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Dynamic *In Vivo* Biocompatibility of Angiogenic Peptide Amphiphile Nanofibers

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Abstract

Biomaterials that promote angiogenesis have great potential in regenerative medicine for rapid revascularization of damaged tissue, survival of transplanted cells, and healing of chronic wounds. Supramolecular nanofibers formed by self-assembly of a heparin-binding peptide amphiphile and heparan sulfate-like glycosaminoglycans were evaluated here using a dorsal skinfold chamber model to dynamically monitor the interaction between the nanofiber gel and the microcirculation, representing a novel application of this model. We paired this model with a conventional subcutaneous implantation model for static histological assessment of the interactions between the gel and host tissue. In the static analysis, the heparan sulfate-containing nanofiber gels were found to persist in the tissue for up to 30 days and revealed excellent biocompatibility. Strikingly, as the nanofiber gel biodegraded, we observed the formation of a *de novo* vascularized connective tissue. In the dynamic experiments using the dorsal skinfold chamber, the material again demonstrated good biocompatibility, with minimal dilation of the microcirculation and only a few adherent leukocytes, monitored through intravital fluorescence microscopy. The new application of the dorsal skinfold model corroborated our findings from the traditional static histology, demonstrating the potential use of this technique to dynamically evaluate the biocompatibility of materials. The observed biocompatibility and development of new vascularized tissue using both techniques demonstrates the potential of these angiogenesis-promoting materials for a host of regenerative strategies.

Keywords

angiogenesis; biocompatibility; peptide amphiphile; regenerative medicine; self-assembly

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1. INTRODUCTION

The potential of nanotechnology in regenerative medicine has gained momentum in recent years [1,2]. Rational bottom-up design of biomaterials, beginning at a molecular level, makes possible the delivery of signals on a cellular and subcellular level, potentially mimicking the sophisticated native biological signaling machinery. Supramolecular self-assembly has been employed frequently to synthesize biomaterials with controlled spatial display and density of bioactive signals through attention to molecular design. Stupp et. al. developed a broad class of molecules known as peptide amphiphiles (PA) capable of such supramolecular self-assembly into bioactive nanofibers [3,4]. These PAs consist of a hydrophobic alkyl segment covalently linked to a peptide sequence containing at least two domains: an amino acid sequence that promotes self-assembly of the molecules through the formation of β -sheets [5] and a bioactive domain that can be customized to interact with specific receptors, ligands, cellular targets, or biopolymers. Charged residues in the peptide sequence are always part of the design for solubility and, more importantly, to use electrolytes in physiologic fluid for electrostatic screening to trigger PA self-assembly into high aspect-ratio nanofibers. These nanofibers can entangle into gel networks at concentrations on the order of 1% by weight in aqueous media [6–8]. The dimensions of these assembled PA nanofibers range from approximately 6 to 10 nanometers in diameter with their length on the order of microns, thereby mimicking the size scale of filamentous structures in natural extracellular matrix. The supramolecular architecture of these nanofibers allows them to display the bioactive peptide domains on their surfaces with an orientation normal to the long axis of the fiber. The forces governing their self-assembly involve formation of hydrogen bonds among amino acid residues as well as hydrophobic collapse of their alkyl segments in high ionic strength aqueous media.

These peptide amphiphiles have been used previously for many biological applications, both *in vitro* and *in vivo*. PAs presenting the laminin-derived IKVAV peptide epitope promote selective differentiation of neural progenitor cells *in vitro* [9] and inhibit glial scar formation in a mouse spinal cord injury model [10]. Other PAs have been developed as biomaterials presenting the RGDS peptide epitope with several covalent architectures [11–13]. Some PAs have been combined with metallic implants to form bioactive hybrid structures [14–16], and used to coat traditional tissue engineering scaffolds [17]. PAs have also been designed to include MRI contrast agents [18,19]. One particular PA under active development for biomedical applications is termed a heparin-binding peptide amphiphile (HBPA). This PA was designed with a Cardin-Weintraub heparin-binding domain to specifically bind and self-assemble in the presence of heparan sulfate-like glycosaminoglycans (HSGAG), allowing for the capture of growth factors that contain heparin-binding domains and resulting in biomimetic growth factor display [20,21]. Importantly, heparan sulfate plays a role as a cofactor in angiogenesis, specifically in vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) molecular biology through factor binding, receptor stabilization, and protection from proteolysis [22–25]. When combined with nanogram quantities of these angiogenic growth factors, this material exhibited prolonged protein release and substantial vascularization in a rat corneal angiogenesis model [21]. In ongoing studies, this material is being explored in applications for ischemic tissue regeneration, where the development of new blood vessels limits healing. Islet transplantation, which is limited by low vascular density leading to poor engraftment in the transplant site, was improved through the use of HBPA loaded with growth factors, showing an enhancement of peri-transplant vasculature and significant normoglycemic restoration in diabetic mice. [26] Also, small molecule nitric oxide (NO) donors, when combined with HBPA, showed a prolonged NO release profile leading to reduced neointimal hyperplasia in a rat carotid artery balloon injury model. [27] Though several PA systems, including HBPA, have demonstrated promise as biomaterials for regenerative medicine, the tissue reaction and biodegradation of these materials has not been explicitly assessed using *in vivo* models.

