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Multidomain Peptide Hydrogel Accelerates Healing of Full-Thickness Wounds in Diabetic Mice

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Abstract

In vivo, multidomain peptide (MDP) hydrogels undergo rapid cell infiltration and elicit a mild inflammatory response which promotes angiogenesis. Over time, the nanofibers are degraded and a natural collagen-based extracellular matrix is produced remodeling the artificial material into natural tissue. These properties make MDPs particularly well suited for applications in regeneration. In this work, we test the regenerative potential of MDP hydrogels in a diabetic wound healing model. When applied to full-thickness dermal wounds in genetically diabetic mice, the MDP hydrogel resulted in significantly accelerated wound healing compared to a clinically used hydrogel, as well as a control buffer. Treatment with the MDP hydrogel resulted in wound closure in 14 days, formation of thick granulation tissue including dense vascularization, innervation, and hair follicle regeneration. This suggests the MDP hydrogel could be an attractive choice for treatment of wounds in diabetic patients.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

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Characterization of the peptide, live-dead cell culture and additional in vivo histology, and quantification from histology (PDF)

Author Contributions

N.C.C. contributed to the conceptual design, experimental execution, interpretation of results, and writing of the manuscript. A.N.M. contributed to conceptual design, experimental execution, and interpretation of results. T.L.L.S. contributed in interpretation of results and creation of figures. D.G.L. contributed to experimental execution. I.L. contributed to experimental execution. D.R.W. contributed to experimental execution. J.D.H. contributed to the conceptual design and writing of the manuscript and supervised the entire project.

The authors declare the following competing financial interest(s): J.D.H. has shares in NangioTx, which aims to commercialize some aspects of the work presented.

Keywords

peptide; hydrogel; self-assembly; diabetic ulcer; wound healing

INTRODUCTION

Diabetes Mellitus is one of the most common metabolic disorders in the world, representing a significant health problem and economic issue.¹ Approximately 15% of diabetic patients develop chronic wounds in their lower extremities due to complicating symptoms, such as neuropathy, ischemia, retinopathy, and cardiovascular disease.¹ Wounds in diabetic patients have relatively longer healing periods than normal wounds.² Specifically, diabetic wounds exhibit delayed wound closure, prolonged inflammation, poor vascularization, cellular infiltration, and granulation tissue formation.² The gold standard of treatment for diabetic patients with chronic wounds consists of debridement of the wound, infection control, offloading of weight, and patient education.³ Despite these efforts, chronic wounds frequently lead to amputation, an outcome that strongly motivates the development of new treatments. Successful wound healing requires a highly organized interplay of numerous resident and recruited cell types, growth factors, and cytokines. Clinical strategies for tissue regeneration in diabetic patients include topical application of important growth factors;^{4,5} however, there are significant drawbacks to this approach. For example, growth factors administered to a patient rapidly diffuse away from the site of interest resulting in low local concentrations and often necessitating multiple dosages.⁶ Not only is this expensive, but multiple dosages can lead to undesired side effects from drug diffusion into healthy tissues.⁷ One approach to address these issues is to load cells, growth factors, or other biomolecules into scaffold materials to control release kinetics and achieve treatment localization.^{5,8} The difficulty here is the complex interplay between delivery material, one or more diffusing soluble agents, and loaded cells. The wound healing process consists of a delicate sequential cascade of cell types and growth factors² and deviation from the proper sequence can adversely affect the biological efficacy of wound healing. A more attractive solution would be to create a single component hydrogel to initiate a healthy healing response without the aforementioned complexity.

There has been considerable focus on the use of hydrogels as scaffolds for tissue engineering applications because of their widely tunable mechanical properties, surface characteristics, biocompatibility, and rate of degradation.^{9–19} Hydrogels have been explored for modern wound dressings due to their high-water content, which provides a moist environment ideal for granulation tissue and epithelium formation.^{18–23} Herein, we describe multidomain

peptides (MDPs), which are self-assembling peptides that form a nanofibrous hydrogel containing 99% water.

MDPs self-assemble to form a nanofibrous hydrogel when the peptide sequence contains a core of alternating hydrophilic and hydrophobic amino acids flanked by charged amino acids (Figure 1A). This primary sequence design results in the formation of nanofibers with a bilayer of β -sheets (Figure 1B). The addition of multivalent salts, such as those found in common buffers, like PBS or HBSS, allows nanofiber elongation and cross-linking to form a viscoelastic hydrogel.²⁴ The hydrogel is thixotropic and syringe deliverable which was previously demonstrated by oscillatory rheometry^{25,26}(Figure 1C).

