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Nanofibrous Peptide Hydrogel Elicits Angiogenesis and Neurogenesis without Drugs, Proteins, or Cells

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Abstract

The design of materials for regenerative medicine has focused on delivery of small molecule drugs, proteins, and cells to help accelerate healing. Additionally, biomaterials have been designed with covalently attached mimics of growth factors, cytokines, or key extracellular matrix components allowing the biomaterial itself to drive biological response. While the approach may vary, the goal of biomaterial design has often centered on promoting either cellular infiltration, degradation, vascularization, or innervation of the scaffold. Numerous successful studies have utilized this complex, multicomponent approach; however, we demonstrate here that a simple nanofibrous peptide hydrogel unexpectedly and innately promotes all of these regenerative responses when subcutaneously implanted into the dorsal tissue of healthy rats. Despite containing no small molecule drugs, cells, proteins or protein mimics, the innate response to this material results in rapid cellular infiltration, production of a wide range of cytokines and growth factors by the infiltrating cells, and remodeling of the synthetic material to a natural collagen-containing ECM. During the remodeling process, a strong angiogenic response and an unprecedented degree of innervation is observed. Collectively, this simple peptide-based material provides an ideal foundational system for a variety of bioregenerative approaches.

Keywords

biomaterials; self-assembly; peptide; hydrogel; innervation; vascularization

Introduction

Biomaterials for regenerative medicine are designed to effectively integrate into the host tissue and accelerate or augment the body's natural healing ability while evoking a minimal inflammatory response. In order to achieve the necessary bioactivity to promote healing,

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these materials are frequently designed to deliver small molecule drugs, cells, or proteins. Serving as a delivery agent, the biomaterial provides controlled release and/or localization of bioactive components in order to drive a specific biological response. Commonly delivered small molecules include antibiotic (1, 2), anti-inflammatory (3), and anti-cancer drugs (4), while a variety of cell types have been delivered to regenerate tissues such as skin and bone (5). Proteins such as vascular endothelial growth factor (VEGF), β -nerve growth factor (β -NGF), and bone morphogenetic protein-2 (BMP-2) are frequently incorporated to promote angiogenesis, axon sprouting, and bone mineralization respectively (6). Developed as an alternative approach to exogenous protein loading, many materials are covalently modified with short biomimetic peptides. These peptides are typically derived from growth factors or critical components of the extracellular matrix (ECM), and they are specifically designed to activate important regulation cascades by binding the same receptor as their natural counterpart. The most commonly utilized biomimetic peptide, “RGD”, is a simple tripeptide which promotes cell attachment, spreading, and proliferation by mimicking a binding site on fibronectin (7). More recently, “QK,” a short peptide mimic of VEGF, has been shown to drive angiogenesis and improve material vascularization (8, 9). In order to achieve the desired biological response, many tissue regeneration strategies require the incorporation of multiple bioactive components. Collectively, these design methodologies have resulted in a wide range of impressive results in tissue regeneration (10–12).

However, multicomponent systems are complex, often leading to challenges in both design and synthesis. Additionally, the multiplicity of components makes it difficult to predict the biological response to the material, and, further, raises regulatory barriers for translation to the clinic. Thus, an ideal material for tissue regeneration exerts the necessary bioactive effects without depending upon the delivery of exogenous bioactive components. In this report we describe a simple, single component multidomain peptide (MDP) hydrogel with the amino acid sequence $K_2(SL)_6K_2$ which does not contain small molecule drugs, cells, nor proteins and does not present any known bioactive sequence. Despite its simplicity, we will show that this syringe deliverable peptide hydrogel 1) is rapidly infiltrated by host cells, 2) provokes an inflammatory response which resolves over a few days, 3) does not undergo fibrous encapsulation, thereby allowing communication between the implant and the host body, 4) results in a dense, mature vascular network, 5) recruits neural fascicles, and 6) predictably degrades over approximately six weeks. Analysis of the cellular infiltrate and its secretome shows the production of critical cytokines and growth factors such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-4 (IL-4) to recruit and convert monocytes to a “prohealing” phenotype; VEGF and platelet-derived growth factor (PDGF) to drive angiogenesis; ciliary neurotrophic factor (CNTF) and β -NGF to recruit Schwann cells and neural fascicles; and matrix metalloproteinase-8 (MMP-8) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) to mediate material remodeling. These results suggest that the nanofibrous MDP hydrogel creates an optimal healing environment for a wide variety of applications through the use of the simple peptide hydrogel alone.