This study examines the tissue reaction to HBPA nanofiber gel networks *in vivo* and furthermore the effect heparan sulfate presentation has on this tissue reaction. Static assessment, using the subcutaneous implantation model, was done to characterize any inflammatory tissue reaction to the implanted nanofiber gel and characterize the cells involved in its degradation. This model also allowed for quantitative histological evaluation of the vascularization of the material. Dynamic assessment with the skinfold chamber model allowed us to monitor the same tissue site in a dynamic mode using a living animal over a 10 day period. This enabled direct examination of the microcirculatory response to the material, such as microvessel diameter changes, integrity of the endothelium, and leukocyte-endothelial interactions. This work represents a novel application of the skinfold chamber model to test the early tissue reaction to biomaterials. Therefore, an additional objective apart from specifically determining the tissue reaction to PA nanofiber gel networks was to explore the utility of this dynamic model in conjunction with a more conventional subcutaneous implantation model.

2. MATERIALS AND METHODS

2.1 Peptide Amphiphile Synthesis and Purification

Heparin-Binding Peptide Amphiphile (**HBPA**, palmitoyl-AAAAGGGLRKKLGKA, $M_w = 1606.1$ g/mol) shown in Figure 1A was described previously [21] and was prepared for this study in the laboratories of Nanotope Inc. (Skokie, IL, USA) on a 3 mmole scale, using automated solid-phase peptide synthesis (CS Bio Co. 136XT synthesizer). Purification of this molecule by reverse-phase HPLC obtained HBPA as the chloride salt with 98% peptide purity in an overall yield of 3.72 g (77%). 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, MBHA Rink amide resin, and HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) were purchased from EMD Chemicals Inc. All other reagents and solvents were purchased from VWR or Sigma-Aldrich Inc. and used as received. Fmoc deprotection was performed with 30% piperidine in dimethylformamide (DMF) for 10 min, repeated twice. Amino acid couplings were done with 4 equivalents of Fmoc protected amino acid, 3.75 equivalents HBTU, and 6 equivalents of DIEA (N,N-diisopropylethylamine) in DMF for 1 h. The reaction was performed under nitrogen gas with constant agitation. Acetylation of any unreacted amines after each coupling step was performed with 10% acetic anhydride in DMF for 10 minutes, repeated twice. Palmitic acid was coupled to the N-terminus of the peptide as above except using an 80:20 DMF/DCM mixture. The coupling reaction was repeated until a negative result for free amine was obtained using the Kaiser test. The crude peptide was cleaved and side-chain protecting groups were removed by agitation of the resin in 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% water. Excess TFA was removed by rotary evaporation and the resulting viscous peptide solution was triturated with cold diethyl ether. A white precipitate was collected and dried *in vacuo*, redissolved in water and purified using reversed-phase high-performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1200 series preparatory scale system fitted with a Waters Inc. column (250×30 mm) using the Atlantis™ 5 μ m, 110A dC₁₈ stationary phase. HBPA was eluted using a mobile phase gradient consisting of water and acetonitrile, each containing 0.1% v/v TFA, with the product monitored by UV absorption at 220 nm. The collected product was shell-frozen in a dry ice/isopropanol bath and the solvent removed by lyophilization. Trifluoroacetate counter-ions were subsequently exchanged by sublimation from 0.01 M hydrochloric acid at a peptide concentration of approximately 5 mg/ml. The purified peptide was dissolved at 10 mg/ml in sterile USP Water for Injection, aseptically filtered through a 0.8/0.2 μ m Supor® Membrane filter (hydrophilic polyethersulfone, Pall Life Sciences), aliquotted into vials, and lyophilized using a Labconco 6L FreeZone™ system. Secondary drying was performed by heating vials to 40 °C for six hours *in vacuo*. Vials were back-filled with sterile, dry nitrogen gas and stoppered *in situ*. The

freeze-dried peptide was then stored at -20°C until use. The identity, purity and peptide content of the product were confirmed by analytical HPLC (Nanotope Inc., Skokie, IL), matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Northwestern University, Evanston IL), and amino acid analysis (Commonwealth Biotechnologies, Richmond, VA). Content uniformity was confirmed by the weight variation method (USP <905>, Nanotope Inc.) Salt content of the product was determined to be predominantly the chloride salt by ion chromatography (8.26 wt% Cl^{-} as compared to only 0.074 wt% TFA^{-}) (Galbraith Laboratories, Knoxville, TN). Residual water content was determined by Karl Fisher titration per USP <921> to be 2.8 wt% (Galbraith Laboratories). The molar absorptivity was found to be $\epsilon_{220\text{nm}} = 4940 \pm 220 \text{ cm}^{-1} \text{ M}^{-1}$ and the specific optical rotation was $[\alpha]_{\text{D}} = -0.673^{\circ} \pm 0.002^{\circ}$ (Nanotope Inc.).

The FITC PA used for visualization, shown in Figure 1B, was synthesized by similar methods. Briefly, the peptide and alkyl portion of the molecule (palmitoyl- $\text{V}_3\text{A}_3\text{K}_3\text{G}_2\text{KGKGK-NH}_2$) was synthesized by automated peptide synthesis. The ϵ -amine of the C-terminal lysine was protected with a 4-methyltrityl group (Mtt), while the remaining lysines were Boc protected. Mtt was selectively removed through agitation of the resin in 4% TFA and 4% TIS in DCM for five minutes, repeated four times. Subsequently 4 equivalents of fluorescein isothiocyanate (FITC) in DMF was added to the resin and agitated for three hours. The peptide was cleaved and purified in the same way as HBPA. For studies utilizing the FITC PA for visualization, it was combined with the HBPA at a ratio of 2 mol%.

