scaffolds, as determined by a standard curve, was 114,000 cells/cm², or 80,000 cells per scaffold. Loaded scaffolds were rinsed twice with fresh medium, seeded with cells and incubated at 37°C and 5% CO₂. All wells were rinsed with fresh medium at 4 hours to wash away non-adhered cells. After 24 hours, wells were rinsed twice with medium and refreshed with 10% v/v AlamarBlue[®] in medium. The fluorescent signal was read as described in section 2.4. A group of unloaded scaffolds without cells, wells with cells only, and medium only were included as negative controls.

2.11. Immunohistochemistry (IHC) and Staining

Immunohistochemistry (IHC) using anti(human)-LL37 was performed on sections of scaffolds harvested at different release times (described in section 2.9) both exposed to *E. coli* for 12 hours and unexposed to bacteria. Scaffold replicates collected immediately after rinsing

and after 14 days of release were exposed to bacteria and stained. Scaffold replicates at 1 hour, 1 day, 4 days, 7 day and 14 days of release were stained without exposure to bacteria.

All IHC images were taken with a Leica Upright Fluorescence Microscope, DMLB2 (Wetzlar, Germany). Scaffolds were transferred into histology cassettes, fixed in 10% v/v formaldehyde in for 24 hours, processed (ATP1 tissue processor, Triangle Biomedical Sciences, Durham, NC) and embedded in paraffin. Scaffolds were cut into 5 μ m sections, de-paraffinized in xylene, hydrated in a series of 100% to 70% ethanol, and left in water for 5 minutes.

Samples were then probed for the presence of peptide using anti(human)-LL37 primary antibody (Anaspec; cat. no. AS-55927), by first boiling under pressure with an antigen retrieval solution (SigmaAldrich) followed by blocking with 5% v/v goat serum in PBS for 30 min at 25°C. Samples were incubated overnight at 7°C with 10 μ g/mL primary rabbit polyclonal anti(human)-LL37 (Anaspec) in 3% v/v goat serum. Then, AlexaFluor[®]488 goat-anti-rabbit antibody (LifeTechnologies, Carlsbad, CA; cat. no. A11008), diluted 1:50 in 3% v/v goat serum was added for 1 hour at 25°C prior to dehydration, xylene clearance, and adding coverslips.

IHC was also performed on peptide-loaded scaffolds collected 24 hours after fibroblast seeding (described in section 2.10). Scaffolds were first stained as described above with anti(human)-LL37 and then counterstained with Hoechst 33342 (LifeTechnologies; diluted 1:6,000 in water) for 6 minutes to stain nuclei.

2.12. Statistical Analyses

For MIC and cytotoxicity assays in solution and scaffold cytotoxicity assays, all experiments were performed in triplicate with n=3 replicates per sample group. For MIC, statistics were performed to compare *c*CBD-LL37 and *f*CBD-LL37 to LL37 and for viability assays, statistics were performed to compare peptide-treated samples versus untreated controls;

all were performed using one-way ANOVA and *post-hoc* Tukey Tests. For all experiments with antimicrobial activity applied to scaffolds, n=4 replicates per sample group were measured in triplicate for *S. aureus* and *P. aeruginosa* and in quadruplicate for *E. coli*; statistical significance of peptide-treated scaffolds versus PBS-treated scaffolds negative controls was determined using one-way ANOVA and a *post-hoc* Dunnett Test. For cumulative release studies, experiments were performed in duplicate for n=3 replicates per sample group; statistical significance of the release between two time points for each peptide was determined using a one-way repeated measures ANOVA and *post-hoc* Tukey Test.

3. RESULTS

3.1. Chimeric CBD-LL37 design

The active human AMP LL37 [44] was functionalized on its C-terminus with a CBD, and separated by a FLAG linker (DYKDDDDK) [45]. The peptides (**Fig. 1a**) were named *c*CBD-LL37 (for collagenase-derived CBD) and *f*CBD-LL37 (for fibronectin-derived CBD). The *c*CBD [35] and *f*CBD [37] domains were chosen for their abilities to bind to collagen and for their short, unique sequences (**Fig. 1b**).

