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Supramolecular Self-Assembling Peptides to Deliver Bone Morphogenetic Proteins for Skeletal Regeneration

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Abstract

Recombinant human bone morphogenetic proteins (BMPs) have shown clinical success in promoting bone healing, but they are also associated with unwanted side effects. The development of improved *BMP carriers* that can retain BMP at the defect site and maximize its efficacy would decrease the therapeutic BMP dose and thus improve its safety profile. In this review, we discuss the advantages of using *self-assembling peptides*, a class of synthetic supramolecular biomaterials, to deliver recombinant BMPs. *Peptide amphiphiles* (PAs) are a broad class of self-assembling peptides, and the use of PAs for BMP delivery and bone regeneration has been explored extensively over the past decade. Like many self-assembling peptide systems, PAs can be designed to form nanofibrous supramolecular biomaterials in which molecules are held together by non-covalent bonds. Chemical and biological functionality can be added to PA nanofibers, through conjugation of chemical moieties or biological epitopes to PA molecules. For example, PA nanofibers have been designed to bind heparan sulfate, a natural polysaccharide that is known to bind BMPs and potentiate their signal. Alternatively, PA nanofibers have been designed to synthetically mimic the structure and function of heparan sulfate, or to directly bind BMP specifically. In small animal models, these bio-inspired PA materials have shown the capacity to promote bone regeneration using BMP at doses 10 – 100 times lower than established therapeutic doses. These promising results have motivated further evaluation of PAs in large animal models, where their safety and efficacy must be established before clinical translation. We conclude with a discussion on the possiblity of combining PAs with other materials used in orthopedic surgery to maximize their utility for clinical translation.

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CLINICAL CHALLENGES IN BONE HEALING

Despite the relatively robust capacity for bone to regenerate after injury, delayed healing or non-union (failure to heal) is a significant clinical challenge in orthopaedic surgery. Non-union rates of >10% are commonly reported, with much higher rates in at-risk populations such as smokers, diabetics, and osteoporotic patients[1–11]. Treatment of the nearly 8 million fractures that occur in the U.S. annually is estimated to cost $21 billion/year[1].

Autologous bone grafting is the historical “gold standard” for surgical bone repair, delivering the three requirements for successful bone regeneration: 1) osteoinductive signals; 2) an osteoconductive matrix; and 3) osteogenic cells[12–14]. However, the procedure requires a longer operative time and is associated with greater blood loss, increased infection risk, and donor site pain[15–21]. Morbidity (e.g., bleeding and hematoma) is not uncommon, and significant acute and chronic donor site pain at the iliac crest have been reported[15, 22, 23], although the incidence rates of these complications have more recently been disputed[24, 25]. Graft volume/availability is also a limitation, and despite its historical use, autologous bone grafting still results in non-unions (failed healing), reportedly in 5–30 % of patients, depending on the injury or defect as well as patient-specific conditions[2, 10, 26–29]. As a result, the use of iliac crest autograft bone (ICBG) has dropped dramatically in the past two decades[7, 16, 30], and interest has risen in developing alternative approaches to augment bone healing. An ideal solution would be a synthetic bone graft substitute that safely promotes bone regeneration and healing while obviating the need to harvest iliac crest or local vertebral bone.

THE BONE HEALING CASCADE

Bone healing occurs through a highly orchestrated sequence of events involving both biochemical and biomechanical cues[31]. The initial disruption of blood vessels after injury results in the formation of a clot, which provides hemostasis. In closed fractures, a non-infectious inflammatory response is then initiated, whereupon inflammatory cells are recruited to the defect[32]. This is followed by a fibrovascular phase involving recruitment of mesenchymal stem cells (MSCs) via chemotactic signals. These cells undergo proliferation for several days, and then condense and differentiate into chondroblasts or osteoblasts, depending on the microenvironment and growth factor signals received[33, 34]. In the case of endochondral ossification, a cartilaginous intermediate first forms, which undergoes hypertrophy after 10–14 days[35]. The cartilage is gradually replaced by bone, which becomes ossified after several weeks. Finally, osteoclasts are recruited to the ossified bone, which then undergoes remodeling to produce mature, lamellar bone[32]. Fracture healing is ultimately considered complete with the restoration of functional and biomechanical properties[36].