Heparan Sulfate (HS) used for this study was purchased from Celsus Laboratories (Cincinnati, OH USA). Heparan sulfate, shown in Figure 1C, is a naturally occurring sulfated glycosaminoglycan with similar chemical structure to medicinal heparin, but with a lower extent of sulfation [28]. This structural difference contributes to reduced antithrombin binding for heparan sulfate as compared to heparin, decreasing the likelihood of complications related to excessive localized anticoagulation in the tissue surrounding the biomaterial. For this reason, we used heparan sulfate for the studies performed here. The heparan sulfate was resuspended at 20 mg/ml in phosphate buffered saline, aliquotted into vials, and lyophilized using a Labconco 6L FreeZoneTM system. Secondary drying was performed by heating vials to 40°C for six hours *in vacuo*. Vials were back-filled with sterile, dry nitrogen gas and stoppered *in situ*. The freeze-dried heparan sulfate was then stored at -20°C until use.

2.2 Animals

All experiments were performed with approval of the Committee on the Use of Live Animals in Teaching and Research, Rhineland-Palatinate, Germany. For these studies, female CD-1 mice (6-to 8-week old, 20–25 g body weight, Charles River Laboratories, Sulzfeld, Germany) were used and housed one per cage, kept with water *ad libitum*, an artificial light-dark regime, and fed with regular mouse pellets (Laboratory Rodent Chow, Altromin, Germany) at the Laboratory Animal Unit of the Institute of Pathology, Johannes Gutenberg University, Mainz, Germany.

2.3 Subcutaneous Implantation Model

For static assessment of the tissue reaction to HBPA nanofiber gels, 66 mice were randomly assigned to four groups. Treatments for each group were as follows: HBPA-heparan sulfate (HBPA-HS) nanofiber gel, HBPA-phosphate nanofiber gel, heparan sulfate alone, and a sham-operated phosphate buffered saline (PBS) control. For the nanofiber gel groups, animals were prepared for 'early response' time points of 3 (n=7) and 10 (n=7) days as well as for 'late response' time points of 30 (n=3) and 60 (n=4) days. For the heparan sulfate and PBS groups, a reduced number of animals (n=3) was used for each of the study time points. Anaesthesia with a mixture of ketamine (90 mg/kg) and xylazine (25 mg/kg) was administered by

intraperitoneal injection. Nanofiber gels were prepared as follows. HBPA and heparan sulfate were resuspended from lyophilized powder at 3 w/v% and 2 w/v%, respectively, using sterile USP Water for Injection. For the group receiving HBPA-HS nanofiber gels, 100 µl of each component were mixed within a 1 ml syringe, for 200 µl total gel volume. For the group receiving HBPA-phosphate nanofiber gels, 100 µl of HBPA was mixed within a 1 ml syringe with 100 µl of 0.1 mM Na₂HPO₄ to form a 200 µl HBPA nanofiber gel in the absence of heparan sulfate. For the group receiving heparan sulfate alone, 100 µl of the heparan sulfate solution was combined in a 1 ml syringe with 100 µl of sterile water. The sham operated PBS control received 200 µl of PBS. The skin of the rostral portion of the interscapular region was shaved and each substance was injected percutaneously in the subscapular region using a 20-gauge needle under sterile conditions following a previously described method [29]. All animals survived the indicated time periods without any complications.

2.4 Histological Preparation of the Implantation Bed

The animals were euthanized by an overdose of Ketamine and Xylazin at the indicated time points. Immediately after sacrifice, the implantation bed was explanted together with the surrounding peri-implant tissue and fixed in 4% buffered formalin for 24 h prior to histological and immunohistochemical analysis. The fixed tissue was cut into seven segments of identical dimensions covering the margins and center of the implantation bed. They were dehydrated in a series of alcohol, transferred to xylene, and embedded in paraffin. From each segment, three consecutive 3–4 µm thick slices were deparaffinized and rehydrated. These slices were stained with Mayer's hematoxylin and eosin (H&E) and examined to establish which segment contained the most representative view of the nanofiber gel. This was selected for qualitative histopathological evaluation. From the corresponding histological block, seven consecutive slices were prepared for further histological analysis. Two sections were used for immunohistochemical detection of murine vessels and macrophages to monitor the cellular tissue response to the implanted nanofiber gels. Endogenous peroxidase activity was quenched by treatment with 4% H₂O₂ in methanol. After blocking with horse serum, one section was stained with rabbit polyclonal vWF antibody for vessels and the other with rabbit polyclonal CD 68 for macrophages (GeneTex, Inc., USA, diluted 1:200). The visualization procedure was performed using the DAKO REALTM EnVisionTM detection system (DAKO, Glostrup, Denmark). As negative control for these two immuno-stains, the next consecutive sections were treated similarly, but with the primary antibody omitted. All control stains were negative. Finally, the samples were counter-stained with haemalaun and viewed by light microscopy.

2.5 Histochemistry for Connective Tissue Ingrowth

The remaining three slides were prepared to visualize connective tissue ingrowth within the implantation bed using histochemical staining procedures for Azan, Movat's pentachrome and Sirius red [30,31]. These stains are commonly used for the detection of reticular and collagen fibers. Azan stain indicates collagen and reticular fibers blue. Sirius red highlights collagen fibers red while Movat's pentachrome stains collagen green. All chemicals were purchased from (Sigma-Aldrich, Germany) and used without further purification. The histopathological assessment was conducted using images from all four histochemical staining protocols, though only H&E and Azan are shown in the accompanying figures since they most clearly illustrated the effects we observed.

2.6 Histological Analysis for Systemic Effects

In order to establish any potential systemic effect of the nanofiber on cardiovascular relevant organs, these organs were also harvested at each indicated time point. After explantation of the implantation bed the heart, brain, lung, liver, and kidneys were harvested and cut in 2–3 mm cross sections. From each segment, a 3–4 µm thick paraffin slice was then deparaffinized,

rehydrated and subsequently stained with H&E in order to detect any systemic effect of the injected materials on these organs, examining for histological differences compared to the sham operated PBS control.

