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Bone Regeneration with Low Dose BMP-2 Amplified by Biomimetic Supramolecular Nanofibers within Collagen Scaffolds

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Abstract

Bone morphogenetic protein-2 (BMP-2) is a potent osteoinductive cytokine that plays a critical role during bone regeneration and repair. In the extracellular environment, sulfated polysaccharides anchored covalently to glycoproteins such as syndecan and also non-covalently to fibronectin fibers have been shown to bind BMP-2 through a heparin-binding domain and regulate its bioactivity. We report here on a synthetic biomimetic strategy that emulates biological BMP-2 signaling through the use of peptide amphiphile nanofibers designed to bind heparin. The supramolecular nanofibers, which integrate the biological role of syndecan and fibronectin, were allowed to form gel networks within the pores of an absorbable collagen scaffold by simply infiltrating dilute solutions of the peptide amphiphile, heparan sulfate, and BMP-2. The hybrid biomaterial enhanced significantly bone regeneration in a rat critical-size femoral defect model using BMP-2 amounts that are one order of magnitude lower than required for healing in this animal model. Using micro-computed tomography, we also showed that the hybrid scaffold was more effective at bridging within the gap relative to a conventional scaffold of the type used clinically based on collagen and BMP-2. Histological evaluation also revealed the presence of more mature bone in the new ossified tissue when the low dose of BMP-2 was delivered using the biomimetic supramolecular system. These results demonstrate how molecularly designed

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materials that mimic features of the extracellular environment can amplify the regenerative capacity of growth factors.

Keywords

Peptide Amphiphile; Heparin; Heparan Sulfate; BMP-2 (bone morphogenetic protein-2); Regenerative Medicine; Bone Regeneration

1. Introduction

Bone is a dynamic tissue that continuously regenerates and has the ability to heal following fractures [1]. However, sufficiently large defects resulting from traumatic bone loss, tumor resections, or infection cannot heal without intervention, and thus the treatment of these skeletal complications presents difficult challenges in orthopaedic medicine [2]. Bone grafting is widely used in clinical procedures to promote healing of non-unions and large defects. In this strategy the iliac crest autologous graft has been the gold standard due to its osteogenic, osteoinductive, and osteoconductive capacity [3]. However, certain drawbacks including limited tissue availability, pain, and donor-site morbidity have limited the use of iliac crest grafts [4]. Allogeneic bone grafting is an alternative, but this strategy has limitations that include sub-optimal osteoactivity compared to autografting, donor incompatibility, and an increased risk of disease transmission [2, 3]. With the limitations of graft-based approaches, there have been many efforts to develop synthetic scaffolds to stimulate the natural healing process of bone. A variety of scaffolds have been investigated, including polymer and oligopeptide-based hydrogels [5], porous scaffolds with controlled architecture [6], and polymer-netted hydroxyapatite microcrystals [7, 8] (for reviews, see [9-11]). One of the main problems with previously developed scaffolds [12, 13] is the fact that they often contain components that will be difficult to resorb and remodel and thus they will be replaced by hybrids of bone and residual minerals in their original formulation. The field of bone regeneration would benefit from biomaterials that will disappear completely and be replaced by pure bone only.

Another emerging alternative to grafting is therapies based on delivery of growth factors such as bone morphogenetic proteins (BMPs) [14, 15]. Recombinant human BMP-2 and BMP-7 delivered within an absorbable collagen sponge have been used clinically, and have demonstrated improved bone healing at a therapeutic dose as high as 12 mg per treatment [16, 17]. However, the collagen scaffold has no specific binding affinity for BMPs, resulting in 30% burst release upon initial implantation [15]. In recent clinical reports, the supraphysiological amount of the exogenous protein required for improved healing has demonstrated issues with generalized hematomas in soft tissue [18], exaggerated inflammatory response in proximal humeral fractures [19] and unicameral bone cysts [20], as well as infections due to an elevated anti-BMP-2 antibody level in open tibial fractures [21]. In order to improve the efficacy of BMP-based therapies and limit adverse responses, new types of protein carriers must be able to prolong protein retention and in turn reduce the therapeutic dose of growth factors [15, 22-24].