Despite the generally robust capacity for skeletal tissue to regenerate, delayed healing or failure to heal (non-union) are not uncommon. The FDA defines a non-union as a fracture that has not healed by 9 months after injury, with radiography showing no improvement over the final 3 months[37]. The risk for non-union is determined by many patient-specific factors.

Smokers, diabetic patients, and elderly patients with or without osteoporosis are at increased risk for delayed healing and non-union[1–8, 11, 37]. Other factors include the type of bone involved, mechanics of the fracture, degree of initial bone loss, time since initial injury, and the extent to which the adjacent soft tissue is disrupted. Consideration of these individual factors is important in determining the ideal approach for the initial treatment or in addressing delayed healing and non-unions.

THE HISTORICAL ROLE OF BMPs IN BONE REGENERATION

The three major mechanisms of osteoconductivity, osteoinductivity, and osteogenesis are well-established requirements for bone healing. The first refers to the capacity of a material to serve as a scaffold that allows for bone ingrowth and mineral deposition. Examples of osteoconductive materials include hydroxyapatite and other calcium phosphate ceramics, allograft, and to a lesser degree, demineralized bone matrix and other collagen-based scaffolds. Osteoinductivity refers to factors with the ability to signal progenitor cells to differentiate into bone-forming cells (osteoblasts). Classic examples of osteoinductive factors are BMPs, reviewed in this section[35, 38–40]. Finally, osteogenic materials are those that contain cell types capable of forming new bone, or osteoprogenitor cells. Examples of osteogenic materials include autologous bone and bone marrow.

Bone morphogenetic protein (BMP) was first discovered in 1965 by Marshall Urist, a bone biologist, orthopaedic surgeon, and pioneer in the field of bone regenerative medicine[39–41]. In his original 1965 publication, Dr. Urist showed that demineralized bone matrix (DBM)—prepared from human cadaveric bone and processed to remove mineral—could induce new bone formation when implanted at non-bony sites in animal models[39]. Urist postulated that this result was indicative of a previously unknown substance present in bone, which he termed, *bone morphogenetic protein*. He and others then undertook the challenge over the next 30 years of isolating BMPs and determined that these proteins play a critical role in several steps of the bone healing cascade, including mitogenic, chemotactic, and osteoinductive actions, as well as promoting callus formation and mineralization[33, 34, 42]. Since then, many studies have validated the role of BMPs in the growth, recruitment, attachment, proliferation, and differentiation of mesenchymal progenitor cells, ultimately resulting in new bone formation[41, 43].

BMP-2

Although eight of the BMPs have established osteochondral functions[44], BMP-2 appears to be the most potently osteoinductive and has therefore garnered the most interest. BMP-2 was first cloned in 1988 by a team led by John Wozney, which led to its classification as a member of the TGF-β superfamily[34]. The clinical use of the growth factor for augmentation of bone healing soon followed[45]. Over the next several decades, the newly available recombinant human BMPs (rhBMPs) were evaluated in pre-clinical animal models for safety and efficacy[35, 41, 46–56]. With the ability to induce progenitor cells to both the chondrogenic and osteogenic lineages, BMP-2 thus has utility in both endochondral and intramembraneous ossification applications[32, 57]. Initial *in vivo* studies typically evaluated the efficacy of rhBMP-2 using simple carriers, such as an absorbable type 1 collagen

sponge, although advances in the past two decades have enabled more sophisticated delivery modalities. BMP-2 shows osteoinductivity *in vivo*, as evidenced by the formation of ectopic bone after implantation in non-bony sites (i.e., subcutaneous and intramuscular implantation)[58–64]. Bony defect models in which rhBMP-2 has shown pre-clinical efficacy include spinal fusion (rats, rabbits, goats, sheep, non-human primates)[65–75], extremity segmental defects (rats, rabbits, dogs, non-human primates)[62, 76–83], and cranial defects (mice, rats, rabbits, dogs, and goats)[84–90], among others. Dosing for these studies is highly variable, and is dependent upon not only the carrier, but also the species, anatomy of the defect, and implant size[57].