With the amino acid sequence $K_2(SL)_6K_2$, the simple peptide described in this study is a member of the broader class of self-assembling amphiphilic peptides termed MDPs (Fig. 1A) (13). MDPs consist of an alternating sequence of hydrophilic and hydrophobic amino acids flanked by charged residues at each peptide termini (Fig. 1B). As a consequence of

this design, MDPs exhibit a β -sheet secondary structure, high solubility, and supramolecular assembly in aqueous solution to form nanofibers with structural similarity to the native ECM (Fig. 1C&D) (14, 15). Previously, we have demonstrated that MDP hydrogels can be loaded to effectively deliver bioactive molecules including drug molecules (16–18), growth factors (19–22), and cytokines (19, 23). The robust mechanism governing self-assembly and hydrogelation of MDPs allows for significant customization of the peptide sequence. Taking advantage of this, we have incorporated bioactive sequences into the MDP to create a tissue engineering scaffold capable of driving specific cellular processes. These sequences include the RGD motif to support cell adhesion (20, 24–26), LRG to accelerate enzymatic degradation of the peptide by matrix metalloproteinase 2 (MMP-2) (20, 24–26), and the “QK” sequence derived from VEGF to drive angiogenesis (22, 27).

Previous studies performed by our laboratory have used the MDP $K_2(SL)_6K_2$ as a baseline self-assembling system and in vitro studies showed no special bioactivity yielding modest results in cell viability, spreading, and morphology when compared to related hydrogels with designed bioactivity (24, 25). This had led us to focus on delivering biologically active molecules using MDP hydrogels, or modifying the MDP hydrogel with biomimetic peptide sequences to gain bioactivity. Those hydrogels which were deemed to be successful in in vitro experiments progressed to more complex in vivo experiments, while peptides showing less success in vitro were eliminated from consideration for further studies. Therefore, before the current study we had not tested $K_2(SL)_6K_2$ in vivo. However, there is a wide and growing consensus in the scientific community that in vitro results often fail to accurately predict the in vivo effect of a material, largely due to the absence of systemic processes (28). In this report, we show that the MDP $K_2(SL)_6K_2$ supports this hypothesis, as in vivo results described here contrast sharply with predictions based on our in vitro work.

In this study, we use a subcutaneous injection model to examine the physiological response to the MDP $K_2(SL)_6K_2$. This in vivo model has many advantages in determining the interaction between a biomaterial and a living system. First, it is a relatively simple and inexpensive experiment to carry out. Second, this system allows interpretation of biomaterials properties outside of the complicating influences of injury or disease (Fig. 1E). Despite its simplicity, this model offers valuable insight into the interaction of the MDP with systemic processes such as the immune, vascular, and nervous systems which are impossible in in vitro systems. As with any biomaterial, the inflammatory response triggered by the MDP is critical in defining its success or failure, and this response can be determined through examination of the response to subcutaneous injection. Because this model is performed on healthy tissue, the ideal result is ultimately degradation of the MDP and remodeling into native tissue. Until complete degradation is achieved, the physiological response to the MDP can be evaluated.

Materials and Methods

Peptide Synthesis

$K_2(SL)_6K_2$ was synthesized using a Focus XC Automated Peptide Synthesizer with N-terminus acetylation and C-terminus amidation, by methods previously reported by our laboratory (14). Successful synthesis was confirmed through MALDI-TOF MS (SI Figure

1). After cleavage of the peptide from the synthesis resin, the peptide was dialyzed against H₂O for one week to remove any remaining cleavage scavengers. Tubing with a MWCO of 100–500 Da was used for dialysis, and daily exchanges of the dialysis bath were performed. The peptide solution was then sterilized using a 0.2 µm filter, and lyophilized to yield a peptide powder. The peptide powder was redissolved to a concentration of 20 mg/ml in sterile H₂O containing 300 mM sucrose, and hydrogelation was induced by mixing this solution 50:50 with 1X HBSS. The final concentration of peptide in the hydrogel used for experimentation was therefore 1 wt%.