MDPs have shown significant promise for tissue engineering applications due to their customizable chemical design and desirable biocompatibility. MDP hydrogels are made simply from amino acids, have predictable degradation in vivo, and can be easily delivered by a syringe.^{26,27} It is possible to tailor both the chemical^{25,28,29} and biological properties^{30–34} of the material for a given application by substituting the amino acids of the MDP. With compositional and structural similarity to the native extracellular matrix (Figure 1C), MDP hydrogels are readily infiltrated by cells, vascularized, and innervated.^{26,27,33–37} Because of these excellent biomaterial properties, in this report, we have investigated the effect of the MDP hydrogel with sequence $K_2(SL)_6K_2$ on the healing of full-thickness dermal wounds in genetically diabetic mice (Figure 1D). As described in detail below, we find wound healing is substantially accelerated with the use of this MDP hydrogel compared to a clinically used hydrogel, IntraSite, or a buffer-only control. The work presented herein shows the MDP hydrogel supports cells present in the wound healing process and thereby accelerates the healing of diabetic wounds by increasing granulation tissue formation, re-epithelialization, vascularization, and innervation.

MATERIALS AND METHODS

Peptide Synthesis

$K_2(SL)_6K_2$ was synthesized using a Focus XC Automated Peptide Synthesizer via solid phase peptide synthesis on a low loading Rink Amide MBHA resin with an Fmoc protection strategy as previously published.²⁴ Briefly, amino acids were used in a 4:1 molar ratio with HATU and DiEA as coupling agents. HATU was used in a 4:1 molar ratio and DiEA was used in a 6:1 molar ratio with DMF as the solvent. Deprotection of Fmoc groups was performed using 25% piperidine in a 50/50 mixture of DMF and DMSO. The N-terminus was acetylated and the resulting peptide was cleaved from the resin using TFA and a cleavage cocktail of ethanedithiol, water, triisopropylsilane, and anisole in a 36:1:1:1:1 volume ratio. The cleavage cocktail was removed using a rotovap, and the peptide was triturated using cold diethyl ether and collected by centrifugation. The peptide was dried overnight, dissolved in Milli-Q water, and pH adjusted to yield a neutral solution. MALDI-TOF mass spectrometry was used to verify successful synthesis (Figure S1). The peptide was dialyzed against deionized water using dialysis tubing with a MWCO of 100–500 Da. Following dialysis, the peptide solution was pH adjusted to neutrality and passed through a 0.2 μ m filter. This solution was lyophilized to yield a sterile peptide powder. The resulting peptide powder was dissolved in sterile 298 mM sucrose to yield a concentration of 20

mg/mL followed by 1:1 dilution with 1× Hanks Balanced Salt Solution (HBSS) to yield a hydrogel with final concentration of 1 wt % peptide, 149 mM sucrose, and 0.5× HBSS.

Cell Culture

NIH-3T3 fibroblasts were cultured in 75 cm² tissue culture treated flasks in an incubator at 37 °C with 5% CO₂. Flasks were seeded with 300,000 cells and allowed to grow to 80–90% confluency in DMEM complete media (DMEM media supplemented with 5000 U/mL penicillin-streptomycin, fetal bovine serum, 100 mM sodium pyruvate and 200 mM L-glutamine). To passage, cells were incubated with Trypsin-EDTA. Trypsin-EDTA was neutralized with twice the amount of media. Cells were pelleted via centrifugation and supernatant was removed. Cells were resuspended in 1 mL of DMEM complete media. Ten microliters of cell suspension was added to 190 µL of Trypan blue to count the number of cells using a hemocytometer. A new tissue culture treated flask was seeded with 300 000 cells.

3D Cell Culture in Hydrogels

Cells were passaged as stated above once 80–90% confluency was reached; however, cells were suspended with 1 mL of HBSS instead of 1 mL of DMEM complete media. Cells were counted using a hemocytometer and brought to a final concentration of 1.0×10^6 cells/mL in HBSS. Twenty milligrams of peptide was dissolved in 1 mL of 298 mM sucrose to make a 2 wt % peptide solution. One milliliter of the 1.0×10^6 cells/mL in HBSS was added to the 2 wt % peptide solution to create a 1 wt % hydrogel with 500,000 cells/mL encapsulated. Seventy microliters of the hydrogel was pipetted into wells of a 16-well breakable slide with well surface area of 0.4 cm² resulting in a cylindrical gel with height of approximately 1.75 mm. Gels were allowed to set for 30 min before 200 µL of DMEM complete media was added on top. Cells were cultured within the hydrogels in an incubator at 37 °C with 5% CO₂. Media was replaced daily.