Clinical studies evaluating the efficacy of BMP-2 have been performed in several orthopaedic settings, including open tibial shaft and other traumatic extremity fractures[91, 92]. The growth factor has also been extensively utilized in interbody and posterolateral spine fusion procedures[93–100]. In 2002, rhBMP-2 (Infuse™) was approved for open tibial shaft fractures as well as in anterior lumbar fusions in the setting of degenerative disc disease[91]. Many of the clinical studies leading up to and following FDA approval suggest that rhBMP-2 performs comparably to autogenous bone graft, and in some cases promotes more efficient bone healing while obviating the need for graft harvest[94, 96, 100]. However there are a number of more recent studies which suggest that initial reports of efficacy were exaggerated, and adverse events associated with its use underreported[97, 101, 102]. Complications which are well-established to result from supraphysiologic rhBMP-2 dosing include ectopic and heterotopic bone formation, exacerbated inflammation and seroma formation, bone resorption, urogenital complications, and dysphagia when used in the cervical spine[97, 103]. Induction of cancer was also a concern by some, although this has been much debated[97, 104, 105]. Although more judicious use of the growth factor—in terms of both dose and clinical indications—has now been adopted by many surgeons, the delivery of rhBMP-2 using a more efficient carrier would both reduce the necessary dose to achieve high rates of union and reduce or potentially even eliminate the complications associated with its clinical use.

BMP-7

When delivered using a collagen carrier, rhBMP-7 (originally referred to as Osteogenic Protein-1, OP-1) was originally shown in 1992 to induce ectopic bone formation in intramuscular and subcutaneous models[106]. Following that discovery, BMP-7 was investigated extensively to assess its capacity to promote bone and cartilage regeneration, resulting in a large body of pre-clinical research that has validated its use as a means to augment bone healing in orthopaedic applications. The growth factor is sufficiently osteoinductive to promote healing of critical sized defects in rabbits, dogs, and non-human primates, and could promote spinal fusion in large animals after delivery using collagen matrix and other similar carriers[54, 107–116]. Some of these studies found that BMP-7 delivery surpassed even autogenous bone graft in some functional outcomes, such as biomechanical strength[109, 116].

The promise of these pre-clinical results led to the first prospective randomized controlled trial on a BMP, which compared *OP-1 Device* (rhBMP-7) to autograft bone in the capacity

to heal established nonunions of the tibiae[117]. The experimental group, which received 3.5 mg rhOP-1 with 1g bovine type I collagen matrix ("OP-1 Device"), achieved comparable clinical outcomes to the autograft control group, while obviating the morbidity associated with autograft harvest (donor site pain, increased blood loss, and increased infection risk).

In 2001, rhBMP-7 (rhOP-1) was approved by the FDA for the treatment of tibial nonunions, where it performed similarly to autogenous bone graft[117]. RhBMP-7 was also investigated as an adjunct to allograft bone as well as hydroxyapatite, where it enhanced osseointegration and new bone formation[118, 119]. Although rhBMP-7 is not approved generally for spinal fusion indications and requires a humanitarian device exemption in that setting, based on equivalence in clinical and radiographical outcomes, the combined use of rhBMP-7 and local autograft has been recommended as a viable alternative to iliac crest autologous bone graft (ICBG) for single-level instrumented spinal fusion procedures[93]. However, due to conflicting evidence for the use of rhBMP-7 delivered with an absorbable collagen sponge as a bone graft substitute for spine fusion, no recommendation has been made for its use in this clinical application[93]. Furthermore, similar to the supraphysiologic dosing of rhBMP-2, bone resorption associated with rhBMP-7 use has been reported[120, 121].