Inspired by biological systems, several biomaterials have been developed to contain key components involved in receptor-ligand interactions such as heparin [25] and fibronectin [26]. In the extracellular environment, sulfated macromolecules bind and localize cytokines and control their signaling [27]; heparan sulfate-like glycosaminoglycans potentiate BMP signaling by facilitating the BMP-BMP receptor complexation [28-30]. Benoit *et al.* modified fluvastatin-releasing poly(ethylene glycol) (PEG) hydrogels with heparin to sequester endogenous BMP-2 produced by human mesenchymal stem cells (hMSCs) in

response to the released drug, resulting in augmented expression of osteogenic proteins *in vitro* [25]. In addition, fibronectin consists of several domains for binding to other fibronectin molecules, integrin, heparin, as well as growth factors [31, 32]. Martino *et al.* investigated a multifunctional recombinant fragment of fibronectin that contained both integrin and growth factor binding domains, and a fibrin matrix with the recombinant fragments covalently attached exhibited increased bone regeneration in a rat critical-size calvarial defect model with small quantities of BMP-2 and platelet-derived growth factor (PDGF) [26].

Our laboratory has developed molecular building blocks known as peptide amphiphiles (PA) that are programmed to self-assemble into high-aspect-ratio nanofibers that are 6-10 nanometers in diameter and microns in length [33, 34]. When dilute solutions of these PAs are injected into physiological environments they can form networks of nanofibers as charges on their surfaces are screened by natural electrolytes. A PA molecule typically contains two main segments, a peptide sequence covalently linked to a hydrophobic alkyl tail or other similar non-peptidic but hydrophobic segments. The self-assembly of PA molecules in an aqueous environment is driven by the hydrophobic collapse of alkyl tails and hydrogen bonding down the length of the nanofibers due to β -sheet propensity in specific domains of the peptide segment adjacent to the hydrophobic tails [35]. On the terminus of the peptide sequence away from the alkyl tail, PA molecules may contain biological signals as epitopes for receptors [36-39], peptide sequences that bind specific proteins [40], or even covalently bound therapeutic drugs that are hydrolytically released over time [41, 42]. The supramolecular nanofibers are designed to present a high surface density of biological signals or a tuned concentration of signals and may therefore be described as artificial extracellular matrices (ECM) [43, 44]. These nanofibers can also form self-supporting gels or infuse into porous titanium scaffolds to create bioactive implants [45, 46].

In previous works we investigated non-covalent interactions between PAs and sulfated polysaccharides to form biologically functional gels [47, 48]. A PA molecule was developed with a Cardin-Weintraub heparin-binding peptide domain to specifically bind to heparin with moderate affinity so it could form nanofibers that display non-adhered “loops” of the polysaccharide [48]. These chains of heparin could localize growth factors through their respective heparin-binding domains [47]. The heparin-binding peptide amphiphile (HBPA) nanofibers can entangle to form gel networks in the presence of sulfated polysaccharide chains. These nanofiber gels demonstrated prolonged release of angiogenic growth factors and induced substantial neo-vascularization in a rat cornea using only nanogram quantities of basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) [47]. Furthermore, these HBPA gels containing heparan sulfate (HS) were found to promote *de novo* formation of a highly vascularized connective tissue with minimal inflammation when implanted subcutaneously [49]. Recently, a hierarchical membrane made of HBPA, heparin, and hyaluronic acid was shown to promote enhanced growth factor retention and increased angiogenesis in the chicken chorioallantoic membrane assay [50].

We report here on the use of these systems with an osteogenic protein such as BMP-2. Biological BMP-2 signaling involves transmembrane glycoproteins such as syndecan, which contain covalently bonded strands of heparan sulfate that localize the cytokine during its interaction with receptors [51]. At the same time, these polysaccharide strands interact non-covalently during signaling with fibronectin fibers, which in turn have both heparin- and integrin- binding domains [31, 51]. In the work reported here, we have investigated the consequence to bone regeneration through BMP-2 signaling using the fibronectin-like peptide amphiphile nanofibers with a strong capacity to bind heparan sulfate chains. These nanofibers could therefore localize both BMP-2 and fibronectin fibers. The nanofibers

displaying heparan sulfate strands were infiltrated in the pores of an absorbable collagen scaffold to test the regenerative capacity of this biomimetic system in a rat femoral critical-size defect model.