3.2. Peptides retain broad-spectrum antimicrobial activity after CBD modifications

The minimum inhibitory concentrations, or the lowest concentrations of agent that reduce visible growth of bacterial strains, were determined against several Gram-negative and Gram-positive bacterial species (**Table 1**; $\mu\text{g/mL}$ values are also provided in **Table S1**). The MICs of LL37 and *c*CBD-LL37 were within an order of magnitude of the reported values for LL37 [42]. *c*CBD-LL37 showed similar antimicrobial activity to LL37, and was slightly more effective against Gram-negative organisms *E. coli* (both laboratory and clinical isolates) and *P.*

aeruginosa compared to LL37. However, the mean MIC values for *c*CBD-LL37 compared to LL37 against Gram-negative bacteria were not significantly different (**Table 1**). *c*CBD-LL37 also showed equal potency to LL37 against the biofilm-forming *S. epidermidis* strain at a very low concentration of 0.53 μ M.

In general, *f*CBD-LL37 demonstrated weaker activity than *c*CBD-LL37 and LL37, particularly against *E. coli*, *P. aeruginosa*, *S. aureus*, *MRSA* and *B. subtilis*, suggesting that adding the *f*CBD sequence to LL37 decreased its antimicrobial activity. Interestingly, *f*CBD-LL37 demonstrated increased activity against multiple antibiotic-resistant *MRSA* and *M/GRSA* strains at 5.83 μ M versus 8.33 and 7.50 μ M for *c*CBD-LL37 and LL37, respectively. MIC values for *f*CBD-LL37 did not vary significantly against any Gram-positive organisms and were consistently between 5 and 10 μ M. In contrast, *c*CBD-LL37 and LL37 demonstrated similar changes in MIC against Gram-positive compared to Gram-negative strains. Overall, all MICs were lower than 10 μ M, which suggests that adding CBDs had a minimal effect on the broad-spectrum antimicrobial activity of LL37 in solution.

3.3. *f*CBD-LL37 is less cytotoxic in solution than *c*CBD-LL37 and LL37

At 8 and 24 hours, LL37 and *c*CBD-LL37 cytotoxicity correlated well with the reported cytotoxic range [44] (**Fig. 2a-b**), with significantly reduced relative AlamarBlue[®] signals, directly correlated to the numbers of viable fibroblasts at peptide concentrations above 10 μ M. *f*CBD-LL37 followed a similar profile at low concentrations but had significantly attenuated cytotoxicity compared to LL37 and *c*CBD-LL37 at concentrations up to 20 μ M for both 8 and 24 hours. By 24 hours (**Fig. 2b**), there was no statistically significant difference between the *c*CBD-LL37 or LL37 samples and the medium-only control ($p < 0.05$) at the highest concentration (20 μ M), which indicates that no viable cells remained. Similar cytotoxicity profiles were observed

with AMP treatment for 8 hours in a primary human dermal fibroblast line CT1005, derived from the dermis of a woman undergoing panniculectomy (**Fig. S1**).

3.4. All peptides bind to PURACOL[®] and are active against Gram-negative bacteria

PURACOL[®] was selected as the wound dressing to study CBD-LL37 delivery from collagen scaffolds, for its 100% native bovine collagen type I composition and ease of handling as 5 mm disks (**Fig. 3a**). SEM images (**Fig. 3b**) demonstrated high porosity of PURACOL[®], consistent with its reported porosity of approximately 80% of scaffold volume [55], which is important for promoting cell adhesion and wound healing [56].

LL37-, *c*CBD-LL37- and *f*CBD-LL37-loaded scaffolds significantly reduced *E. coli* burden after 12 hours ($p < 0.001$ for LL37 and *c*CBD-LL37; $p < 0.05$ for *f*CBD-LL37) compared with PBS-loaded scaffolds (**Fig. 3c**). This suggests that all peptides retained antimicrobial activity when adsorbed to collagen. Peptide retention on collagen scaffolds was confirmed in all treatment groups, even after exposure to *E. coli* for 12 hours, by IHC staining for LL37 (**Fig. 3d**).