Staining and Imaging of 3D Encapsulated Cells

At the specified time point, media was removed from the gels. Cells were fixed in 10% buffered formalin overnight. The formalin was removed and replaced with 0.5% Triton X in HBSS for 10 min to permeabilize cell walls, followed by 100 mM glycine in HBSS for 10 min. The glycine solution was replaced with 1% bovine serum albumin (BSA) in HBSS and allowed to sit for 30 min to block nonspecific staining. The cell cytoskeleton was then stained with Alexa488-Phalloidin solution for 30 min followed by staining of the cell nuclei with ProLong Gold DAPI solution for 20 min. The stained hydrogels were placed on a glass coverslip and imaged with a Nikon A1-Rsi confocal system. Fifty micrometer Z-stacks with 5 µm steps were collected at 40× and compressed to give a single image. Images were edited using ImageJ software.³⁸

Live–Dead Staining and Counting

At the specified time point, media was removed from the gels and washed with 1× PBS. One hundred microliters of staining solution (2 µM calcein AM and 4 µM ethidium homodimer-1 diluted to volume with 1× DPBS) was added to the gels and cells were incubated at room

temperature for 15–30 min. Hydrogels were placed on a glass coverslip and imaged with a Nikon A1-Rsi confocal system. Fifty micrometer Z-stacks with 5 μm steps were collected at 20 $\times$ and compressed to give a single 700 μm by 700 μm image covering a volume of roughly 0.025 mm^3 . Imaris imaging software (Bitplane) was used to count live and dead cells within the compressed Z-stack image.

In Vivo Wound Healing

All experimental procedures were approved by the Rice Institutional Animal Care and Use Committee (IACUC) in accordance with the Animal Welfare Act and with the Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health. Male and female diabetic mice (BKS.Cg-Dock7 $< m > + / +$ Lepr $< db > / J$) aged 8–10 weeks were purchased from Jackson Laboratory and housed two to a cage upon arrival. Mice were anesthetized in an induction chamber using 3% isoflurane in oxygen and then placed on a nose cone administering 2% isoflurane with 1.5 L oxygen. The mice were given a subcutaneous injection of meloxicam (1 mg/kg) to control pain. The dorsal surface of the mouse was clipped and a depilatory cream was used. The area was sterilized using betadine and alcohol swabs. Two critical-sized wounds were outlined on either side of the mouse's midline using an 8 mm biopsy punch. Sterile forceps were used to lift the skin in the middle of the outlined wound, and sterile scissors were used to create a full thickness wound extending through the subcutaneous tissue, as well as the panniculus carnosus. Fifty microliters of substance was added to each wound and then covered with a transparent occlusive dressing (Tegaderm). On days 3, 7, and 10, the bandages were removed, substances were reapplied, and the wounds were bandaged. On day 14, mice were sacrificed with an overdose of isoflurane and subsequently asphyxiated using carbon dioxide. The dorsal tissue of the mice was removed and fixed in 10% buffered formalin. To analyze hair follicle regeneration within the wound, the study was extended with a second cohort to day 28. On days 3, 7, 10, 14, 17, 21, and 24, the bandages were removed. If the wound was not fully contracted substances were reapplied and the wounds were bandaged. If the wound was fully contracted, the wound was left untreated and a bandage was not applied. On day 28, mice were sacrificed and the backs were harvested.

Analysis of Wound Contraction and Wound Closure

Macroscopic evaluation of wound contraction and closure was performed on days 0, 3, 7, 10, 14, 17, 21, 24, and 28. A ruler was placed alongside each mouse, and an image was taken from the same height. ImageJ was used to measure wound contraction by tracing the advancing dermal border (outer circumference), visualized by a depth difference between the skin and the underlying tissue, on day X as well as to measure wound closure by tracing the granulation tissue border (inner circumference) on day X (Figure S2). Wound contraction (eq 1) and closure (eq 2) were calculated as a percentage of the initial wound size using the following equations where area on day 0 = A , area of outer circumference on day X = B , and area of inner circumference on day X = C .³⁹