2. Materials and Methods

2.1. PA synthesis and purification

Heparin-binding PA (HBPA) was synthesized using solid phase peptide synthesis methods as previously reported [49]. HBPA molecules were purified using reversed phase high performance liquid chromatography (HPLC) in a water/acetonitrile gradient, each containing 0.1% v/v trifluoroacetic acid (TFA). The purified material was lyophilized and stored at -20 °C until use.

2.2. Cryogenic TEM

Cryo-TEM was performed on a JEOL 1230 microscope according to a previously described protocol [37]. HBPA was prepared at 3 wt% in sterile water, then diluted to 1 wt% for imaging.

2.3. Growth factor release kinetics

Heparan sulfate (HS) was purchased from Celsus Laboratories and BMP-2 from PeproTech. We verified the binding ability of the HBPA nanofiber-heparan sulfate complex to BMP-2 by studying the release kinetics of the cytokine from a gel formed by the nanofibers. The formation of HBPA nanofiber gel was triggered by mixing with either heparan sulfate dissolved in deionized water (HBPA + HS) or phosphate buffered saline (PBS, Hyclone) in order to form a control gel without heparan sulfate (HBPA + PBS). HBPA gels were not incorporated into absorbable collagen sponge for this assay. In order to form the two types of gels (n=3), 100 ng rhBMP-2 were added to solutions of either 2 wt% HS in deionized water or 2x PBS and mixed with 3 wt% HBPA solutions. The final concentrations of HBPA and HS in the HBPA + HS gel were 1.5 wt% and 1 wt%, respectively. This gel was allowed to equilibrate for 2 hr at room temperature before it was soaked with 1 mL of release media (0.1wt% albumin in PBS). Afterwards, 800 μ L of the supernatant was immediately removed and replenished with 800 μ L fresh release media. At 1, 2, 3, 4, 5, 6, 7, and 8 day time points, 800 μ L of the supernatant was similarly removed (stored at -80 °C) and the gels were replenished with new media. For ELISA, plates were prepared using high-binding 96-well plates (CoStar) and an ELISA reagent kit (Peprotech) according to the manufacturer's protocol. After conjugation with avidin-HRP, 100 μ L of HRP substrate (R&D Systems) was added to each well. When sufficient color had developed after 5 min, 50 μ L stop solution (1 M HCl) was added. After incubating for 10 min, maximum absorbance was read at 450 nm with plate correction at 650 nm using SpectraMax M5 Microplate Reader (Molecular Devices).

2.4. Absorbable collagen sponge fabrication

Collagen scaffold was fabricated by a freeze-drying process to yield a uniformly porous collagen matrix [52]. Briefly, a 0.5 wt% collagen slurry was prepared by blending bovine type I collagen (Sigma Aldrich) in 0.05 M acetic acid using a Ultra-Turrax® T-18 homogenizer (IKA) for 2 h over an ice bath. The slurry was degassed with vacuum, and 30 mL of the mixture was cast into 2-7/8" aluminum pans (McMaster-Carr). The pans were frozen to -10 °C using an AdVantage BenchTop Freeze Dryer (VirTis) at a rate of 0.3 °C/min and were lyophilized to yield porous collagen sheets (~7 mm thick). The collagen sheets were sterilized and cross-linked by dehydrothermal treatment at 105 °C overnight under vacuum. Biopsy punches were used to punch out 6 mm diameter cylinders. Scaffolds were

then chemically cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy-sulfosuccinimide (NHS) in 70% ethanol at a ratio of 100:100:1 EDC/NHS/-COOH for 2 h, rinsed in deionized (DI) water, frozen at -30 °C, and lyophilized to yield the final product.