2.7 Morphological Evaluation of the Inflammatory Response

Histopathological evaluation was conducted using a Nikon ECLIPSE 80i microscope (Nikon, Japan) by two independent examiners experienced in histomorphological analysis who were blinded to the experimental protocol. The histological slides of the implantation bed, as well as of the organs, were assessed qualitatively for the following characteristic features: Fibrotic capsules around the biomaterials, fibrosis, hemorrhage, necrosis, vascularization, neutrophils, lymphocytes, plasma cells, macrophages, giant cells and degradation. Microphotographs were taken using a Nikon DS-Fi1/Digital camera and a digital sight control unit (Nikon, Japan). While only H&E and Azan are displayed in the paper, images from all four staining protocols listed in section 2.5, in addition to vWF and CD68 immunostains, were used to verify all qualitative assessments resulting from our histological evaluation.

2.8 Histomorphometric Analysis

Histomorphometric analysis was performed using the software *NIS-Elements* (Nikon, Tokyo, Japan) according to the manufacturer's instructions. Briefly, images were obtained with a DS-Fi1 digital camera connected to an *Eclipse 80i* histological microscope (Nikon, Tokyo, Japan), equipped with an automatic scanning table (Prior, USA). A total scan, one large image assembled from 100–120 images (Figure 2) of the region of interest containing the nanofiber gel and the corresponding peri-implant tissue, was taken by using a 100x magnification at a resolution of 2500×1200 pixels. For each animal the slide that was stained with the anti-rabbit vWF antibody (see histological preparation) was used to detect the host vascularization of the scaffold. Using the *NIS-Elements* "annotations and measurements" tool, the visible nanofiber gel area and blood vessels were marked separately by the "area" tool. The total number of vessels on each slide was determined. The number of vessels/mm² was calculated from the total vessel number found within the visible segments of the nanofiber gel. For each time point, a mean number of vessels/mm² was determined. While blood vessels were quantified using vWF immunostaining, they are also evident using H&E and Azan staining at higher magnification. We have presented these two stains to illustrate the formation of new vasculature in the accompanying figures because, in addition to showing vasculature, these stains also provide information about the nanofiber gel along with the morphology of cells within, and in the periphery of, the nanofiber gel.

2.9 Skinfold Chamber Model

The dorsal skinfold chamber technique was used for repeated microcirculatory analysis of living animals over a period of 10 days [32,33]. Four CD-1 mice were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (25 mg/kg) administered by intraperitoneal injection. The dorsal skin was shaved and two symmetrical titanium frames were implanted to sandwich an extended double layer of the dorsal skin. The epidermis and dermis of the topside of the skin was completely removed in a circular area 15 mm in diameter. HBPA and heparan sulfate were resuspended from lyophilized powder at 3w/v% and 2w/v%, respectively, using sterile USP Water for Injection. The HBPA was supplemented with 2mol% FITC PA for visualization and 10 µl of HBPA+FITC was combined with 10 µl of heparan sulfate in a sterile tube to form the nanofiber gel. A pipette was used to transfer 10 µl of this gel to the center of the prepared circular subdermal region. After applying the material, a removable coverslip was placed over the exposed tissue and fastened into one of the titanium frames. Saline was used to eliminate air bubbles from within the chamber. Figure 6C shows a digital photograph of the fluorescent nanofiber gel within the skinfold window. Each surgical procedure was carried out

under strictly aseptic conditions. The animals tolerated the chambers well and showed no signs of discomfort or changes in sleeping or feeding habits. The macroscopic and microscopic appearance of the skinfold chamber preparations was monitored daily. Intravital fluorescence microscopic assessment of angiogenesis, microhemodynamics, and leukocyte-endothelial cell interaction was performed immediately after implantation and then daily for the subsequent 10 days, always performed on awake animals.

2.10 Intravital Fluorescence Microscopy

For *in vivo* microscopic observation, the live animal was placed unimpeded by anaesthesia in a plexi-glass tube with the dorsal skinfold chamber frame protruding from the tube and attached to the microscope stage. Using a fluorescent microscope with a 100 W mercury lamp (Nikon ECLIPSE 80i microscope) equipped with standard blue/green/red filters in addition to a brightfield source, the striated muscle microcirculation was imaged. For tracking the FITC-incorporated HBPA nanofiber gel, the 4x objective (40x magnification) was used. The same region of interest (ROI) was captured each day, acquiring green fluorescence and brightfield images sequentially at each timepoint. The exposure time was kept constant for each day to note any changes in the gel density over time.

For intravital tracking of leukocytes, rhodamine 6G (Sigma-Aldrich, Germany) was prepared at 1wt% in saline and 0.1 ml of this solution was injected intravenously into the tail vein. This dye localizes in the mitochondria of viable leukocytes and enables detection of these cells in vital microvasculature even at high flow rates [34]. Images for leukocyte tracking were collected using a 20x objective (200x) magnification, acquiring brightfield, green fluorescence (HBPA) and red fluorescence (leukocytes) sequentially in the same area.

2.11 Microcirculatory Analysis

Quantitative offline analysis of the digital images collected was performed by computer-assisted image analysis systems *NIS-Elements* (Nikon, Tokyo, Japan) and *Image J* (a public domain, Java-based image processing program developed at the NIH, USA). Directly after implantation, regions of interest (ROI) containing the murine microcirculation at the interface of the HBPA-HS nanofiber gel were established. Within this ROI, the average diameter of microvascular post-capillary or collecting venules was measured for each animal at each day and compared as a percent of the baseline day 0 vessel diameter to assess the effect of the material on the adjacent microcirculatory dilation. Additionally, the microcirculation was assessed qualitatively for vascular integrity and the chamber was evaluated for the presence of any edema.