Subcutaneous Injection

All experiments were performed in accordance with an IACUC protocol approved by Rice University. Wistar rats weighing 226–249 g (Charles River Laboratories) were given 200 µl subcutaneous injections on the dorsal aspect. The animals were anesthetized using isoflurane (3% carried by oxygen) and maintained on a nose cone (2% isoflurane). Prior to injection, hair was clipped with a power trimmer, and the injection sites were sterilized with an alcohol swab. After 3, 7, 14, 21, or 42 days, the animals were euthanized by CO₂ overdose, and the dorsal tissue was harvested and fixed in 4% paraformaldehyde for histological analysis.

Histology & Immunohistochemistry

All samples were processed, embedded, and sectioned at Baylor College of Medicine's Breast Cancer Pathology core, and all staining was performed on 5 µm thick tissue sections. Hematoxylin and eosin and Masson's trichrome staining were performed according to manufacturer's instructions. For immunostaining, tissue sections were deparaffinized and rehydrated through xylene and ethanol washes. Slides were boiled in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes for antigen retrieval. Slides were allowed to cool to room temperature, and 0.5% Triton-X in HBSS was applied for 5 minutes to permeabilize cells. Slides were rinsed with HBSS and blocked with 1% BSA in HBSS for 30 minutes. Antibodies were diluted as shown in SI Table 1 in 1% BSA in HBSS and applied overnight at 4 °C. After rinsing with HBSS, secondary antibodies were applied at a 1:500 dilution for 1 hour followed by mounting with a DAPI-based mountant.

Quantification of Histology

Implants from each animal were harvested and quantified for cellular infiltration, hydrogel degradation, neural density, and blood vessel density. The data shown in Figures 2–5 uses an n=8 animals and 2 implants per rat. Values obtained for implants harvested from the same animal were averaged to avoid bias.

Quantification of Cellular Infiltration

Images of H&E stained tissue sections were taken at 20X magnification at the center of the hydrogel and processed for cell counting using ImageJ (29). Images were converted to greyscale (16-bit), and threshold adjusted to highlight only the cell nuclei. To avoid merged nuclei, the Watershed ImageJ plugin was used and cells were counted using the Particle Analyzer option. The number of nuclei was divided by the area of the implant to yield cells per mm².

Hydrogel Degradation

Panorama images were obtained of each tissue section containing MDP hydrogel. The total area of the hydrogel was calculated in ImageJ to approximate a degradation profile of the material.

Blood Vessel Density

Using tissue sections immunostained for α SMA, the number of blood vessels clearly located within the $K_2(SL)_6K_2$ hydrogel was counted manually. Only blood vessels showing a complete ring of α SMA staining were counted in order to avoid counting non-specific staining or undeveloped vasculature. The total area of the $K_2(SL)_6K_2$ hydrogel was calculated using ImageJ for each tissue section, and the number of blood vessels counted was divided by this area. The value shown for control tissue was calculated from dorsal fascia tissue of untreated rats, where the number of blood vessels counted was divided by the total area of the tissue harvested.

Neural Density

In H&E stained tissue sections, both the hydrogel and remodeled tissue immediately surrounding the hydrogel are easily identifiable. Using ImageJ, this area was calculated, and the number of neural fascicles located within the implant and remodeled tissue was counted. The total number of neural fascicles was divided by the area determined using ImageJ to give neural fascicle density per mm^2 of tissue. The value shown for control tissue was calculated from dorsal fascia tissue of untreated rats, where the number of neural fascicles counted was divided by the total area of the tissue harvested.

Statistical analysis

All error bars indicate the standard error of the mean. Differences between groups were determined using a one-way analysis of variance (ANOVA) and a Tukey's multiple comparisons test using GraphPad Prism v7.0A. P-values < 0.05 were considered significant. Values that are not significantly different from one another are indicated with the same Greek letter(s). Statistically significant differences are shown with different Greek letters.