2.5. Scaffold assembly for the in vivo study

Stock solutions and scaffolds were freshly prepared prior to surgeries. A 3.6 wt% HBPA stock solution was prepared by dissolving lyophilized HBPA in sterile water under sonication for 15 min. A 0.1 mg/mL rhBMP-2 (PeproTech) stock solution was prepared according to the manufacturer's instructions. First, a 60 μ L mixture comprised of 3 wt% HBPA and 1 μ g rhBMP-2 was prepared. This mixture was added drop-wise on top of a dry cylindrical collagen scaffold and allowed to soak in for 5 min. Afterwards, 60 μ L of 2 wt% HS was added drop-wise, and this hybrid scaffold was allowed to gel for 10 min. The final concentrations of HBPA and HS in the scaffold were 1.5 wt% and 1 wt%, respectively, and this scaffold is abbreviated as Coll + HBPA + HS + BMP-2. Scaffolds missing the HS chains (Coll + HBPA + BMP-2) were prepared by triggering gel formation of 60 μ L 3 wt% HBPA solution containing 1 μ g rhBMP-2 with 60 μ L 2x PBS instead of HS within a collagen sponge. Scaffolds without HBPA nanofibers (Coll + HS + BMP-2) were prepared by adding 120 μ L 1 wt% HS solution containing 1 μ g rhBMP-2 onto a collagen sponge. Scaffolds with 1 μ g rhBMP-2 only (Coll + BMP-2) were prepared by adding 120 μ L PBS containing 1 μ g rhBMP-2 onto a collagen sponge. Finally, scaffolds containing HBPA nanofiber-HS gel without rhBMP-2 (Coll + HBPA + HS) were prepared by the same method used to prepare Coll + HBPA + HS + BMP-2 scaffolds but without using BMP-2. Bare collagen scaffolds without any additional components (Coll) were also prepared as controls for all other samples.

2.6. Scanning electron microscopy

SEM was used to visually analyze the supramolecular nanofibers gelled in the pores of collagen scaffolds. Collagen + HBPA + HS scaffolds were prepared as described above. Scaffolds were then fixed in 4% PFA for 1 h, washed with DI water, and dehydrated in a graded ethanol series. To preserve the PA structure, liquid ethanol was removed by using critical point drying (Tousimis, Samdri®-795). Cross-sections of the scaffolds were made, mounted, and coated with 12 nm OsO₄ (Filgen, OCP-60A). Samples were imaged with a Hitachi S-4800 II FE-SEM.

2.7. Fluorescence imaging of assembled hybrid scaffold

Collagen + HBPA + HS scaffolds were prepared using FITC-conjugated HS (FITC-HS). FITC-HS was synthesized through conjugation of fluorescein isothiocyanate (FITC) to the carboxyl groups on HS via an aldehyde/isocyanide route [53]. First, 25 mg HS was dissolved in 20 mL DI water overnight, followed by an addition of 20 mL dimethyl sulfoxide (DMSO). Afterwards, 12.5 μ L acetaldehyde, 12.5 μ L isocyanide, and 12.5 mg FITC were dissolved in 500 μ L DMSO and added to the prepared HS solution for 2 h at room temperature. Products were purified by dialysis in 4 L DI water over 2 days with 2 exchanges per day, lyophilized, and stored at -20 °C until use. Scaffolds were assembled as described above, fixed in 4% PFA for 1 h, and thin slices were sectioned with a scalpel for fluorescence imaging with Nikon inverted fluorescent microscope with the appropriate filters.

2.8. Animal surgeries

An established rat critical-size femoral defect model was used in this study [45]. All surgical and animal care procedures were reviewed and approved by the Institutional Animal Care