2.12 Histological Preparation of the Implantation Bed

Following the 10 day evaluation period for the skinfold chamber model studies, the animals were sacrificed by an overdose of Ketamine and Xylazin. Histological examination of the tissue within the chamber was performed similarly to that described for the subcutaneous implantation model. The skin fold and accompanying titanium frame were surgically removed and submerged in fixative for 24 hours. After fixation, the tissue within each chamber was cut in 2–3 mm cross sections, dehydrated, transferred to xylene, and embedded in paraffin. Each segment was sectioned to produce a 3–4 μ m thick paraffin slice, which was subsequently deparaffinized and rehydrated. These sections were stained with H&E and examined for any effect of the implanted material on underlying tissue or adjacent vasculature.

3. RESULTS

3.1 Subcutaneous Implantation of HBPA-HS Nanofiber Gel

Three days following subcutaneous implantation of the HBPA-HS nanofiber gel, large-scale gel fragments were apparent within the implantation bed, as shown in Figure 2 and Figures 3A and 3B. These pieces of gel appeared internally homogeneous with no signs of penetration by macrophages, granulocytes, or other inflammatory cells. Overall, the HBPA nanofiber gel, while acting as a barrier to infiltration of peri-implant cells, remained well integrated at the peri-implant interface, with no signs of any fibrotic capsule formation. At this time point, the area of this visible gel on the entire histological slide was 3.3 mm². (Figure 4A) There were no blood vessels found within the HBPA-HS nanofiber gel at this early time point. Finally, there were no signs of acute infection resulting from the implantation of the nanofiber gel or from the surgical procedure used to implant it.

Ten days after implantation, the overall structure of the HBPA-HS nanofiber gel remained visible as homogenous bulk fragments, as shown in Figure 3C and 3D, similar to those observed at day 3. At this time point, however, the nanofiber acted as a matrix for fibroblasts, which had begun to enter the gel from the surrounding peri-implant mesenchyme (see Figure 3C). The extracellular matrix produced by these fibroblasts became the support for newly formed microcirculatory vessels within the nanofiber gel. At 10 days, there was an average of 16 blood vessels per mm² within the gel, with the area of visible gel totalling 1.6 mm², a reduction from the area occupied by visible aggregates at day 3 (Figure 4A and 4B). This biodegradation of the bulk nanofiber gel began at the peri-implant periphery, primarily by macrophages. As these large aggregates of gel were reduced in size through degradation, the matrix began a conversion towards a well-vascularized connective tissue. This conversion began at the periphery of the implanted gel network, initiated from the peri-implant tissue. There was no indication of any multinucleated giant cells or lymphocytes within the nanofiber gel or at the gel-tissue interface, and the overall reaction was consistent with a very mild inflammatory response.

By 30 days after implantation, the majority of the previously seen gel fragments were either gone or drastically reduced in size, as shown in Figures 3E and 3F. The visible fragments occupied only an average of 1.1 mm² of a total histological slide (see Figure 4A). However, the process that began at day 10 had continued, with the nanofiber gel and its former implantation bed converted into a well-vascularized connective tissue containing 32 blood vessels per mm² (Figure 4B). Some macrophages remained at day 30, but there was still no sign of any multi-nucleated giant cells or lymphocytes.

By day 60 after implantation, there was no longer any visible sign of nanofiber gel within the implantation bed. What remained of the once well-vascularized connective tissue seen at day 30 was a thin, minimally vascularized scar tissue consistent with the very mild inflammation we had seen at earlier timepoints. There was no sign of either acute or chronic inflammation, with no macrophages, lymphocytes, or multinucleated giant cells present in the peri-implant region.

3.2 Subcutaneous Implantation of HBPA-phosphate Nanofiber Gel

Formation of HBPA with phosphate ions produced nanofiber gels with similar qualitative consistency prior to implantation as those prepared by the addition of heparan sulfate (HS). By day 3 after implantation, it was apparent from histopathological evaluation that the tissue reaction to this nanofiber gel without HS was substantially different from that for the nanofiber gel containing HS (see Figures 5A and 5B). While gel fragments not infiltrated by cells were still present, they appear more mosaic-like in structure, with less interconnectivity and more space between fragments. The space between these gel pieces acts as a conduit for infiltration

of some granulocytes and a large number of macrophages, already beginning the initial phases of nanofiber gel degradation after only 3 days. Additionally, there was no sign of vessel formation within the nanofiber gel.

As shown in Figures 5C and 5D, ten days after implantation of the HBPA-phosphate nanofiber gel, the number and size of visible nanofiber gel fragments was reduced. The mosaic-like appearance remained, with continued degradation of the gel by macrophages. There was still no evidence of vessel formation within the gel at this time, nor were there signs of the formation of any new connective tissue matrix. At no point in these first 10 days was there any evidence of multinucleated giant cells or lymphocytes within the gel or at its periphery.

By day 30 after implantation, there was no longer any visible sign of the HBPA-phosphate nanofiber gel aggregates within the implantation bed. What remained in the implantation bed was a scar tissue similar to that observed at 60 days in the implantation bed of the HBPA-HS nanofiber gel. At no point was there any new vascularization in the implant bed.