The activity of *c*CBD-LL37 and *f*CBD-LL37 peptides against *P. aeruginosa*, a Gram-negative pathogen commonly found in wounds, was similar to levels demonstrated against *E. coli*, with *c*CBD-LL37 demonstrating the highest overall activity against *P. aeruginosa* (**Fig. 3c**). LL37 exhibited higher mean relative turbidity against *P. aeruginosa* than CBD-modified LL37 peptides, suggesting lower activity. In contrast, neither CBD-LL37 peptide showed significant antimicrobial activity against Gram-positive *S. aureus*, although LL37 exhibited antimicrobial activity against *S. aureus* compared with PBS-loaded control scaffolds ($p < 0.05$). These data suggest that collagen-tethered LL37 peptides are more effective against Gram-negative than Gram-positive organisms, and that CBD-LL37 peptide antimicrobial activity may have a higher sensitivity to differences in bacterial membrane structure.

3.5. Modification with CBD improves peptide retention on collagen scaffolds

We hypothesized that unmodified LL37 may be non-specifically adsorbed onto collagen and quickly released into the surrounding media, while CBD-LL37 peptides would have a slower release profile over time facilitated by specific binding of CBD to collagen type I [36, 57]. To test this hypothesis, we measured peptide release from collagen scaffolds and calculated the cumulative release profiles for each peptide (**Fig. 4a**). Overall, the total mean cumulative release of *c*CBD-LL37 and LL37 after 14 days was 18.5% and 26.9%, respectively. However, by 1 hour, 12.9% of the adsorbed *c*CBD-LL37 was already released. Though LL37 release was only 8.36% after 1 hour, its release was at a faster rate between subsequent time points, to 14.8% at 4 hours and 19.9% at 1 day, respectively. *c*CBD-LL37 release appeared comparatively slower at these later time points. Interestingly, significantly greater ($p < 0.05$) amounts (micrograms) of LL37 were released between each time interval compared to *c*CBD-LL37 and *f*CBD-LL37. Cumulative *f*CBD-LL37 release from collagen scaffolds was significantly lower than LL37 and *c*CBD-LL37, with only 0.91% of the loaded peptide released over the course of 14 days. The concentration of *f*CBD-LL37 detected in the release medium was significantly different ($p < 0.001$) compared to release medium-only negative control samples for each time point. Immunostaining of peptide-adsorbed scaffold samples harvested at day 14 supports the observed retention of all peptides during the release assay (**Fig. 4b**). Scaffolds harvested at different time points during the release study (without performing an initial rinse) were stained in the same way and also suggest that peptides are retained on the scaffold (**Fig. S2**).

ELISA analysis was also performed on the solution collected from preceding scaffold rinses to determine the percentage of peptide initially rinsed off the scaffold. Rinsing accounted for $2.83\% \pm 0.296\%$, $5.35\% \pm 0.305\%$ and $0.023\% \pm 0.008\%$ LL37, *c*CBD-LL37 and *f*CBD-

LL37 of the initial peptide adsorbed onto the scaffolds, respectively (mean of three rinses with $n > 3$ replicates per group $\pm$ S.E). Thus, for all peptides, greater than 94% ($> 310 \mu\text{M}$) of the initial peptide is adsorbed to the collagen scaffolds. After 14 days, 73%, 81%, and 99% of LL37, *c*CBD-LL37 and *f*CBD-LL37, or approximately 240, 270, and 325 μM , respectively are retained within the scaffold. This suggests that by using CBDs, LL37 can be initially retained at concentrations 24 times higher than its cytotoxic threshold *and* released in increments at least 10x lower than its cytotoxic threshold between 1 hour and 14 days.

3.6. CBD-LL37 peptides have improved retention compared to unmodified LL37

The primary rationale for modifying LL37 with CBDs is to improve retention of the peptide over time on collagen-based scaffolds, thus improving its longer-term antimicrobial activity while reducing the total amount of peptide required to treat wound infections. Thus, after observing a high retention of all peptides after 14 days in release medium (**Fig. 4a-b; Fig. S2**), we tested 14-day release scaffold activity against *E. coli* (**Fig. 5a**) and then probed for LL37 to assess peptide retention (**Fig. 5b**). After 14 days, both CBD-LL37 peptides were retained on collagen scaffolds and exhibited similar levels of antimicrobial activity as samples tested after 12 hours in release medium. In contrast, LL37-loaded collagen scaffolds had lower antimicrobial activity at 14 days compared to 12 hours. IHC results showed lower LL37 staining on collagen scaffolds, whereas staining remained strong on collagen scaffolds treated with either *c*CBD-LL37 or *f*CBD-LL37 (**Fig. 5b**). The reduction in LL37 staining is consistent with the observed loss of antimicrobial activity on LL37-treated collagen scaffolds after 14 days. Together, these data indicate that CBD modification facilitates retention of both binding and activity of LL37 on collagen scaffolds.