and Use Committee at Northwestern University. Thirty-six 21-24 week old male Lewis rats (Harlan, Indianapolis) were used for this experiment. All rats were anesthetized by inhalation of 1-3% isoflurane, and for an analgesic, Buprenex injections (0.5 mg/kg) were given prior to and following the surgeries for pain management. Briefly, the animal was placed on its side, the hair around the incision area was shaved, and a lateral incision was made in the skin to circumferentially strip the subcutaneous tissues, exposing the femoral diaphysis of the left hindlimb. A 2.5 cm long titanium bone plate (Synthes) with five 1.5 mm diameter holes was placed on the anterior exposed cortex of the femur to ensure appropriate positioning of the drill holes. A 5 mm segmental defect was created using a high-speed oscillating saw (Stryker), and the proximal and distal segments of the femur were fixed by the bone plate with four 1.1 mm diameter screws. The center screw hole covered the defect gap. After flushing the fracture site with normal saline solution, the scaffolds described above were inserted in the defect to completely fill the gap. The wound was closed by suturing a layer of fascia and the skin separately. Buprenex was administered once every 12 h for the first 36 h after implantation. Animals were sacrificed at 6 weeks, and the femurs were harvested for analysis.

2.9. Micro-computed tomography analysis

Harvested rat femurs were fixed in 10% neutral buffered formalin for at least 48 h, dehydrated in graded ethanol and xylene, and embedded in paraffin in order to remove the Ti bone plates and screws without significantly disturbing the tissue within the defect site. Excess paraffin was cut off around the samples. Femurs were placed in the sample holder parallel to the scanner's rotation axis with the proximal side located up. A μ CT 40 system (Scanco Medical) operated at 45 kV and 177 μ A was used to collect data for the volume of the reconstructed bone encompassing the 5 mm defect gap. Previously described methods were used to identify and reconstruct the femoral diaphysis in the μ CT 40 system [45]. The amount of new bone formation in the gap was quantified using the Scanco software suite as followed. First, the two slices where the bone gap began and ended were visually identified. The volume of mineralized tissue within these two slices were captured using a threshold of $\mu=1.35/\text{cm}$ (value of 170 on software). As the planes of the cut edges were slightly tilted with respect to the rotational axis of the sample holder, some of the slices contained both the original diaphysis of the femur ("old bone") and newly formed bone. Old bone was distinguishable from the new bone by the regular shape of the round cortex, as well as the greater degree of mineralization in the old bone relative to the new bone. To exclude signals measured from the old bone in the original measurement, regions of interest (ROIs) were tightly drawn around the visually differentiated old bone through a manual process and with the aid of morphing functions provided in the software. The volume of mineralized tissue within these ROIs were captured using the same $\mu=1.35/\text{cm}$ threshold. Furthermore, the regular shape of the round cortex also served as a guide to visually differentiate between old bone and the more irregular mass of new bone. During analysis, bridging was defined as a continuous bone formation between proximal and distal ends of the femur.

2.10. Histology

After μ CT analysis, rat femurs were soaked in xylene to remove the paraffin, rehydrated in graded ethanol, and decalcified with Decal® (Decal Chemical Corporation) overnight. Femurs were then dehydrated in graded ethanol and xylene, embedded in paraffin, and 10 μm sections were stained with Goldner's Trichrome. Entire femur sections were imaged at 20x magnification with a TissueFAXS System.

2.11. Statistics and data analysis

Error bars for new bone formation indicate the standard error of the mean. For new bone formation measurement from μ CT analysis, differences between groups were determined

using a one-way analysis of variance (ANOVA) with a Newman-Keuls multiple comparisons post-hoc test.

3. Results

3.1. Enhanced BMP-2 retention by the display of heparan sulfate

We first verified the ability of the HBPA (Fig. 1, A and B) and heparan sulfate (HS) complex to bind BMP-2 by studying the release kinetics of the growth factor from gels of this material. A solution containing both HBPA and BMP-2 was gelled by mixing with a second solution of either HS or phosphate salts to form self-supporting gels. Using ELISA to quantify the amount of protein released, we observed a slower release of BMP-2 in the presence of HS (Fig. 1C). After 24 h, $45.4 \pm 8.8\%$ of the initially loaded BMP-2 was released from the gel prepared without HS, while gels that contained HS released only $22.7 \pm 3.9\%$ of BMP-2. After 8 days, the cumulative amount of BMP-2 released in the absence of HS was approximately $84.0 \pm 18.9\%$, which was more than double the amount of protein released from the HBPA + HS gel ($34.5 \pm 8.1\%$).