BMP-6

Although not yet FDA-approved, BMP-6 is another osteoinductive BMP that has received significant attention for its potential for clinical translation. The capacity to promote osteogenic differentiation is well-established, and its ability to promote bone formation and healing have been tested in a number of pre-clinical models[122–126]. Studies have been performed to compare the osteoinductivity and bone forming capacity of rhBMP-6 with that of either rhBMP-7 or rhBMP-2, with conflicting results[125, 127–129]. Despite this, continued investigations exploring the utility of rhBMP-6 in combination with a variety of carriers for bone regenerative medicine are ongoing[125, 126, 130–132].

RhBMP-6 is the biologic component of a product currently under development for clinical translation for bone regenerative purposes. The carrier in this OSTEOGROW device, is autologous blood coagulum, which has a greater affinity for rhBMP-6 than does the FDA-approved rhBMP-2 and rhBMP-7 carriers for their respective growth factors. This property is expected to eliminate the burst release and enable delivery of a significantly lower therapeutic dose of the growth factor[133]. OSTEOGROW is currently being evaluated in GLP and GMP studies for safety and efficacy in both acute radial fracture and high tibial osteotomy (HTO) indications, and in the first report of Phase I results from the HTO trial, no serious side effects were reported[125].

SYNTHETIC CARRIERS FOR BMP

The safety of BMPs for clinical use came into question after serious side effects were noted with use of rhBMP-2 in spine surgery applications[97, 105], and its use subsequently dropped dramatically. The adverse outcomes are attributed to the supraphysiologic dosing required for successful healing, and the need for such high doses is due to the inefficiency of growth factor delivery with the use of the FDA-approved carriers. This understanding highlights the

importance of the carrier in delivering GF for optimal efficacy while maintaining an acceptable safety profile. In recent years, a wide variety of delivery vehicles have been investigated for improved efficiency in growth factor delivery. One such approach is the use of self-assembling peptides.

To obviate the requirement for supraphysiologic doses of recombinant growth factor, *improved carriers* for BMP should more efficiently bind and retain the growth factor. This enables the maintenance of local concentrations sufficient to induce signaling while preventing unwanted diffusion and off-target effects such as heterotopic/ectopic bone growth or uncontrolled inflammation. In addition to slowing BMP release, the ideal carrier would also provide an osteoconductive scaffold that can support the infiltration of bone-forming cells and bony ingrowth. Researchers have loaded BMP onto a wide variety of materials, including minerals such as hydroxyapatite[134] and tricalcium phosphate[135], proteins such as collagen[136] and fibrin[137], and natural polymers such alginate[138], hyaluronic acid,[139] and chitosan[140]. BMP carriers have also been crafted from *synthetic* polymers, usually *covalent* polymers such as polyethylene glycol (PEG)[141], poly(ethyl acrylate) (PEA),[142] and polylactic-co-glycolic acid (PLGA)[143].

The use of completely synthetic materials can offer improved control over materials properties, since the chemical structures can be rationally tuned. Historically, synthetic carriers for BMPs and other growth factors have been traditional polymers in which structural units are linked through covalent bonds. However, polymers may take a long time to biodegrade or be cleared, and thus compromise biocompatibility of the carriers. At the same time, formation of ordered structures designed to optimize function of carriers, particularly for bioactivity, is rather challenging with covalent polymers. Over the past two decades supramolecular materials have emerged[144], in which structural units or monomers interact through non-covalent bonds. An early example of a supramolecular biomaterial with liquid crystalline properties was reported by the Stupp laboratory[145], and many of these materials are known as *supramolecular polymers*.[146] Thus, the use of *supramolecular biomaterials* based on formation of *non-covalent* bonds among their constituent molecules may offer distinct advantages as osteoconductive carriers for BMP delivery. In supramolecular biomaterials, the individual molecular building blocks are not tethered together by covalent bonds, but instead *self-assemble* through non-covalent interactions such as hydrogen bonds, hydrophobic interactions, metal chelation, van der Waals forces, or π-π stacking.