3.3 Subcutaneous Implantation Controls

As expected, there was no sign of any histological abnormality at any point during the study for the sham operated PBS group, indicating no issues with sterility in the implantation procedure. Additionally, no histological abnormality and no signs of haemorrhage were detectable for the group receiving heparan sulfate alone as a control. Specifically, this group showed no increase in new blood vessel formation in or near the implantation bed. In the systematic autopsy study, histology of all cardiovascular-relevant organs showed no histological abnormalities in any of the treatment groups for any of the organs examined. There was no detectable difference between the sham-operated group and the groups implanted with either of the nanofiber gels, indicating no systemic effect as a result of the administration of the nanofiber gel.

3.4 Skinfold Chamber Model

The HBPA-HS nanofiber gel, mixed with 2 mol% FITC-PA, was implanted within a dorsal skin fold chamber (see Figure 6C). Tracking this nanofiber gel and the adjacent vasculature using intravital fluorescence microscopy, the gel was found to persist in the chamber through the end of the study at 10 days, as shown in Figure 6A. The same region of interest was tracked over the 10-day study in each animal examined (n=4), allowing for the same microcirculatory features to be repeatedly monitored. The exposure time for image capture was kept constant throughout the study and the gel showed no noticeable decrease in fluorescence, suggesting no appreciable loss of gel volume over these first 10 days within the chamber. The adjacent microcirculation exhibited no signs of pathophysiological dilatation, demonstrated through monitoring the diameter of microcirculatory collecting vessels over time, as shown in Figure 6B. Some dilation was evident in the first few days following implantation, eventually subsiding and returning to approximately the pre-implantation diameter. However, mild dilation is not inconsistent with expectation for the surgical procedure of inserting the dorsal skinfold chamber, and thus not indicative of a pathophysiological response. There was no sign of microvessel leakage or edema within the chamber.

Leukocytes, stained with rhodamine, were monitored within the skinfold chamber at the interface of the HBPA-HS nanofiber gel. This technique permitted these cells to be tracked in real time as they moved through the microcirculation. Some leucocytes were seen to be adherent to the endothelium within the first 5 days, though most moved freely with blood flow within the vessel lumen, as shown in Figure 7. The presence of adherent cells subsided after day 5 until the end of the study. At no point during the study was there any evidence that these cells contributed to microvascular leakage.

Conventional histology of the skinfold chamber tissue at the day 10 endpoint of the study revealed an occurrence similar to that seen for the subcutaneous implantation study. As shown in Figure 8, the HBPA-HS nanofiber gel applied on top of the underlying tissue within the chamber was enveloped in a newly vascularized connective tissue arising from the underlying mesenchyme. A newly formed vasculature could be seen within the nanofiber gel, embedded in a new connective tissue matrix. Degradation by macrophages was apparent at this time point, and there was no sign of any giant cells, lymphocytes, or severe immune response.

4. DISCUSSION

Biomaterials formed from supramolecular assemblies have generated excitement for their ability to incorporate well-defined molecular-level signalling to produce bioactive scaffolds for regenerative medicine [1,2]. One such system, the heparin-binding peptide amphiphile (HBPA), was designed to specifically bind heparan sulfate-like glycosaminoglycans during nanofiber self-assembly [21]. Through these associated glycosaminoglycans, the supramolecular assembly creates a gel network that is able to bind angiogenic growth factors, such as VEGF and FGF-2, in a biomimetic fashion. These gels promote angiogenesis *in vivo*, as demonstrated through extensive blood vessel formation in a rat cornea. Slow or inadequate vascularization is frequently a limiting factor in the selection of biomaterial scaffolds for applications in regenerative medicine. From the treatment of chronic non-healing wounds to therapeutic cell delivery, accelerating vascularization has the potential to enhance the efficacy of many regenerative therapies [35,36]. For this reason, its potential to induce or modulate angiogenesis makes this HBPA system one of the most exciting of a host of PA systems currently under active development. The work reported here offers the first detailed study of the biocompatibility of these PA systems, specifically examining the biocompatibility of the angiogenic HBPA system. but, until now, little was known about the inflammatory tissue reaction elicited by such a synthetic material *in vivo*.

In this study, we analysed the inflammatory response to HBPA nanofiber gels formed with heparan sulfate. We employed a subcutaneous implantation model to characterize the tissue reaction to these gels. This permitted a traditional static histological assessment of the inflammatory response resulting from application of these materials within tissue. In this way, the tissue reaction could be assessed qualitatively, tracking via histology the integrity of the gel, infiltration by inflammatory cells, degradation and detection of the cells involved in this process, interaction with the peri-implant tissue, fibrous capsule formation, haemorrhage, and vascularization. Additionally, this model facilitates quantitative analysis of the histological preparation to measure the total nanofiber gel area and extent of vascularization. The information gained from such an intensive histological study is necessary to truly understand the tissue reaction to this material prior to further clinical development.

Subcutaneous implantation of HBPA-HS nanofiber gels results in a very favorable tissue reaction. In the first three days, the gel serves a barrier-like function, as cells in the peri-implant region do not penetrate the interior portion of the gel. Though acting as a barrier, there is no fibrous capsule formation and the gel is well integrated into the peri-implant tissue. The nanofiber gel appears primarily homogenous, a trait that remains even as it begins to be infiltrated from the periphery by fibroblasts and macrophages. Interestingly, the HBPA-HS nanofiber gel prompts these infiltrating fibroblasts to produce matrix that serves as a foundation for the development of microvessels within the gel, a phenomenon first observed at the 10-day timepoint. After 30 days, the transformation of the implant bed into a well-vascularized connective tissue is complete. By day 60, the nanofiber gel as well as the *de novo* vascularized tissue had disappeared, with the only evidence of their existence being remaining scar tissue, indicating reorganization of this vascularized tissue. At no time during the study were there signs of a severe inflammatory response, and no giant cells or lymphocytes were evident.