3.2. Collagen-peptide amphiphile nanofiber hybrid scaffold

For the *in vivo* study, we designed a hybrid scaffold system where the HBPA + HS gel network is allowed to assemble within the pores and on surfaces of a collagen scaffold by infusing solutions of the peptide amphiphile and polysaccharide chains. In order to verify that the HBPA + HS gel was dispersed throughout the interconnected pores of the collagen scaffold, the presence of self-assembled nanofibers on the exterior and interior of the scaffold was evaluated using scanning electron microscopy (SEM). The bare scaffold had an apparent pore size of approximately 200-300 μm in diameter, and the surface of the pores showed the presence of collagen fibrils with their characteristic periodic banding structure (Fig. 1D). After gelation of HBPA by HS within the scaffold, the external surface of these hybrid scaffolds contained a network of nanofibers (Fig. 1E). A cross-section of the hybrid scaffold showed that the porous structure of the scaffold was still maintained with an apparent pore size of approximately 100-200 μm in diameter, and a network of short nanofibers were also present on the surface of these pores (Fig. 1F). These findings confirmed that the solution of HBPA was able to penetrate throughout the interconnected pores of the collagen scaffold. Furthermore, fluorescently tagged HS was used to verify its dispersion throughout the scaffold during gelation, and a cross-sectional slice revealed that HS was indeed present throughout (Fig. 1G). This confirms that the glycosaminoglycan chains can diffuse into a porous scaffold soaked in HBPA to trigger formation of peptide amphiphile nanofiber networks.

3.3. In vivo bone regeneration

The well-established rat critical size femoral defect model was used to assess the ability of the collagen-nanofiber hybrid scaffold to heal and bridge a 5 mm defect gap. Six weeks following treatment, femurs were harvested and analyzed using μCT in order to evaluate bone growth within the gap. Representative images from 3-D μCT rendering showed that treatment with the collagen + HBPA + heparan sulfate + BMP-2 scaffold resulted in significantly increased growth of new bone relative to the other groups (Fig. 2A). Over half of all animals treated with this hybrid scaffold demonstrated continuous bone formation between the proximal and distal femur (Fig. 2B), a clear sign of bone union healing. Conversely, only one animal treated with the collagen + HBPA + BMP-2 scaffold showed bridging and bridging was never observed in other controls.

Quantitative analysis of μCT reconstructions of the defect demonstrated that animals treated with the collagen-nanofiber scaffolds with low dose of BMP-2 have by far the highest mean

volume of ossified tissue within the callus relative to all other treatments, effectively tripling the volume of regenerated bone ($20.3 \pm 4.2 \text{ mm}^3$ vs. $6.6 \pm 0.3 \text{ mm}^3$) (Fig. 2C). This mean volume of new ossified tissue was significantly greater ($p < 0.001$) than those observed in untreated animals ($2.4 \pm 0.1 \text{ mm}^3$), or in those treated with collagen scaffolds with ($8.2 \pm 1.7 \text{ mm}^3$) or without BMP-2 ($3.5 \pm 0.8 \text{ mm}^3$), with PA nanofibers displaying heparan sulfate but without BMP-2 ($6.7 \pm 1.4 \text{ mm}^3$), with heparan sulfate and BMP-2 ($5.9 \pm 1.3 \text{ mm}^3$), or with PA nanofibers and BMP-2 but without the polysaccharide for biomimetic signaling ($7.5 \pm 1.2 \text{ mm}^3$).

A qualitative histological assessment confirmed the results from μCT measurements (Fig. 2D). Standard Goldner's Trichrome staining was used to indicate bone formation within the surgically produced defect. There was no evidence of a local inflammatory response in any of the defects. The untreated animals showed a complete separation of the diaphysis. Higher magnification images revealed the presence of periosteal bone formed near the end of the bone, but only fibrous tissue was present across the gap. Samples that were treated with collagen scaffolds with BMP-2, with heparan sulfate and BMP-2, or with HBPA nanofibers and BMP-2 in the absence of the polysaccharide chains showed evidence of new woven trabeculae adjacent to the proximal and distal ends, but this new bone formation did not bridge the gap. The respective higher-magnification images from these groups demonstrated woven bone forming sparsely within the fibrous tissue, suggesting immature healing. Consistent with the findings from μCT , the treatment of the collagen-nanofiber scaffold with low dose BMP-2 exhibited the greatest amount of ossification, showing histological evidence of complete fusion of the gap. At higher magnification, it can be seen that the trabeculae of the ossified tissue have a healthy appearance indicated by the thickness of the well-organized woven bone.