Cytokine Array

Cytokine array experiments used an n of 3 rats per time point with 1 implant per rat. Subcutaneous implants were recovered from the dorsal tissue of the rats, added to 1X cell lysis buffer with protease inhibitors, and vortexed to extract proteins. The supernatant was collected and centrifuged at 15,000 rpm at 4 °C for 5 min. After centrifugation, the supernatant was collected and the total protein concentration of each sample was determined using the Bradford Assay. Final protein concentration was adjusted to 650 μ g/mL using 1X blocking buffer. Cytokine array membranes were processed according to the supplied kit instructions. Briefly, each membrane was blocked with 1X blocking buffer for 30 min. 1 mL of 650 μ g/mL extracted protein sample was added to the membranes and rocked overnight at 4 °C. The membranes were incubated with 1X Biotin-Conjugated Anti-Cytokines for 2 h at room temperature followed by HRP-Conjugated Streptavidin for 2 h at room temperature. A chemiluminescent detection buffer was added to the membrane, followed by imaging of the

membrane using an LAS 4000 Imager. Quantification of cytokine array results was performed using ImageJ software following manufacturer's guidelines. Results were semi-quantitative and allowed for comparison of signal intensity to a positive control rather than numerical determination of protein concentration. For each membrane, all cytokine signals were normalized to the signal intensity of the positive control.

Results

Cellular Infiltration

In comparison to several other biomaterials of both natural and synthetic sources including cellulose (30), gelatin (31), urea based bolaamphiphiles (32), and methacrylic acid beads (33), the $K_2(SL)_6K_2$ hydrogel shows rapid and dense infiltration by host cells without necessitating the incorporation of bioactive molecules to drive this response. At the earliest time point examined, 3 days post-injection, the MDP hydrogel is observed by histology to be completely infiltrated by cells, with cells present in both the periphery and core of the hydrogel (Fig. 2A). H&E staining shows a non-uniform distribution of cells at day 3, with outer areas of the hydrogel densely populated and the inner region more sparsely populated. By week 1, cells appear more homogeneously distributed throughout the hydrogel, and this remains true for the remainder of the time points examined (Fig. 2A, Fig. S2 & S3). The number of cell nuclei counted per mm^2 consistently remains near 10,000 (Fig. 2B).

Histological analysis of $K_2(SL)_6K_2$ implant sections stained with H&E revealed that mainly inflammatory cells infiltrate the material (Fig. S4). Three days after implantation, polymorphonuclear cells, particularly neutrophils and monocytes, are present in the material. In addition to their main role of phagocytosis, these cells initiate the acute inflammatory response and are responsible for the release of cytokines and signaling molecules to regulate the immune response (34). One week after implantation, macrophages arrive and fewer neutrophils are observed, which implies the progression of the immune response. At 2 weeks and 3 weeks after implantation, lymphocytes and macrophages are the main cell populations within the implant. By week 6, lymphoid cells are not easily observed by histology and mostly mononuclear cells (which are expected to be primarily macrophages) are observed.

Although no chemoattractant cytokines were loaded into the $K_2(SL)_6K_2$ hydrogel prior to implantation, a cytokine array experiment confirmed the presence of multiple chemokines within the hydrogel implant at day 3, 1 week, and 2 weeks post-injection (Fig. 2C). The detection of these chemokines mechanistically explains the infiltration shown in Fig. 2A, where cells are recruited from the host tissue into the hydrogel. The two families of chemokines identified, CC and CXC chemokines, are mainly implicated in immune and inflammatory cell trafficking and recruitment (35). CC chemokines examined include MCP-1 and macrophage inflammatory protein 3 α (MIP-3 α), while CXC chemokines include cytokine induced neutrophil chemoattractant 1 (CINC-1), cytokine induced neutrophil chemoattractant 2 α (CINC-2 α), cytokine induced neutrophil chemoattractant 3 (CINC-3), LIX, and CXCL7. These chemokines are present at all time points, with MCP-1, CINC-2 α , and CXCL7 showing comparatively higher signal intensities. From 3 days to 1 week post-injection, the relative signal intensity of all chemokines increases, while

trends from 1 week to 2 weeks post-injection depend on the identity of the chemokine examined.

Scaffold Degradation and Remodeling

Histological tissue sections were examined to obtain a degradation profile of the implants (Fig. 3A&B, Fig. S5). Weeks 3 and 6 show a statistically significant decrease in hydrogel area from the area calculated at day 3. A slight and statistically insignificant swelling of the hydrogel is apparent at week 1, which we attribute to general inflammation at the injection site. This swelling resolves by week 2, and a continual decline in average hydrogel area occurs over the remaining time points (Fig. 3D). Despite substantial degradation, all implants were identifiable at the 6 week time point. This is in notable contrast to a previous study of ours which examined MDP hydrogels containing an MMP susceptible degradation site which are nearly entirely degraded within two weeks (22).