$$\% \text{wound contraction} = \frac{A - B}{A} 100 \quad (1)$$

$$\% \text{wound closure} = \frac{A - C}{A} 100 \quad (2)$$

Histology and Immunohistochemistry

After removal of the dorsal tissue for wound retrieval, the dorsal tissues were fixed in 10% buffered formalin. Samples were processed and embedded at Baylor College of Medicine's Breast Center Pathology Core. Paraffin blocks were faced until the wound was greater than 4 mm in length to ensure analysis took place as close as possible to the center of the wound. Although 8 mm wounds were initially created, tissue shrinks down to as little as half the original size when fixed in 10% buffered formalin. Samples were sectioned to create 5 μm thick tissue sections. Sections were then stained following Hematoxylin and Eosin (H&E) and Masson's Trichrome standard procedures to examine cellular infiltration, re-epithelialization, granulation tissue formation, and collagen deposition. For immunohistochemistry, tissue sections were deparaffinized and rehydrated followed by boiling in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 min. Cells were permeabilized using 0.5% Triton X in PBS for 5 min on room temperature slides. Slides were rinsed with PBS and nonspecific staining was blocked with 1% BSA in PBS for 30 min. Primary antibodies (α -SMA 1:300, neurofilament protein (NFP) 1:100, and β III-tubulin 1:100) were applied and incubated overnight at 4 °C. Slides were washed with PBS, and secondary antibodies (Anti-Rabbit AF 568 1:500 and Anti-Mouse AF 488 1:500) were applied for 1 h. Slides were rinsed a final time and coverslips were mounted using a DAPI-based mountant.

Quantification of Re-epithelialization

Tissue sections from day 14 and day 28 stained with Masson's Trichrome were imaged using a light microscope. In ImageJ, the edges of the wound were marked and the total length of the wound was determined (dermal gap). The distance between the right and left epithelial tongues was measured to determine the epithelial gap. The epithelial gap was divided by the dermal gap and multiplied by 100 to give the percentage of the wound unepithelialized.

Quantification of Granulation Tissue Depth

Tissue sections from days 14 and 28 stained with Masson's Trichrome were imaged using a light microscope. At the measured center of the observable wound, the depth of the granulation tissue was measured in mm using ImageJ.

Quantification of Collagen

Tissue sections from day 14 stained with Masson's Trichrome were imaged using a light microscope. ImageJ was used to split RGB images into a red, green, and blue channel. The threshold on the blue channel was adjusted until only blue was selected allowing the total area of collagen to be measured. The percentage of collagen in the granulation tissue was determined by dividing the area of collagen by the total area of granulation tissue and multiplying by 100%.

Quantification of Blood Vessel Density, Neural Fascicle Density, and Hair Follicles

Tissue sections from day 14 stained with α -SMA were imaged using an EVOS Fl fluorescence microscope. The area of the granulation tissue in the wound bed was measured using ImageJ in mm². Blood vessels forming a complete closure were manually counted within the entire area of the granulation tissue using the ImageJ cell counter plugin. The number of blood vessels counted was divided by the total area of the granulation tissue in the wound bed to yield the number of blood vessels per mm². Neural fascicles were counted within the entire area of the granulation tissue. The number of neural fascicles was divided by the total area of the granulation tissue in the wound bed to yield the number of neural fascicles per mm². Tissue sections from day 28 stained with Masson's Trichrome were used to count hair follicles within the entire area of the granulation tissue. Hair follicles in the adjacent native unwounded tissues were not included in the counting.

Statistical Analysis

One-way ANOVA was conducted for multiple comparisons of parametric data, with a Tukey posthoc analysis for all pairwise comparisons of the mean responses to the different treatment groups. All samples for day 14 data were tested in 12 mice (6 female and 6 male) with 2 wounds each ($n = 24$). All samples for day 28 data were tested in 6 mice (3 female and 3 male) with 2 wounds each ($n = 12$). Values of $p < 0.05$ were considered statistically significant ($*p < 0.05$; $**p < 0.01$). Data are represented as average $\pm$ standard error of the mean.

RESULTS AND DISCUSSION

The gelation process of the MDP allows cells to be 3D encapsulated within the MDP hydrogel. In this study, we utilized murine NIH-3T3 fibroblasts due to the large presence of fibroblasts in the granulation tissue of healing wounds.⁴⁰ Fibroblasts were seeded in the MDP hydrogel for 3, 7, 10, and 14 days. After the predetermined period, cells were fixed and stained within the hydrogel using Alexa488-phalloidin and DAPI to visualize the actin cytoskeleton and cell nuclei. Confocal z-stack images show by 3 days, cells proliferated and exhibited a spread morphology characteristic of fibroblasts (Figure 2A). At day 7, all cells exhibited the spread morphology of fibroblasts, and the cells rapidly grew within the hydrogel matrix creating networks via cell to cell junctions (Figure 2B). Through day 10 (Figure 2C) and day 14 (Figure 2D), the fibroblasts continued to grow and create extensive networks completely saturating the hydrogel matrix. Live-dead staining and confocal z-stack images were utilized to determine viable cells (Figure S3). Fibroblasts exhibited high cell viability within the MDP hydrogel. After 3 days, the percentage of viable cells was 77% (Figure 2E, I, and J). This number increased to 93% by day 7 (Figure 2F, I, and J) at which point cells began to undergo apoptosis (Figure S4), most likely due to lack of nutrients and space. By day 14, 80% of cells were viable (Figure 2H–J).