4. Discussion

In the work reported here, a supramolecular nanofiber network of heparin-binding PA (Fig. 1, A and B) and heparan sulfate was evaluated as a delivery system for BMP-2 to promote bone regeneration. As expected, we observed that the nanofiber gel enhanced BMP-2 retention *in vitro* (Fig. 1C). The protein localization observed here is consistent with previous studies from our group using this material to release angiogenic growth factors [47]. In other reports, retention of BMP-2 was observed when the growth factor was attached on a surface of heparin/hyaluronic acid (HA) gel particles prepared via an inverse emulsion crosslinking technique [54] or on a chemically modified crosslinked heparin/HA/poly(L-lysine) film [55]. The supramolecular gel studied here also shows retention of BMP-2, but does so without requiring chemical cross-linking of its components, which could be an attractive attribute for a bone graft with adequate biodegradation rate as new bone forms.

The collagen-nanofiber hybrid scaffold used here was rationally designed for the clinical applications of tissue repair (Fig. 1, E-G). Collagen-based scaffolds have several key attributes that make them an attractive system for regeneration, including biodegradability, mechanical stability, as well as amenability to sterilization [56]. Most importantly, collagen-based materials have been approved for clinical use in a number of applications [16-18]. Porous collagen scaffolds have also been reported to provide a suitable environment for cellular attachment, infiltration, and proliferation [57]. Therefore, the biocompatibility and structural benefits of the scaffold used here [56, 57] combined with previously demonstrated biocompatibility of HBPA nanofibers [49] could impart this hybrid scaffold with the benefits of both components. From a biological point of view, the networks of long nanofibers within the pores could serve to guide cell migration within the scaffold. At the same time, presentation of growth factor bound in biomimetic fashion by the heparan sulfate

chains in the interior of the scaffold could optimize signaling for regeneration with low dose of BMP-2. From a physical point of view, forming the supramolecular nanofiber networks within the pores of the scaffold naturally helps with retention of the nanofiber gel within the bone defect. In this context, in a previous investigation from our laboratory, we found it was hard to localize a segment of a bare gel from a different PA within the defect [45]. Nonetheless, we still observed in this previous study efficacy from a bioactive gel confined to the defect.

The model used for preclinical evaluation of the hybrid scaffold requires a dose of 11 μg BMP-2 to promote effective bridging of the defect [58]. We have demonstrated that less than one tenth of the required dose promoted bridging of the surgically created gap when the growth factor was delivered through a biomimetic supramolecular system embedded in a conventional collagen scaffold (Fig. 2). We hypothesize that in the biological environment there are dynamic and spatial effects that optimize signaling and that our system captures some of them as depicted in Fig. 3. If it were simply a matter of entrapping and localizing the growth factor, we would have expected heparan sulfate or the nanofibers alone to be effective relative to controls. However, the sharp transition to statistically significant efficacy when all components are present suggests that signaling efficiency is also provided by our biomimetic system. On the surface of the cell membrane, heparan sulfate chains are covalently linked to the transmembrane glycoprotein syndecan. The sulfated strands bind non-covalently to BMP-2, but also at the same time to fibronectin fibers via their heparin-binding domains. We speculate that the synergy of these two interactions must favor ligand-receptor binding and that our supramolecular system has the capacity to recreate this configuration. This explains in our view why in the absence of peptide amphiphile nanofibers, the heparan sulfate alone is incapable of promoting significant bone regeneration.