To ensure scaffold remodeling was occurring without fibrous encapsulation, Masson's trichrome staining allowed visualization of the collagen extracellular matrix surrounding and within the hydrogel implant. As evidenced by the lack of dark blue staining at the periphery of the implants, fibrous encapsulation of the hydrogel is not seen at any time point (Fig. 3C, Fig S5). However, beginning at week 1, light blue staining can be detected within the implant, indicating collagen deposition and scaffold remodeling. By the final time point, 6 weeks post-injection, extensive collagen deposition and hydrogel remodeling are evident both within the remaining hydrogel as well as in its immediate vicinity where the hydrogel has already degraded and been remodeled into native ECM. Fibrous encapsulation of the $K_2(SL)_6K_2$ hydrogel was not seen in any of the samples, regardless of time point.

Analysis of the protein content of the $K_2(SL)_6K_2$ implants confirms endogenous expression of two cytokines specifically involved in ECM remodeling by 3 days post-injection. MMP-8 is a matrix metalloprotease enzyme responsible for proteolytic degradation, while TIMP-1 functions as an inhibitor of matrix metalloproteases. A high signal intensity for MMP-8 is detected at all time points, with the highest signal intensity occurring at week 1. TIMP-1 is also detected within the implant, although there is no significant difference in intensity between time points (Fig. 3E). Histological results suggest that these cytokines are present at concentrations appropriate to avoid fibrous encapsulation as an inappropriate balance of MMP and TIMP enzymes has been reported to cause fibrous encapsulation when an excess of TIMP is present, or degradation of healthy native tissue when an excess of MMP is present, neither of which were evident histologically (36).

Angiogenesis and Vascularization

Although the $K_2(SL)_6K_2$ hydrogel is not designed to induce angiogenesis, the hydrogel quickly becomes highly vascularized after subcutaneous injection (Fig. 4). At 3 days post-injection, the implant is surrounded by a high density of blood vessels (Fig. S7). At this early time point, blood vessels are occasionally identifiable in the periphery of the implant, while few to no blood vessels are seen in the center of the hydrogel (Fig. 4C). However, the density of vascularization continually increases over time, and many blood vessels are identifiable at the center of the hydrogel implant by 2 weeks post-injection (Fig. 4D, Fig.

S8). Quantification of blood vessels identified by α -SMA immunostaining produces the graph shown in Fig. 4E and Fig. S9. Although the subcutaneous space is not a highly vascularized tissue, the MDP hydrogel becomes densely vascularized. At day 3 the hydrogel is observed to have lower vascular density compared to the native tissue, but this density increases at every time point assessed with the maximal density at six weeks.

Two key growth factors associated with angiogenesis, VEGF and PDGF-aa, are detectable within the implants at all time points (Fig. 4F). Both growth factors show a statistically significant increase in signal from the 3 day to 1 week time point, and this correlates with the histological appearance of blood vessels within the hydrogel implant. Additionally, the CXC chemokines identified within the implant (Fig. 2C) have also been linked to angiogenesis (37, 38).

Innervation

The identification of neural fascicles within subcutaneously implanted materials has rarely been reported in the literature; nevertheless, neural fascicles are easily identifiable within the $K_2(SL)_6K_2$ hydrogel as early as 3 days post-injection. Fig. 5A provides a cartoon depiction of nerve structure, while Fig. 5B and Figs. S10 & S11 show the histological appearance of neural fascicles identified within the hydrogel implant when stained with H&E.

Immunohistochemistry for positive expression of neurofilament protein (NFP) and β -III tubulin confirms the identity of neural fascicles within the implant, and positive staining for myelin basic protein (MBP) and S100 demonstrates that the neural axons are myelinated and supported by Schwann cells (Fig. 5C&D). Fig. 5E shows the number of neural fascicles identified per mm^2 of implant at each experimental time point. Although the hydrogel is not loaded with any neurotrophic factors, the implant has reached the neural density of native subcutaneous tissue by 3 days post-injection. At the 2 and 3 week time points, the hydrogel contains a statistically higher density of neural fascicles than native tissue. By 6 weeks post-injection the implant is resolving, and the density of neural fascicles decreases to a value similar to native tissue.