SELF-ASSEMBLING PEPTIDES FOR BMP DELIVERY

Within supramolecular biomaterials, *self-assembling peptides* have gained attention and shown promise in a variety of applications[147]. Since signal transduction is largely mediated by proteins, which requires peptide chains to engage in non-covalent interactions, self-assembling peptides are an attractive platform for designing bioactive materials. The non-covalent connections among the monomers facilitate the formation of ordered structures that may be important for bioactivity and biodegradation rates might be much faster than those associated with covalent polymers[148]. The library of natural and unnatural amino acids

offers great combinatorial diversity, and many amino acids have hydrogen bonding or hydrophobic regions that can participate in non-covalent self-assembly.

PuraMatrix™ is a hydrogel scaffold comprised of a 16 amino acid peptide sequence called RADA16, which forms non-covalent β-sheeted structures to self-assemble into peptide nanofibrils[149] (Figure 1A). In one study, PuraMatrix™ mixed with recombinant human BMP-2 successfully regenerated calvarial bone in New Zealand white rabbits, while PuraMatrix™ alone and BMP-2 alone did not[150]. In another study, researchers created an injectable hydrogel of RADA16 mixed with BMP-2, which transitions from a solution to a gel in an *ex-vivo* pig femoral head model[151]. The RADA16 hydrogel successfully slowed BMP-2 release, and *in vitro* experiments demonstrated that the BMP-2 released retained its bioactivity[151]. The RADA16 peptide may also be modified to incorporate biological motifs, in particular short peptide sequences that can signal cells[152] (Figure 1B). One study found that the incorporation of cell adhesion motifs into RADA16 hydrogels improved osteoblast cell adhesion and migration into the scaffolds[152] (Figure 1B). Another self-assembling peptide system is SPG-178, a 13-amino acid sequence that forms nanofibrous structures[153] (Figure 2A). Similar to RADA16, SPG-178 self-assembles into nanofibers due to β-sheet structures among the peptide monomers[153] (Figure 2A). A network of SPG-178 nanofibers can form hydrogels, which have been shown to have some inherent osteoinductive properties (Figure 2B)[154, 155]. While RADA16 and SPG-178 are both relatively short peptide sequences (16 and 13 amino acids long, respectively), longer peptide chains can also be the basis for self-assembling peptide materials. For example, Poly$(VPAVG)_{220}$ is an thermoresponsive elastin-like polymer with the sequence VPAVG repeated 220 times[156]. Poly$(VPAVG)_{220}$ self-assembles into spherical nanoparticles (Figure 3), which are capable of encapsulating and delivering both BMP-2 and BMP-14[156]. These examples show the potential of self-assembling peptides for BMP delivery and bone regeneration as well as the diversity that is possible with the use of self-assembling peptide biomaterials. However, peptides can be further improved by modification with other types of molecules, similar to the post-translational modification of proteins in biology. One example is *peptide amphiphiles (PAs)*, a class of synthetic molecules that contain a short peptide chain conjugated to an aliphatic tail.[148, 157–159]

PEPTIDE AMPHIPHILE SCAFFOLDS FOR BMP DELIVERY

In canonical form, peptide amphiphile (PA) molecules that can self-assemble into supramolecular nanofibers and form hydrogels contain a single aliphatic tail covalently bonded to a peptide sequence that induces hydrophobic collapse. The peptide segment commonly is one that leads to the formation of β-sheets with high densities of intermolecular hydrogen bonding[148, 157–161]. These non-covalent interactions can be tuned to enable self-assembly into a variety of structures, making PAs a versatile platform for biomaterials design.[148, 157–159] Since the first report in 2001[157], work on PA nanofibers over the past two decades has demonstrated their ability to regenerate a variety of tissues including bone[162–165], cartilage[166], muscle[167], vasculature[168], and neural tissue[169]. For bone regeneration specifically, PA-based biomaterials have successfully healed bone defects with low doses of recombinant BMP-2.[163–165].