When designing these experiments, it was thought that the biomimetic supramolecular system without exogenous BMP-2 could also be therapeutic through the binding and localization of endogenous factors present in the injury site. This was based on previous work from our group, which showed that the PA-polysaccharide network alone promoted the formation of new vasculature in a mouse dorsal skinfold chamber model [49]. However, since cortical bone and marrow have low levels of blood flow in comparison to other types of bone [59], it is possible that there were insufficient levels of relevant endogenous factors in response to injury in the rat femoral diaphysis. Nonetheless, the transition to highly significant efficacy when treated with the complete collagen-nanofiber hybrid system with the addition of BMP-2 encourages further evaluation of our biomimetic strategy in other uses such as spinal fusion or in challenging defects as a result of trauma or cancer. Furthermore, since other heparin-binding proteins, such as TGF- β , VEGF, PDGF, and Wnt proteins have also been found to enhance bone repair [27, 60-63], the supramolecular system could be used to synergistically incorporate multiple factors for a broad use in bone reconstruction.

5. Conclusions

We have developed a hybrid biomaterial for bone regeneration that integrates a conventional collagen scaffold with supramolecular nanofibers designed molecularly to bind heparan sulfate and other similar biopolymers. This system promotes large volumes of regenerated bone and a high probability of bridging in a rat critical defect with 1 μg of BMP-2. We propose that this clinically important finding is rooted in the fact that the scaffold system emulates the binding of heparin-like segments in glycoproteins to growth factors and extracellular matrix fibers. This translational system suggests generally that by mimicking

certain aspects of the extracellular environment in biomaterials it may be possible to amplify the regenerative capacity of growth factors.

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Figure 1.

(A) Chemical structure of heparin-binding peptide amphiphile used. (B) Cryogenic transmission electron micrograph showing the filamentous nature of nanostructures formed by the heparin-binding peptide amphiphile. (C) *In vitro* analysis of BMP-2 release from nanofiber gels with or without heparan sulfate ($n = 3$) (ELISA data presented as mean $\pm$ standard error of the mean). (D) Scanning electron micrographs of a plain collagen scaffold before infiltration with peptide amphiphile and heparan sulfate solutions. (E and F) Scanning electron micrographs of the surface (E) and interior (F) of hybrid scaffolds. (G) Fluorescence imaging of a cross-section of collagen scaffold containing fibers of heparin-binding peptide amphiphile and FITC-tagged heparan sulfate.

Figure 2.

Analysis of the *in vivo* bone regeneration capacity of heparan sulfate-presenting heparin-binding peptide amphiphile nanofibers. (A) Representative femur reconstructions from micro-computed tomography are shown for the various treatment groups. (B) The number of animals used per condition and the number of animals with a bridged femur after treatment. (C) Quantitative analysis of new bone volume (mm³) within the defect measured by micro-computed tomography. Data are presented as mean $\pm$ standard error of the mean; *** $P < 0.001$. (D) Representative histological longitudinal sections of demineralized femora stained with Goldner's Trichrome. The presence of ossification is characterized by collagen (green) with cytoplasm (red), erythrocytes (orange), and nuclei (dark violet). In the upper box,

arrows indicate the proximal (top) and distal (bottom) edges of the defect (scale bars=1 mm). The lower box shows a higher magnification image of the inset indicated above (scale bars, 200 μm).

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Figure 3.

(A) Schematic representation of extracellular matrix components involved in ligand-receptor interactions during BMP-2 signaling. Syndecan-4 is a transmembrane proteoglycan containing covalently bonded strands of sulfated polysaccharides. These sulfated macromolecules interact non-covalently with fibronectin fibers via their heparin-binding domains and also localize BMP-2 cytokines to facilitate interaction with their cell-surface receptors (27, 51). (B) Schematic representation of our biomimetic strategy to emulate certain aspects of BMP-2 signaling. A supramolecular nanofiber biomimetic of fibronectin displays sulfated polysaccharide strands on its surface, which can localize heparin-binding cytokines such as BMP-2 to facilitate ligand-receptor interactions. The sulfated polysaccharide strands can also bundle with fibronectin fibers through their heparin-binding domains and consequently recruit integrins during signaling (not shown).