We hypothesized the ability of the MDP hydrogel to support cell growth and proliferation in vitro, as well as promote cellular infiltration and angiogenesis, demonstrated in a subcutaneous in vivo model,^{27,34–37} would aid in tissue regeneration in a diabetic wound healing model. Also, our MDP hydrogel is thixotropic, allowing direct delivery to the site of

interest through a syringe needle, and upon application conforms to the shape of the wound. In this study, we utilized genetically diabetic mice (BKS.Cg-Dock7^m + /+ Lepr^{db}/J or db/db), exhibiting delayed wound healing compared to normal mice.³⁹ Wounds were created on the dorsal surface of mice (Figure 1D–F), and the effects of the MDP hydrogel were compared to IntraSite,⁴¹ a current diabetic ulcer hydrogel treatment, as well as a simple rinsing with buffer, composed of Hanks buffered salt solution (HBSS) and 298 mM sucrose (matching the nonpeptide portion of the MDP hydrogel).

The diabetic mouse model has many benefits, including reproducibility, straightforward and cost-efficient materials, and techniques including examination through histological analysis. However, the healing of murine wounds does not directly parallel the healing of human wounds. The combination of the panniculus carnosus, a striated muscle layer in the subcutaneous tissue (Figure 1E), with a loose skin layer allows rapid contraction of murine wounds which does not occur in humans.⁴² Published studies frequently rely on the use of silicone splints to prevent the process of wound contraction from occurring, therefore allowing wound closure to outpace wound contraction.^{39,42} This allows the slower “human-like” wound healing to be observed more readily.^{39,42} In our study, wound contraction and wound closure were evaluated separately using macroscopic images of the wounds (Figure 3A–C and Figure S5). Wound contraction is defined as the measurement of the contractility of the skin closing the wound (outer circumference), while wound closure is defined as the measurement of granulation tissue covering the wound bed (inner circumference).³⁹

Figure 3 shows the photographic monitoring of wound closure and contraction along with its quantification. By day 14, contraction was observed to be 49, 35, and 34% in the MDP hydrogel, IntraSite, and buffer respectively (Figure 3D–F and Figures S6 and S7), but these differences were not found to be significantly different. However, the treatments examined did have important and statistically significant effects on wound closure. By day 14, the MDP hydrogel resulted in 95% closure, whereas IntraSite and buffer demonstrated only 68 and 38%, respectively (Figure 3D–F and Figure S6 and S8). While IntraSite showed statistically significant acceleration of wound closure over buffer control, mice treated with IntraSite required twice the time (day 28) to reach the same degree of closure observed by mice treated with MDP. Mice treated with buffer alone were not able to reach the level of healing observed in MDP treated mice by day 14 during the 28-day course of our study. Notably, treatment with buffer alone showed healing taking place through contraction exclusively (Figure 3F). In our study, the use of a silicone splint was unnecessary as the MDP hydrogel and IntraSite resulted in faster wound closure than murine contraction allowing observation of “human-like” healing (Figure 3D, E and Figure S6). In addition to the observed acceleration in wound closure, healed wounds of MDP treated mice were also observed to be regrowing hair from the center and periphery of the original wound starting at day 14. A more detailed analysis of hair follicle regeneration is described below.

Mice were sacrificed after day 14 and 28 for wound site histology. Day 14 histology was used to assess granulation tissue formation, collagen synthesis, vascularization, and innervation, whereas day 28 histology was primarily used to quantify hair follicles which require a longer period of time to develop. Day 14 wound sections were analyzed using Masson’s Trichrome staining to assess the newly formed granulation tissue in the wound bed

and re-epithelialization. Wounds treated with the MDP hydrogel resulted in the greatest depth of granulation tissue in the wound bed and a high cellular density (Figure 4A, E and Figures S11 and S15). In contrast, wounds treated with IntraSite result in a moderate depth of granulation tissue irregularly spaced around acellular pieces of remaining IntraSite hydrogel (Figure 4B, E and Figures S11, S15, and S16). Wounds treated with IntraSite resulted in patchy granulation tissue due to the lack of degradation and poor cell infiltration, whereas the degradation of the MDP hydrogel resulted in homogeneous granulation tissue regeneration throughout the wound bed (Figure 4A, B and Figures S11 and S16). At this time point, wounds treated with buffer alone demonstrated minimal granulation tissue, and frequently wound beds showed no observable tissue regeneration (Figure 4C, E and Figures S11 and S15). A wound was considered to be fully granulated when the granulation tissue has filled the wound bed to the level of the adjacent native tissue (Figure 1H).⁴³ At day 14, most wounds treated with the MDP hydrogel were observed to be fully granulated. In contrast, wounds treated with IntraSite or buffer had minimal granulation tissue. In addition to assessing the depth of the granulation tissue, Masson's Trichrome enables visualization of collagen within the wound bed. As a wound matures, it is remodeled and replaced with collagen to increase the stiffness and tensile strength of the wound (Figure 1G, H).⁴⁴ Wounds treated with the MDP hydrogel demonstrate a robust collagen staining across the wound bed, signifying more collagen within the granulation tissue (Figure 4A and Figures S11–S13). In contrast, wounds treated with IntraSite or buffer have minimal observable collagen within the granulation tissue (Figure 4B, C and Figures S11–S13).