3.7. Collagen-bound peptides are non-cytotoxic, even at high concentrations

An additional rationale for tethering AMPs to surfaces using covalent and non-covalent methods is to increase peptide stability and retain antimicrobial activity, while decreasing cytotoxicity [12]. Thus, we loaded peptides onto collagen scaffolds and assessed the viability of fibroblasts seeded on the scaffolds after 24 hours. CRL-2352 cell viability was not statistically different on peptide-loaded scaffolds compared to PBS-loaded scaffold controls (**Fig. 6**). These data suggest that the same concentrations of AMPs applied to collagen scaffolds that killed bacteria (**Fig. 3c**) were not cytotoxic to mammalian fibroblasts. To verify fibroblast seeding and verify peptide adsorption on collagen scaffolds, sections were immunostained for LL37 and counter-stained with Hoechst to visualize nuclei. In all scaffold sample groups (peptide-adsorbed and unloaded controls), nuclei were observed along the edges of the scaffold, as well as within the scaffold material 24 hours after seeding. These data demonstrate the presence of cells on the scaffolds with or without peptide adsorption (**Fig. S3**).

4. DISCUSSION

4.1. Considerations for AMP tethering

Tethering AMPs such as LL37 to collagen could enable clinical application of broad-spectrum AMPs to treat wound infections without cytotoxicity. Tethering or controlled release approaches may mitigate AMP cytotoxicity while retaining antimicrobial activity, and may serve to increase *in vivo* stability. While covalent tethering via flexible tether molecules could increase stability, a lack understanding of how tethering affects peptide structure and mechanisms could limit antimicrobial activity of these systems [12, 23]. For example, short tether lengths may not allow proper peptide structure and orientation to kill bacteria, but different lengths and properties are required for different peptides that utilize different mechanisms of action [17, 18, 26, 30].

Tethering may also cause alterations to AMP local charge and surface density [15, 18, 23, 26, 30, 58], aggregation behavior and distribution [23], flexibility [17, 19], orientation [17, 20, 24, 25], conformation and secondary structure [20, 22, 23] which may all play roles in altering mechanisms required for antimicrobial activity [12, 15, 18, 20]. The complexity of these systems also limits their clinical use, requiring optimization of tether properties such as length [18], surface density [15, 23], and grafting method [30]. Some grafting methods may be cytotoxic [30] or involve highly specific, complex, or costly synthesis [21]. These systems often have unclear clinical endpoints for regulatory pathways (e.g. FDA clearance) leading to trial failure [13]. At the fundamental level, there is a lack of consistent understanding of tethered AMP structure-function relationships, leading to varying success of AMPs in the clinic [21, 23]. Although several approaches have been developed for covalent and non-covalent tethering of AMPs to many different substrates, this is the first report of using a CBD to tether a chimeric AMP to an extracellular matrix biopolymer.

More recently, Cassin *et. al.*, investigated LL37 incorporation into collagen and hyaluronic acid biopolymer polyelectrolyte multilayers (PEMs) via physisorption and covalent immobilization, and characterized LL37 release, antimicrobial activity, and cytotoxicity [30]. All PEMs effectively reduced *E. coli* adhesion, but the exposure of mammalian cells to chemically-immobilized LL37 resulted in morphological changes, which may indicate some degree of cytotoxicity [30]. Activity against other bacteria types was not investigated. This highlights one of the biggest challenges in the field of tethered AMPs today, choosing a tethering scheme that will allow the best balance of AMP properties to kill a broad spectrum of bacteria without demonstrating toxicity. By investigating a new non-covalent collagen tethering strategy using

CBDs, we hope to provide additional insights to the use of tethering to reduce the cytotoxicity of peptides delivered from biopolymers.