By contrast, when the nanofiber gel was prepared with phosphate instead of heparan sulfate, the tissue reaction was quite different. The morphology of the gel after 3 days appeared less as a homogenous structure, and more a mosaic of aggregates with spaces in between. These aggregates were surrounded by granulocytes and macrophages that entered the implant bed and had already begun degradation of the nanofiber gel. At day 10, the degradation of the gel continued, primarily by macrophages. By day 30, there was no sign of the nanofiber gel, and what remained was a scar tissue similar to that seen at day 60 for the HBPA-HS nanofiber gel. This nanofiber gel, produced without heparan sulfate, no longer has the barrier-like function within the first three days that the heparan-containing gel demonstrated. Additionally, this gel never induced the formation of new vasculature. Though there were also no signs of a severe inflammatory response. Even though the HBPA-phosphate nanofiber gel did not elicit a strong inflammatory response, its tissue reaction differed substantially from that of the heparan sulfate system.

This study demonstrates good biocompatibility for the HBPA material, both in the presence and absence of heparan sulfate. The PA is composed of a fatty acid tail and amino acids, both naturally occurring constituents. This is combined with heparan sulfate, a natural glycosaminoglycan present in the endothelium. However, the supramolecular assemblies of these small molecule precursors are not naturally occurring. These assemblies, while producing quite robust gelled networks, were degraded by macrophages and did not elicit a strong foreign body response, nor were giant cells required for degradation. Therefore, confirmation that there was no observable toxicity attributable to either the monomer or its supramolecular assembly with and without heparan is an encouraging and important finding for future clinical applications.

The most striking finding is the development of *de novo* vascularized tissue in the implantation bed when HBPA-HS nanofiber gels were injected. This result was not observed in nanofiber gels prepared without heparan sulfate, even though this gel persisted in the tissue for at least 10 days. Additionally, controls receiving only heparan sulfate showed no enhanced vascularization in the implantation bed. The incorporation of heparan into the nanofiber gel also leads to a more histologically homogenous appearance, suggesting that the interconnectivity of the gel is enhanced by the incorporation of heparan, preventing the formation of the mosaic-like aggregate arrangements seen in the phosphate nanofiber gel. Both of these observations are consistent with the design strategy of the HBPA system. The HBPA, possessing a heparin-binding sequence, serves to create a nanofibrous scaffold upon interaction with heparan sulfate-like glycosaminoglycans. However, the designed bioactivity of this system, its ability to concentrate and properly display signalling domains of angiogenic factors to their receptors, is linked to the presence of heparan sulfate [20,21]. Additionally, it is reasonable to expect that HBPA nanofiber gels containing a polyanion such as heparan sulfate would possess greater interconnectivity than gels formed through the addition of divalent phosphate ions. Therefore, the differences observed between the HBPA-HS nanofiber gel and the gel produced with phosphate can likely be attributed to the presence of heparan sulfate.

The mechanism for the formation of this *de novo* vascularized connective tissue is not entirely understood. Histology indicates that the neo-angiogenic process converting the nanofiber gel into vascularized tissue is initiated by fibroblasts. This is consistent with the mechanism for the formation of microcapillary networks, where matrix produced by fibroblasts contributes to the architecture of a new microcirculation [37]. Additionally, fibroblasts are known to secrete paracrine factors, such as VEGF and FGF-2, that stimulate endothelial cells and signal angiogenesis [38]. However, since the vascularization was not seen for the HBPA-phosphate nanofiber gel or for the heparan sulfate control, the observed effect is attributed to the presence of heparan sulfate as a component of the nanofiber gel. The precise activity of heparan sulfate presented on our nanofibers is not known in this instance, though this glycosaminoglycan does

play several roles as a co-factor in angiogenesis that could be contributing to the response observed. Heparan sulfate binds a variety of angiogenic growth factors, including VEGF and FGF-2, promotes and stabilizes the interaction of these factors with their corresponding receptors, enhances receptor dimerization, and protects these factors against enzymatic degradation [22–25]. Additionally, heparan sulfate is known to induce endothelial cell proliferation. Therefore, the heparan sulfate presented on the surface of the HBPA-HS nanofiber could be contributing to this fibroblast-driven process through binding and retaining the growth factors produced by the fibroblasts, potentiating these factors, and contributing to the rapidly proliferating endothelium necessary to form the observed *de novo* vascularized tissue. Further, it is possible that the HBPA enhances the bioactivity of heparan sulfate by prolonging its tissue exposure in the injection site.

Importantly, this study demonstrated a new application of the dorsal skinfold chamber model to dynamically assess the tissue reaction to an implanted gel scaffold. This model allows repeated tracking of the same microcirculatory segments over a period of time, enabling examination of inflammatory indicators within the microcirculation of an awake mouse unimpeded by anaesthesia. Unlike the extensive histological methods employed with the subcutaneous implantation model, the skinfold chamber model drastically reduces the number of animals required for a thorough study. Although the surgical procedure used to implant the skinfold chamber is more time consuming than a subcutaneous injection, each animal can be used for a number of timepoints, thus allowing data to be obtained from fewer animals in real time and without having to terminate the animal experiment and perform histology before an assessment can be made.

The dorsal skinfold chamber model corroborates the results of the subcutaneous model with respect to the biocompatibility of the HBPA-HS nanofiber gels. The gel persists in the chamber throughout the 10-day study with no noticeable change in its overall structure or volume. We observed few signs of inflammation in the microcirculation adjacent to the implanted nanofiber gel, with minimal vessel dilatation, no indication of edema, and very few leukocytes adherent to the endothelium in the vicinity the gel. This type of response within this model is indicative of very good tissue compatibility of HBPA-HS nanofiber gels.