The formation of granulation tissue within the wound bed allows re-epithelialization to occur, forming a barrier between the wound and the outside environment.⁴⁵ Masson's Trichrome stained sections were utilized to measure the epithelial tongue migrating across the granulation tissue (Figure 1G, H), marked by small arrow heads, which stained a dark pink (Figure 4A–C). At day 14, wounds treated with the MDP hydrogel were found to have resulted in 40% re-epithelialization, compared to IntraSite or buffer which resulted in only 25 and 23% re-epithelialization respectively (Figure 4A–D and Figures S11 and S14). For re-epithelialization to occur, keratinocytes must migrate and proliferate.⁴⁵ This suggests the MDP hydrogel creates a more suitable environment for the migration and proliferation of keratinocytes, allowing re-epithelialization to occur more readily.

Day 14 tissue sections were further analyzed for the extent of vascularization within the wound bed (Figure 5D and Figure S20). Qualitative and quantitative assessment of immunostained images revealed a significantly higher number of α -SMA positive blood vessels in wounds treated with the MDP hydrogel (Figure 5A, E, and F and Figures S21 and S22). Also, the wounds treated with the MDP hydrogel had blood vessels homogeneously distributed throughout the granulation tissue (Figure 5A, D and Figures S17–S19), whereas the blood vessels present in IntraSite or buffer appeared only irregularly in the wound bed (Figure 5B, C and Figures S17–S20). The formation of new blood vessels within the wound bed is important for sustaining the newly formed granulation tissue. It requires a complex interplay between growth factors stimulating angiogenesis, as well as the migration and mitogenic stimulation of endothelial cells. The application of the MDP hydrogel to these murine diabetic wounds recruits and supports the necessary cells required for this complex process.

In addition to finding blood vessels within the granulation tissue, neural fascicles (enclosed bundles of axons) from the peripheral nervous system were also observed (Figure 6D). To the best of our knowledge, this has not previously been reported in the diabetic wound healing literature. Tissue sections were immunostained with neurofilament protein (NFP) and β -III tubulin to confirm the presence of neural fascicles within the wound bed. Although fascicles were found in the granulation tissue of wounds treated with each substance (Figure 6A–D and Figure S23) wounds treated with the MDP hydrogel had a significantly higher density of neural fascicles than those treated with IntraSite or buffer (Figure 6E, F and Figures S24 and S25). In addition, in wounds treated with the MDP hydrogel, neural fascicles were found in large clusters (Figure 6A, D), compared to single neural fascicles in wounds treated with IntraSite (Figure 6B) or buffer (Figure 6C). When the axons of a nerve are severed during an injury, Schwann cells wrapping around the axons begin to proliferate and clear debris from the degenerating nerve.⁴⁶ In addition, Schwann cells guide regeneration by increasing the production of crucial growth factors required for nerve regeneration, ultimately reinnervating the damaged tissue.⁴⁶ It is evident the MDP hydrogel supports the proliferation and mitogenic activity of Schwann cells, allowing ample regeneration of the peripheral nervous system within the wound bed.

Histology of wounds at day 28 reveals later stage healing in the MDP treated wounds (epithelial closure, receding granulation tissue, and hair follicle growth, see below) while healing observed for IntraSite at day 28 largely matches the healing demonstrated for MDP treated wounds at day 14. Buffer controls continue to show healing is nearly exclusively due to panniculus carnosus mediated contraction. Between days 14 and 28 the granulation tissue depth found in MDP treated wounds is observed to decrease to match the surrounding native tissue (Figures S27–S29), whereas the amount of collagen within the wound bed increases (Figure 7D–I and Figures S28–30).