4.2. Effects of CBD addition on LL37 antimicrobial activity and cytotoxicity in solution

LL37 is a short peptide that must adopt an alpha-helical conformation for its antimicrobial activity [59]. Thus, it is crucial for CBD-LL37 function that CBD modification does not interfere with the LL37 domain's physical interaction with bacteria [15, 16, 18]. Peptide sequence and structure has been shown to influence AMP flexibility [17, 19], secondary structure [20, 22], orientation [17, 20, 22, 24, 25], and charge density [18, 58]. Previous work on CBD-mediated collagen tethering has involved either high molecular weight, uncharged or globular molecules such as growth factors [34, 35, 60] and streptavidin [37], or peptides too small to adopt significant structure on their own, such as fibronectin-derived RGD peptides [61, 62]. In these cases, CBD acted like an anchor without influencing peptide activities.

We found that *c*CBD-LL37 exhibited similar antimicrobial activity and cytotoxicity as unmodified LL37, which suggests that the *c*CBD modification does not adversely affect AMP function. In certain cases, we observed a trend toward enhanced antimicrobial activity with *c*CBD-LL37 against Gram-negative microbes compared to LL37, although most of these differences were not statistically significant. However, this is consistent with reported observations of enhanced bioactivity of *c*CBD-modified, collagen-tethered proteins, which was attributed to the high content of charged and polar amino acids in the *c*CBD sequence [63, 64]. Indeed, its amphiphilicity and added charges (net +3) could extend the LL37 helix [17] and promote electrostatic interactions [63, 64] with negatively-charged bacterial membranes of various thicknesses.

In contrast, *f*CBD caused reductions in LL37 antimicrobial activity and cytotoxicity compared to LL37 and *c*CBD-LL37. MIC values for *f*CBD-LL37 were consistent across Gram-negative and Gram-positive organisms, suggesting that *f*CBD-LL37 is relatively insensitive to bacterial type. Its lower cytotoxicity could be related to decreased overall charge of LL37 caused by addition of *f*CBD (net -1), or structural alterations caused by aggregation of *f*CBD moieties [61, 65]. Despite the observed reduction in antimicrobial activity, the minimum therapeutic index of *f*CBD is still 2x ($> 20 \mu\text{M}$ cytotoxicity / $10 \mu\text{M}$ MIC).

4.3. CBD-mediated peptide binding to collagen scaffolds

CBDs may increase the stability and retention of AMPs on collagen, by limiting peptide exposure to proteases [12, 15, 17-19, 66] and providing high affinity [50], specific binding onto collagen [50, 61, 67]. This increased stability is highly sought-after currently in both soluble and tethered AMP technologies [6, 12, 15, 66]. We tested both *c*CBD and *f*CBD sequences and hypothesized that they may differentially affect LL37 tethering and antimicrobial activity due to their variable structures and binding affinities. *c*CBD induces conformational changes in collagen type I in order to accommodate the small collagenase catalytic site ($5\text{\AA}$ binding site vs. $3000\text{\AA}$ length and $15\text{\AA}$ diameter of collagen) [67]. *f*CBD is less specific and has been shown to bind to collagen types I, III and V [37, 61, 62] as well as gelatin [68]. *f*CBD has demonstrated disulfide bond formation, oxidation sensitivity [65] and aggregation leading to dimerization [65, 69], and also undergoes conformational changes upon binding to collagen [62], implying that its AMP-tethering may also result in interesting structural alterations.

Orientation of peptide binding and collagen scaffold structure are important design considerations for CBD-AMP-based antimicrobial collagen scaffolds. Surface-tethered and surface-adsorbed peptides have demonstrated conflicting results and variable success as

antimicrobial agents [12, 15, 16, 18, 19, 58], but in all cases, the peptide structure when bound was important for its function [17-20, 24]. There are no reports of how (or if) CBDs affect AMP antimicrobial activity when tethered to collagen. However, it has been shown that *c*CBD [35, 60, 70] and *f*CBD [37, 61] both improve collagen binding and retention of other molecules, leading to maintained or enhanced [63, 64] *in vitro* or *in vivo* bioactivity.