Cytokine array results demonstrate that cells recruited to the MDP hydrogel secrete two neurotrophic factors in response to the material. Both β -NGF and CNTF are detectable within $K_2(SL)_6K_2$ implants at all time points (Fig. 5F). The detection of β -NGF and CNTF corresponds with the increasing average density of fascicles through week 3, and together these data suggest that neural fascicles are being recruited to the hydrogel.

Inflammatory Cytokine Profile

Because the $K_2(SL)_6K_2$ implants are not loaded with exogenous bioactive components, the biological responses reported here must be due to the host system's response to the material alone. Implantation of most foreign materials results in inflammation, and it is well known that inflammatory cells are capable of secreting the chemokines, cytokines, and growth factors involved in cellular infiltration, scaffold degradation, vascularization, and innervation (39).

Characterization of the inflammatory responses to the $K_2(SL)_6K_2$ hydrogel was achieved by evaluating the expression of multiple pro-inflammatory cytokines and two anti-inflammatory

cytokines (Fig. 6). The pro-inflammatory cytokines, including TNF- α , IL-1 α , IL-1 β , IL-6, and IFN- γ , all show similar trends in expression over time. Comparing the day 3 and 2 week time points, a statistically significant increase in signal is seen for all pro-inflammatory cytokines which illustrates that the hydrogel provokes an inflammatory response. However, none of the pro-inflammatory cytokines show a statistically significant increase from week 1 to week 2, suggesting that the pro-inflammatory response is not escalating over time. To support this, IL-10 and IL-4, two common anti-inflammatory cytokines associated with resolution of the inflammatory response and wound healing, also increase from day 3 to week 2. Overall, the host response to the K₂(SL)₆K₂ hydrogel appears to progress from a pro-inflammatory environment to a pro-wound healing response by week 2.

Discussion

The MDP K₂(SL)₆K₂ hydrogel harnesses the body's innate capacity to heal. Because this peptide hydrogel is recognized as a foreign material, injection leads to an acute inflammatory response that subsequently drives a highly orchestrated healing process. Key to executing this healing process are the many inflammatory host cells that infiltrate the hydrogel rapidly after implantation (Fig. S4). Unlike the K₂(SL)₆K₂ hydrogel, many different biomaterials including polyethylene glycol hydrogels (40) and gelatin cryogels (41), either entirely lack or provoke minimal infiltration by innate cells although infiltration can sometimes be achieved through loading with exogenous chemoattractant cytokines (41, 42). The contrast seen in cellular infiltration between the K₂(SL)₆K₂ hydrogel and these materials is likely due to the physical nature of the hydrogel. As a soft material composed of supramolecular nanofibers, host cells can easily infiltrate the K₂(SL)₆K₂ hydrogel allowing for the extremely high degree of cellular infiltration seen here. Cellular infiltration is critical in defining the degradation profile of the hydrogel in addition to driving the bioactive processes of scaffold vascularization and innervation.

Once the K₂(SL)₆K₂ hydrogel has been infiltrated by cells, the resident cells of the hydrogel begin secreting growth factors and cytokines. The physical structure of the hydrogel again plays a key role in the utility of these soluble factors allowing them to easily diffuse out of the hydrogel where they are able to initiate a cascade which recruits additional cells, drives biodegradation of the scaffold, initiates vascularization, and attracts innervation. Additional cells are recruited to the material through secretion of CC and CXC chemoattractant cytokines within the hydrogel matrix (Fig. 2C). As additional cells arrive to the hydrogel matrix, the high density of cell infiltration triggers host cells to secrete angiogenic signals that bring additional nutrients to the area through the development of vasculature. The secretion of two key angiogenic growth factors, VEGF and PDGF-aa mechanistically explains the development of extensive vasculature within the hydrogel matrix. In comparable studies, the use of bioactive molecules or short peptide mimics of these molecules have been required to promote angiogenesis (43–46). However, with the K₂(SL)₆K₂ hydrogel, these cytokines are secreted by the innate infiltrating cells rather than exogenously loaded into the hydrogel. Also, innately secreted are the neurotrophic factors β -NGF and CNTF which result in peripheral neural fascicles being recruited to the implant. In the few studies we found where peripheral nerves infiltrate a biomaterial, significantly more complex systems were required, for example using stem cell delivery (47, 48).