De novo hair follicle regeneration has been described in the wound bed of wounds larger than 5 mm in diameter after 14 days,⁴⁷ though not from a model of diabetic wound healing. We observed the appearance of hair follicles around and within the wound bed as early as day 14 in MDP treated wounds, while IntraSite and buffer treated wounds revealed hair 1 week later (day 21, Figure 7A). By day 24, 100% of MDP hydrogel treated wounds had hair in or around the wound, whereas 92% and 67% of IntraSite and buffer treated wounds had hair by day 28 (Figure 7A). Because the MDP hydrogel accelerated the appearance of hair, wounds treated with the MDP hydrogel had a larger quantity of hair present at day 28 for both female and male wounds (Figure 7A–C). Masson's Trichrome stained tissue sections were used to analyze the number of hair follicles present within the wound bed (Figure 7B–I and Figures S28–S30). Because the appearance of hair first occurs around the edges of the wound, hair follicles were analyzed both at the edge of each wound (Figure 7D–F and Figure S30) and at the center (Figure 7G–I and Figure S30) to examine the differences between substances. Wounds treated with the MDP hydrogel resulted in a statistically greater number of hair follicles on average at both the edge (Figure 7B) and at the center (Figure 7C) when compared to IntraSite or buffer with the largest differences observed in the center of the wounds.

As demonstrated above, we are able to see rapid wound closure when full-thickness wounds in diabetic mice are treated with the MDP hydrogel. By day 3, wound closure in MDP treated wounds outpaced both IntraSite and buffer treated wounds and continued to do so for the remainder of the experiment. In addition, the granulation tissue grown in our case contains a good network of natural collagen uniformly replacing the synthetic peptide matrix. In contrast, IntraSite failed to degrade and had poor cell infiltration resulting in patchy granulation tissue, and buffer-only treated wounds often produced no granulation tissue at all. MDP treated wound sites are highly cellularized, contain a dense vascular network, become innervated, and contain regenerated hair follicles. Although IntraSite and buffer lead to wounds containing cells, blood vessels, neural fascicles, and even hair follicles, MDP treated wounds show a statistically significant acceleration in all categories. Furthermore, a majority of studies published on wound healing utilize loaded growth factors, cells, or bioactive sequences in scaffolds to achieve comparable results.^{10,18,19,21–23,48} When examined in parallel to these other studies, it is evident the healing we observe is competitive, or better, without the need for exogenous growth factors or cells.

CONCLUSION

MDPs are a class of injectable biomaterials that mimic the nanostructure of the natural extracellular matrix allowing rapid cellular infiltration, and thus are ideal for tissue engineering strategies. Addition of the MDP hydrogel to diabetic mice with full-thickness wounds allows granulation tissue and re-epithelialization to outpace wound contraction without the addition of exogenous growth factors or cells. The MDP hydrogel results in wounds with thick granulation tissue, dense vascularization, innervation by the peripheral nervous system, and hair follicle regeneration. Comparatively, controls showed slower wound closure and poor or absent granulation tissue. The physiochemical properties of the MDP hydrogel, $K_2(SL)_6K_2$, play a central role in facilitating the wound healing process due to its biocompatibility, and ability to promote cellular infiltration, angiogenesis, neurogenesis, and hair follicle regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

MDP forms a nanofibrous hydrogel that is easily applied to wounds in a diabetic mouse model. (a) 16-amino acid MDP, $K_2(SL)_6K_2$, forms (b) nanofibers through the hydrophobic effect and hydrogen bonding. (c) Nanofibers entangle and cross-link after the addition of multivalent salts, eventually forming a hydrogel (inset). Scale bar is equal to 500 nm. (d) Because the hydrogel is syringe deliverable, it can be easily applied to wounds and conforms to their shape. (e–h) Cartoon depiction of the time course of wound healing. (e) Before creation of the wound, the subcutaneous tissue is filled with adipose tissue and the panniculus carnosus muscle layer. (f) Wounding of the dorsal tissue removes epithelium, adipose tissue, and the panniculus carnosus. (g) As the wound heals granulation tissue, collagen, blood vessels, and neural fascicles fill the wound bed and epithelial cells migrate across the granulation tissue creating epithelial tongues. (h) Over time, the amount of granulation tissue, collagen, blood vessels, and neural fascicles increase and the epithelial tongues extend to close the wound.

Figure 2.

MDP hydrogel facilitates 3D cell culture of NIH-3T3 fibroblasts. NIH-3T3 fibroblasts were encapsulated within the MDP hydrogel. Cells were grown in 3D for (a) 3, (b) 7, (c) 10, and (d) 14 days and stained with Alexa488-phalloidin (green) and DAPI (blue) to visualize the actin cytoskeleton and nuclei, respectively. Scale bars are equal to 50 μm . Calcein Am (green) and ethidium homodimer-1 (red) were used for live-dead staining of fibroblasts at (e) day 3, (f) day 7, (g) day 10, and (h) day 14. Scale bars are equal to 100 μm . (i) Number of viable cells per mm^3 increased from day 3 to day 10, at which point cells saturated the MDP hydrogel, whereas (j) the percentage of viable cells decreased after day 7. $n = 6$. All data are presented as average $\pm$ s.e.m.