4.4. Antimicrobial activity of collagen scaffolds loaded with CBD- and unmodified AMPs

All of the LL37 peptides tested, CBD-modified or unmodified, adsorbed to PURACOL[®] collagen scaffolds, both initially and after 14 days in release medium. CBD-mediated tethering may increase peptide stability [15], reflected not only by greater retention of both *c*CBD-LL37 and *f*CBD-LL37 on collagen scaffolds after 14 days and exposure to bacteria, but also by retention of scaffold antimicrobial activity compared to LL37-treated scaffolds. This non-covalent retention correlates well with the reported release profiles of physically-adsorbed and chemically-immobilized unmodified LL37 from ECM materials and falls between the two reported profiles at 96 hours [30]. Further, the negatively-charged *f*CBD domain may cause *f*CBD-LL37 aggregation on collagen [61, 65]. Since aggregation is the first step in the reported antimicrobial mechanism of LL37 [44, 59, 71], this could have contributed to *f*CBD-LL37's stable antimicrobial action over the full 14 day period tested. The formation of intramolecular disulfide bonds by *f*CBD [68] could also play a role in altering the release profile for *f*CBD-LL37 compared to LL37 or *c*CBD-LL37.

Reduction in LL37 antimicrobial activity after 14 days *in vitro* could have been the result of LL37 degradation, which has been reported to be relatively rapid [6]. Alternatively, it is possible that the negative charge of the *E. coli* membranes could have caused LL37 to change its conformation [59, 72, 73] and desorb from the surface [18].

LL37 is believed to act against bacterial membranes via the carpet model, which supposes that peptide molecules aggregate on the membrane surfaces until reaching a critical peptide-to-lipid ratio, followed by intercalation within the membrane, pore formation, and lipid removal in the form of peptide-lipid aggregates [40, 44, 59, 71-73]. In solution, LL37 is globular until it adopts a helical structure in a lipophilic membrane environment [59, 72]. Thus, AMP adsorbed to collagen scaffolds may need to partially or fully desorb and interact with bacterial membranes to mediate effective killing, which has been observed for certain surface-adsorbed peptide systems [17-19, 74]. Alternatively, we have demonstrated previously that tethered AMPs are still able to adopt non-native mechanisms of action (such as membrane charge destabilization) if unable to act through their primary mechanism of action [18].

In general, CBD-LL37 peptides displayed varying activities against different microbes when bound to collagen; for example, improved antimicrobial activity against Gram-negative *P. aeruginosa* but decreased antimicrobial activity against *S. aureus*. Fully permeating the thick peptidoglycan layer of Gram-positive organisms may require different structural considerations, such as CBD and peptide sequence composition [16], or linker sequence lengths [18].

4.5. Collagen-bound AMP reduction in cytotoxicity

We hypothesized, based on findings for covalently-bound peptides [15], that collagen-tethering would reduce peptide cytotoxicity. In our study, all peptide-loaded collagen scaffolds demonstrated biocompatibility based on fibroblast viability 24 hours after cell seeding, confirming this hypothesis and supporting the results of Cassin *et. al*, who observed cytotoxicity of chemically-immobilized LL37 at only 16 μ M and suspected it was due to the immobilization strategy itself [30]. Unexpectedly, physical adsorption alone is enough to also reduce the cytotoxicity of LL37 that is retained on the surface.

4.6. Implications for wound healing

In addition to its antimicrobial properties, LL37 plays a role in wound healing (at concentrations between 0.1 and 5 μM) due to its synergy with other components at the wound site such as beta defensin 2 [11]. Preservation and survival of cells at the wound site may be important for the outcomes of the healing process and these activities of antimicrobial peptides, both in general and when tethered to collagen, need to be investigated further.

Dosing optimization will need to be performed for each CBD-AMP prior to *in vivo* studies. Only a single peptide loading concentration was tested in this study. Although CBD-LL37-loaded collagen scaffolds were effective against Gram-negative bacteria at this concentration, higher doses may be required for activity against Gram-positive microbes. Clinical utility of AMP-loaded wound dressings will depend on *in vivo* efficacy, as well as cost effectiveness. In ongoing studies, we are evaluating recombinant production of CBD-AMPs to reduce production costs and enable additional modifications to peptide sequences, including alternative CBD and ECM tethering domains, alternative AMP domains, and variable linker domain sequences and lengths. Recombinant protein production may be a promising approach for reducing peptide manufacturing costs in future studies [6, 75]. We initially chose the FLAG sequence between LL37 and CBD domains to serve as both a linker [17] and an epitope tag [45] that would provide the possibility of FLAG-based purification of CBD-AMPs. The addition of a linker sequence is important to maintain the flexibility required for membrane interactions of tethered peptides [15, 17, 19], and should be explored in future studies of CBD-AMP design and activity.