Previously we have shown that MDP hydrogel not loaded with growth factors such as VEGF or PDGF or not containing a biomimetic signal for vascularization did not result in significant vascularization (22). However, the peptide used in that case, $K(SL)_3RG(SL)_3K$, contains an MMP susceptible cleavage sequence which results in its comparatively rapid degradation. The difference between that case and the current MDP $K_2(SL)_6K_2$ can be attributed to this difference in degradation rate in which the $K_2(SL)_6K_2$ hydrogel persists long enough to result in a robust biological response.

Successful scaffold remodeling and tissue regeneration requires the coordinated activity of numerous cell types and biological signaling molecules. Without cell infiltration of a biomaterial, interaction between host cells and the material can only occur at the surface of the material. In the case of the $K_2(SL)_6K_2$ hydrogel, however, interaction between the infiltrating host cells and the material occurs throughout the entire implant. This allows for a much larger number of cells to interact with the biomaterial and consequently a greater magnitude of cell signaling to occur which in turn drives the exceptional healing responses observed here without requiring loading of additional signaling molecules or cells. Equally important to the increased interaction, is the aqueous and porous nature of the hydrogel (and lack of fibrous encapsulation) which allows for cell, protein, and nutrient trafficking throughout the $K_2(SL)_6K_2$ hydrogel. Rapid diffusion of signaling molecules enables communication between cells and coordination of corresponding biological responses, all of which are consistent with the initiation and resolution of an acute inflammatory response (Figs. 2–5).

While the inflammatory response can be beneficial for tissue healing and regeneration, it also requires careful monitoring to ensure that it does not escalate to a detrimental level. Persistent escalation of the inflammatory response is associated with chronic inflammation, resulting in deleterious effects on healthy native tissue. In the case of the $K_2(SL)_6K_2$ hydrogel, the inflammatory response to the material allows for the regenerative effects highlighted here without resulting in detrimental effects on the surrounding tissue. Furthermore, the body is able to effectively resolve the inflammatory response invoked by the hydrogel when the material is implanted into a healthy tissue environment. Future studies will be necessary to examine whether a diseased-state will negatively impact the regenerative capacity and properties of this hydrogel. Additionally, the extent to which peptide sequence impacts the healing inflammatory response remains to be seen. In order to understand the crucial aspects of chemical scaffold design, the impact of features such as amino acid identity, scaffold charge, and self-assembly characteristics on these key regenerative properties will be investigated.

Conclusion

As a syringe injectable, nanofibrous hydrogel, the MDP $K_2(SL)_6K_2$ is capable of driving specific biological processes crucial for most tissue engineering strategies without the delivery of exogenous bioactive components. Upon introduction to the biological environment, the hydrogel provokes an inflammatory response and is rapidly infiltrated by a large number of host cells. Upon arrival, these cells begin secreting a wide range of cytokines and growth factors which sets in motion further cell recruitment, angiogenesis,

innervation, degradation, and scaffold remodeling. Many of the detected proteins, especially VEGF and β -NGF, are regularly included in tissue regeneration strategies to specifically drive angiogenesis and innervation respectively. Yet, in the case of $K_2(SL)_6K_2$, these growth factors are innately produced by the host system's cellular infiltrate and provide the same functional responses. We ascribe the surprisingly robust biological response to this relatively simple hydrogel to an inflammatory response coupled with a nanostructure and hydrogel density that is highly conducive to cell infiltration. Collectively, these features make the $K_2(SL)_6K_2$ hydrogel an excellent candidate material for a wide variety of tissue engineering and regeneration applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (A) Chemical structure of MDP, $K_2(SL)_6K_2$ (B) A "hydrophobic sandwich" composed of a dimer of $K_2(SL)_6K_2$ peptides (C) The bilayered nanofiber formation resulting from self-assembly of $K_2(SL)_6K_2$ peptides (D) SEM image of the $K_2(SL)_6K_2$ hydrogel, scale bar is equal to 200 nm. Inset shows the clear hydrogel. (E) Cartoon showing subcutaneous injection of hydrogel into the dorsal tissue of rats. (F-I) Illustration of the time course of hydrogel remodeling beginning with cell infiltration followed by vascularization and innervation and slow degradation. (F) At day 0 the $K_2(SL)_6K_2$ hydrogel contains no cells,