Figure 3.

MDP hydrogel promotes wound closure. Images of wounds were taken for (a) MDP hydrogel, (b) IntraSite, and (c) buffer to measure wound contraction and wound closure (outline) in ImageJ. Scale bars are equal to 5 mm. Percent wound contraction (dashed lines) and percent wound closure (solid lines) were then calculated for (d) MDP hydrogel, (e) IntraSite, and (f) buffer by normalizing to the wound size on day 0. $n = 18$ mice per substance (36 wounds per substance) for day 0–14. $n = 6$ mice per substance (12 wounds per substance) for days 17–28. All data are presented as average $\pm$ s.e.m. Statistical significance performed using one-way ANOVA with a Tukey posthoc multiple comparison test to compare contraction and closure (* $p < 0.05$; ** $p < 0.01$).

Figure 4. MDP hydrogel results in greater tissue regeneration. Masson's Trichrome stained tissue sections of female wounds treated with (a) MDP hydrogel, (b) IntraSite, and (c) buffer. Scale bars are equal to 1 mm. Large arrow heads mark the wound margins while small arrow heads mark the end of the epithelial tongue (stained dark pink) and were used to determine (d) the percent of wound re-epithelialized. (e) At the center of the wound, the depth of granulation tissue was measured. $n = 12$ mice per substance (24 wounds per substance). All data are presented as average $\pm$ s.e.m. Statistical significance performed using one-way ANOVA with a Tukey posthoc multiple comparison test. Different Greek letters indicate statistically significant difference ($p < 0.05$).

Figure 5.

Dense vascularization results from treatment with the MDP hydrogel. α SMA immunostaining of (a) MDP hydrogel, (b) IntraSite, and (c) buffer tissue sections of female wounds. Positive α SMA expression is shown in red and cell nuclei are stained blue with DAPI. Scale bars are equal to 100 μ m. (d) Masson's Trichrome staining of numerous blood vessels found in the granulation tissue of a wound treated with the MDP hydrogel. Scale bar is equal to 100 μ m. (e) Number of blood vessels counted within the entire area of granulation tissue of male and female tissue sections. (f) Number of blood vessels counted per mm² of granulation tissue in male and female tissue sections. $n = 12$ mice per substance (24 wounds per substance). All data are presented as average $\pm$ s.e.m. Statistical significance performed using one-way ANOVA with a Tukey posthoc multiple comparison test. Different Greek letters indicate statistically significant difference ($p < 0.05$).

Figure 6.

Neural fascicles from the peripheral nervous system are found within wounds.

Neurofilament protein (NFP) and β -III tubulin immunostaining of neural fascicles in (a) MDP hydrogel, (b) IntraSite, and (c) buffer tissue sections. Positive NFP expression is shown in green, positive β -III tubulin expression is shown in red, and cell nuclei are stained blue with DAPI. Scale bars are equal to 100 μ m. (d) H&E staining of neural fascicles found in the granulation tissue of a wound treated with the MDP hydrogel. Scale bar is equal to 100 μ m. (e) Number of neural fascicles counted within the entire area of granulation tissue of male and female tissue sections. (f) Number of neural fascicles counted per mm² of granulation tissue in male and female tissue sections. $n = 12$ mice per substance (24 wounds per substance). All data are presented as average $\pm$ s.e.m. Statistical significance performed using one-way ANOVA with a Tukey posthoc multiple comparison test. Different Greek letters indicate statistically significant difference ($p < 0.05$).

Figure 7.

MDP hydrogel results in accelerated hair follicle regeneration within the wound bed. Images of wounds were taken of each mouse to examine the presence of hair in or around each wound. (a) Wounds treated with the MDP hydrogel (blue) had hair appear sooner than wounds treated with IntraSite (red) or buffer (black). Masson's Trichrome stained tissue sections of wounds were used to count the number of hair follicles at the (b) edge of the wound and at the (c) center of the wound. Representative Masson's Trichrome stained tissue sections of female wounds at the edge treated with (d) MDP hydrogel, (e) IntraSite, and (f) buffer as well as female wounds at the center treated with (g) MDP hydrogel, (h) IntraSite, and (i) buffer. Scale bars are equal to 100 μ m. $n = 12$ mice per substance (24 wounds per substance). All data are presented as average $\pm$ s.e.m. Statistical significance performed using one-way ANOVA with a Tukey posthoc multiple comparison test. Different Greek letters indicate statistically significant difference ($p < 0.05$).