Evaluation of the roles of alternative AMP, CBD, and linker sequences, as well as tethering to alternative ECM molecules prevalent in scaffold materials and healing wounds, will

allow us to develop a versatile platform technology for treating infection while optimally stimulating tissue regeneration.

5. CONCLUSIONS

Herein, we demonstrated CBD-tethered LL37 binding to a collagen-based wound dressing, showed retention of antimicrobial activity (up to 14 days), and showed that scaffolds were non-cytotoxic despite adsorbing AMPs at concentrations 24 times higher than their reported cytotoxic threshold. We show that when interacting with collagen, LL37 has a higher therapeutic index. Further, CBD addition and tethering to collagen modulate LL37 retention, release, antimicrobial activity and cytotoxicity. CBDs also allow retention and activity against Gram-negative *E. coli* for 14 days, indicating that CBD mediates higher collagen binding affinity. Importantly, none of the peptides (with or without CBD) demonstrated cytotoxicity against fibroblasts despite high loading concentrations, showing promise for collagen-tethering as a strategy to deliver effective, non-toxic doses of AMPs to kill bacteria and promote wound healing.

Results from this study are important for not only demonstrating proof-of-concept of AMP binding to biopolymers and retaining their efficacy, but also making rational choices of AMP and CBD structures to allow effective and specific binding onto biopolymers without cytotoxicity. Collagen-tethering of AMPs without the harmful side effects of current approaches (e.g., bacterial resistance, general toxicity, scarring) and utilizing their wound healing functionality have important implications for the future of tethered AMPs. This includes elucidating structure-activity relationships and dosage concentrations to allow application-tailored design of biopolymer-tethered AMPs, investigating novel production routes and delivery

strategies, and exploring *in vivo* wound healing and antimicrobial abilities of biopolymer-tethered AMPs.

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FIGURE CAPTIONS

Figure 1: Design of chimeric CBD-LL37. The LL37 antimicrobial peptide sequence at the N-terminus, followed by a flexible FLAGTM linker domain, and collagen binding domain (CBD) on the C-terminus (a); Sequence and properties of CBDs (b); K_D – dissociation constant.

Figure 2: Antimicrobial peptide cytotoxicity. Relative fluorescent units ($\times 10^3$), correlate directly with the number of viable CRL-2352 human fibroblasts, given by AlamarBlue[®] reduction when exposed to LL37, cCBD-LL37 and fCBD-LL37 peptides in solution after 8 hours (a) and 24 hours (b). All values represent means $\pm$ S.D. from at least three experimental replicates. The dotted line represents the average medium-only fluorescence; the vertical line represents the reported cytotoxic threshold of soluble LL37 [11]. Difference between the means: *fCBD-LL37 is different from all other AMPs, $p < 0.001$; **all samples are different from one another, $p < 0.05$.

Figure 3: Binding and activity of peptides on PURACOL[®] scaffolds. (a) PURACOL[®] scaffolds cut using a 5 mm biopsy punch; (b) SEM image of dry and sterilized scaffold disks taken at 10kV; bar = 200 μ m; (c) Relative turbidity after loaded scaffold exposure to Gram negative *E. coli*, Gram positive *S. aureus*, or Gram negative *P. aeruginosa* for 12 hours. All values represent means $\pm$ S.E. from at least three experimental replicates. Statistical significance of peptide-loaded samples against PBS-loaded samples is indicated by: * $p < 0.01$, ** $p < 0.001$ or *** $p < 0.007$; Statistical significance between peptide-loaded samples is indicated by ⁺ $p < 0.05$; (d) representative immunohistochemistry images of scaffold sections stained using anti-human LL37 and visualized with AlexaFluor[®]488-conjugated secondary antibody after exposure to *E. coli* for 12 hours; bar = 100 μ m.