At the endpoint of the study, traditional histology of the tissue within the skinfold chamber implantation bed revealed a newly vascularized connective tissue emerging from the underlying mesenchyme and enveloping portions of the HBPA-HS nanofiber gel. This was similar to the *de novo* vascularized tissue observed in the subcutaneous model, corroborating the results from in the conventional static assessment and once again illustrating the potency of this material to promote the formation of a newly vascularized tissue shortly after implantation.

The results of this study, specifically the capacity of the material to form newly vascularized tissue in the implantation bed, support further the potential of the HBPA-heparan sulfate nanostructures in vascularization and soft tissue regeneration. Chronic wounds in adults, often perpetuated by ischemia, could see a therapeutic benefit through the formation of a densely vascularized granulation tissue [39,40]. Similarly, integration of autologous skin grafts and healing of severely burned tissue may benefit from enhanced vascularization. Additionally, the HBPA-heparan nanofiber gel could be a useful addition to therapeutic cell delivery, where cell viability after transplantation can be very low. In an *in vivo* setting, cells must be within 200 μm of their blood supply, so rapid vascularization of the transplant site is essential to ensure survival of these therapeutic cells over time [41]. Overall, the formation of a *de novo* vascularized tissue, as demonstrated here, could have broad application in the field of regenerative medicine.

5. CONCLUSIONS

Using two rodent models, we have demonstrated excellent biocompatibility for hybrid nanofibers composed of heparan sulfate and a peptide amphiphile capable of binding this biopolymer. When these nanofiber gels were injected subcutaneously, a *de novo* vascularized tissue developed, yielding a physiological microcirculation without increased permeability or persistent inflammation. These findings support the potential of these materials in targets such as healing chronic wound and cell transplantation. We have also demonstrated in this work the ability to dynamically evaluate the biocompatibility of soft material through a novel application of the dorsal skinfold chamber model and intravital fluorescent microscopy. The analysis corroborated observations from our static histological analysis and allows the use of fewer animals.

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Figure 1. Chemical structures of heparin-binding peptide amphiphile (**A**) and a fluorescein-conjugated peptide amphiphile (**B**) used in this study. Additionally, the general chemical structure of heparan sulfate showing the three sites of variable sulfation (**C**).

Figure 2.

Computerized scanning of an entire histological slide produced from an assembly of many high-resolution (100x) single images. This technique allows an for quantitative analysis of the entire implantation bed in a single image. Here, the tissue and material is stained with hematoxylin and eosin, showing that the nanofiber gel structure is maintained at 3 days and demonstrating a favorable peri-implant response.

Figure 3. Representative histology for HBPA-heparan sulfate nanofiber gels implanted subcutaneously. The implant bed and surrounding tissue were excised at 3 days, 10 days and 30 days stained with H&E (left) and Azan (right). At 3 days (**A** and **B**), the nanofiber gel was primarily homogenous and not infiltrated by immune cells. By 10 days (**C** and **D**) the nanofiber gel showed early signs of degradation and converted into a vascularized connective tissue from the periphery, with representative blood vessels indicated by arrows and a macrophage ingesting the nanofiber gel circled in red. At 30 days (**E** and **F**) the implantation bed was a well-vascularized connective tissue, with representative blood vessel indicated by arrows. The staining and magnification for each image is shown and all scale bars represent 100 μm .

Figure 4. Quantification of the degradation (**A**) and vascularization (**B**) of subcutaneously implanted HBPA-heparan sulfate nanofiber gels, determined through analysis of histological images.

Figure 5. Representative histology for HBPA-phosphate nanofiber gels implanted subcutaneously. The implant bed and surrounding tissue was excised at 3 days (**A** and **B**), 10 days (**C** and **D**). The nanofiber gel appears as a mosaic of aggregates and has already been infiltrated with immune cells at day 3, and by day 10 shows significant degradation. There is no evidence of new vasculature within the implant bed. The staining and magnification for each image is shown and all scale bars represent 100 μm.

Figure 6. Intravital microscopy to track the vasculature (**A, left**) and fluorescent nanofiber gel (**A, right**) of the fluorescent HBPA-Heparan Sulfate nanofiber gel implanted within a dorsal skinfold chamber at 2, 6, and 10 days following implantation. Scale bars represent 500 μm . The same region of microcirculation in an awake mouse was examined at each day, as shown, and the diameter of vessels within the dorsal skinfold chamber adjacent to the HBPA-heparan sulfate nanofiber gel were measured and expressed as a percent of the day 0 diameter for the 4 animals (**B**). The material is readily apparent within the dorsal skinfold chamber (**C**), appearing yellow due to the presence of the FITC, with an arrow showing the area of the vasculature displayed in panel **A**.

Figure 7. Representative intravital microscopy showing the green fluorescent HBPA-Heparan nanofiber gel (A), rhodamine-illuminated leukocytes (B), and brightfield showing the microcirculation (C). In this way, leukocytes adhered to the endothelium in the vicinity of the gel could be tracked to observe the nature of the acute immune response to the nanofiber gel. Scale bars represent 200 μm .

Figure 8.

(A) Representative histology from the endpoint of the dorsal skinfold chamber model, displaying the implantation bed stained with H&E. As shown, the HBPA-HS nanofiber gel is enveloped within a newly formed vascularized connective tissue, with arrows indicating representative vessels. (B) High magnification fluorescent image from dorsal HBPA-HS nanofiber gel immediately before sacrifice, with arrows pointing to dark regions indicating blood vessels within the nanofiber gel. Scale bars represent 100 μm (left) and 500 μm (right).