blood vessels, or neural fascicles present. (G) At Day 3 the implant becomes infiltrated by cells. (H) Weeks 1–3 the implant begins to degrade and is infiltrated by cells, blood vessels, and neural fascicles. (I) By week 6 neural fascicles recede and the implant further degrades.

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Fig. 2.

Cellular infiltration of the $K_2(SL)_6K_2$ hydrogel. (A) Hematoxylin and eosin stained tissue sections with images taken near the center of the hydrogel implant. The image for day 3 is taken slightly off center to show the uneven distribution of cells within the hydrogel at this time point. Exact location of each image within the tissue section and week 2 and 3 time points are available in supporting information figures S2 and S3. Scale bars are equal to 50 μ m. (B) Quantification of cell nuclei counted per mm² of hydrogel area. (C) Cytokine array analysis of chemokines identified within the hydrogel implant. For (B) and (C), data with the same Greek letter are not statistically different from one another, while data with different Greek letters are statistically different from one another. Error bars indicate the standard error of the mean.

Fig. 3.

Degradation of $K_2(SL)_6K_2$ subcutaneous implants. (A&B) Masson's trichrome staining of an implant at 3 days (A) and 6 weeks (B) post-injection. Hydrogel implants are demarcated by a dotted line, and scale bars are equal to 500 μ m. (C) Masson's trichrome staining of implants and surrounding native tissue at 3 days and 1, 2, 3, or 6 weeks post-injection. Images are taken at the edge of the implant with the implant demarcated by the dotted black line. Scale bars are equal to 50 μ m. (D) Hydrogel area measured from histological sections at each experimental time point. (E) Detection of MMP-8 and TIMP-1 proteins within the hydrogel implant. For (D) and (E), data with the same Greek letter are not statistically different from one another, while data with different Greek letters are statistically different from one another. Error bars indicate the standard error of the mean.

Fig. 4.

Vascularization of $K_2(SL)_6K_2$ subcutaneous implants. (A) Cartoon depiction of blood vessel structure. (B) H&E staining of a blood vessel found inside the hydrogel at 6 weeks post-injection. Scale bar is equal to 25 μ m. (C&D) α -SMA actin staining of the center of an implant after 3 days (C) and 2 weeks (D). Positive α -SMA expression is shown in red, and cell nuclei are DAPI-stained. Scale bars are equal to 50 μ m. (E) Number of blood vessels counted per mm² of hydrogel area. (F) Relative protein levels of VEGF and PDGF-AA collected from subcutaneous implants. For (E) and (F), data with the same Greek letter are not statistically different from one another, while data with different Greek letters are statistically different from one another. Error bars indicate the standard error of the mean.

Fig. 5.

Interaction with the peripheral nervous system. (A) Cartoon representation of a nerve (B) H&E staining of neural fascicles identified within the subcutaneous implant at 6 weeks post-injection. Scale bar is equal to 50 μ m. (C) Neural fascicle identity is positively confirmed through immunostaining for NFP (green) and β -III tubulin (red). Scale bar is equal to 25 μ m. (D) Axons are shown to be myelinated (MBP: purple) with Schwann cell supporting cells present (S100: cyan). Scale bar is equal to 25 μ m. (E) Quantification of neural fascicles found within the hydrogel. (F) Relative protein levels of β -NGF and CNTF in subcutaneous implants. For (E) and (F), data with the same Greek letter are not statistically different from

one another, while data with different Greek letters are statistically different from one another. Error bars indicate the standard error of the mean.

Fig 6.

Expression of inflammatory cytokines. Pro-inflammatory cytokines include TNF- α , IL-1 α , IL-1 β , IL-6, IFN- γ , while IL-10 and IL-4 are anti-inflammatory cytokines. Data with the same Greek letter are not statistically different from one another, while data with different Greek letters are statistically different from one another. Error bars indicate the standard error of the mean.