Figure 4: The cumulative release profiles for LL37, *c*CBD-LL37 and *f*CBD-LL37, compared to a PBS-loaded negative control (depicted as the percent of total amount of loaded peptide) as a function of time. All values represent mean $\pm$ S.D. from two experimental replicates, n=3 scaffolds per treatment group. Statistically significant differences at each time point were found between *all peptides, $p<0.05$ or ***f*CBD-LL37 and both LL37 and *c*CBD-LL37, $p<0.001$. *f*CBD-LL37 release was significantly higher than the untreated, PBS-only control at all time points, $p<0.001$; (b) representative immunohistochemistry images of scaffold sections harvested at 14 days, stained using anti-human(LL37) antibody and visualized with AlexaFluor[®]488-conjugated secondary antibody; bar = 100 μ m.

Figure 5: Antimicrobial activity of peptide-loaded scaffolds over 14 days. Relative turbidity after scaffold exposure to *E. coli* for 12 hours (black) and 14 days (white) (a). All values represent means $\pm$ S.E. from at least four replicates. Statistically significant difference between means of peptide-loaded scaffolds and PBS-loaded scaffolds is indicated by: * $p<0.01$, ** $p<0.001$, and of *c*CBD-LL37 versus LL37 at 14 days ⁺ $p<0.05$; (b) representative immunohistochemistry images of scaffold sections, harvested after 14 days of release, followed by 12 hour exposure to *E. coli* and stained using anti-human(LL37) antibody and visualized with AlexaFluor[®]488-conjugated secondary antibody; bar = 100 μ m.

Figure 6: Cytotoxicity of antimicrobial peptides tethered to collagen scaffolds. Relative fluorescent units ($\times 10^3$), correlated directly with the number of viable CRL-2352 human fibroblasts, given by AlamarBlue[®] reduction after 24 hours. All values represent mean $\pm$ S.E. from at least three experimental replicates. Statistically significant difference between PBS-, LL37-, *c*CBD-LL37- and *f*CBD-LL37-loaded scaffolds and medium-only, cell-only and no cells, PBS-only scaffold controls, * $p<0.001$.

TABLES

Table 1: Minimum inhibitory concentrations of LL37, *c*CBD-LL37 and *f*CBD-LL37 against different bacteria strains.

Microbe		Published MIC for LL37 (μ M) [42]	LL37 (μ M)	<i>c</i> CBD-LL37 (μ M)	<i>f</i> CBD-LL37 (μ M)
Gram-negative	<i>E. coli</i> HB101	0.42	0.77 ± 0.12	0.67 ± 0.20	$2.92 \pm 1.10^*$
	<i>E. coli</i> B78	--	1.67 ± 0.42	1.15 ± 0.10	6.46 ± 3.03
	<i>P. aeruginosa</i>	1.27	0.69 ± 0.07	0.49 ± 0.06	$9.17 \pm 1.67^*$
Gram-positive	<i>S. epidermidis</i>	0.56	0.50 ± 0.04	0.53 ± 0.22	$10.0 \pm 2.50^*$
	<i>S. aureus</i>	0.80	0.45 ± 0.05	0.64 ± 0.27	$6.67 \pm 0.83^*$
	Methicillin-resistant <i>S. aureus</i> (MRSA)	0.76	0.68 ± 0.07	$8.13 \pm 0.68^*$	$5.83 \pm 0.83^*$
	Methicillin-, Gentamycin-resistant <i>S. aureus</i> (M/GRSA)	2.34	7.50 ± 0.00	8.33 ± 0.83	5.83 ± 0.83
	<i>B. subtilis</i>	0.60	0.53 ± 0.09	2.25 ± 0.49	$5.83 \pm 1.10^{**}$

Mean is significantly different from mean for LL37; * $p < 0.001$ or ** $p < 0.05$ ($n=3$).

STATEMENT of SIGNIFICANCE

Over 6.5 million people annually in the United States suffer chronic wounds; many will become infected with antibiotic-resistant bacteria. Treatments used to prevent and fight infection are toxic and may hinder wound healing. To overcome treatments gaps, we functionalized collagen scaffolds with chimeric antimicrobial peptides (AMPs) with collagen binding domains to create antimicrobial and non-cytotoxic scaffolds that may promote healing. AMPs are broad-spectrum antimicrobials that also promote healing; however, their instability and toxicity are major challenges. This is the first report of CBD-mediated, delivery of AMPs onto collagen scaffolds that demonstrates no cytotoxicity toward fibroblasts. This study also suggests that retention of antimicrobial activity is CBD-dependent, which provides foundations for fundamental studies of CBD-AMP mechanisms and clinical explorations.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6