In 2008 the authors' laboratory developed a "heparin-binding PA," which contained a short peptide sequence that binds heparan sulfate[170], a highly sulfated polysaccharide that regulates growth factor activity. In addition to their heparan sulfate-binding activity, the PA molecules were designed to self-assemble into nanofibrous structures reminiscent of the natural extracellular matrix (ECM)[170]. Heparan sulfate naturally exists as a glycosaminoglycan bound to fibrillar proteins within the ECM, from where it binds a multitude of growth factors and controls their interactions with cell receptors[171]. Since one of these growth factors is BMP-2[64, 172], the ability of the heparin-binding PA to regenerate bone was explored in 2013[163]. In that study, it was postulated that PA nanofibers would bind heparan sulfate, which would in turn bind BMP-2 and present it to cell receptors[163] (Figure 4A). The heparin-binding PA nanofibers were combined with low-dose (1 μg) BMP-2, loaded onto a porous collagen sponge to improve surgical handling properties, and then heparan sulfate was added to this scaffold (Figure 4B–C)[163]. This synthetic biomaterial was implanted in rat critical sized femoral defects, where the combination of PA, BMP-2, and heparan sulfate achieved full bony bridging in over half of animals (Figure 4D)[163]. Both the PA and heparan sulfate were required to achieve this healing rate with use of the low-dose BMP-2 (Figure 4D)[163]. Since heparan sulfate is a natural glycosaminoglycan that can potentiate BMP signaling, its co-delivery with PA nanofibers is an attractive strategy for bone regeneration. However, to avoid the need to source the highly diverse heparan sulfate, supramolecular polymers with "built-in" synthetic ability to potentiate BMP-2 signal would be desirable.

PA nanostructures can be synthetically designed with biological function by adding bioactive epitopes, usually short amino acid sequences that comprise the bioactive portion of natural proteins (Figure 5A)[168, 169, 173, 174]. Compared to natural proteins which have short half lives, these synthetic epitopes that mimic proteins are more stable when embedded in supramolecular assemblies and can remain bioactive for longer periods of time. Furthermore, the presentation of epitopes on PA nanofibers can result in higher bioactivity than the soluble peptides, given the stability of the supramolecular construct with all of its internal cohesive energy relative to the soluble peptide[174] (Figure 5B–C). In addition, since supramoleciular polymers are dynamic[175, 176], the internal structure of bioactive signals may rearrange to optimize effective binding with cells receptors[146] (Figure 5D). The chemical sequence and structural diversity of heparan sulfate allows this natural polysaccharide to interact with many different proteins – one report suggests up to 435 unique proteins[177] - using the proteins' heparin-binding domains that recognize the sulfated moieties with specific chemical sequences. Due to this structural diversity, capturing the function of heparan sulfate presents unique challenges compared to proteins on which short bioactive sequences have been clearly identified.

To mimic the structure and function of natural heparan sulfate, the authors' laboratory synthesized an abiotic tri-sulfated monosaccharide and conjugated it to a PA molecule, thus creating a "glycopeptide PA"[165] (Figure 6A). When the glycopeptide PA self-assembles into nanofibers, the tri-sulfated monosaccharides are presented at the surface of those nanofibers (Figure 6B). Because non-covalent bonds within supramolecular structures can dynamically rearrange,[175, 176] the tri-sulfated monosaccharides can access different configurations and thus adapt to the heparin-binding domains of different proteins.[165] Interestingly, it was

discovered that supramolecular assemblies of the glycopeptide PA were able to bind five different important proteins in biological development and regenerative medicine, BMP-2, BMP-4, FGF-1, FGF-1, and VEGF, which demonstrates its potential for multipotent protein activation[165]. When the tri-sulfated monosaccharide was replaced with a non-sulfated counterpart (Figure 6A), protein binding to PA assemblies did not occur[165]. Furthermore, *in-vitro* experiments measuring alkaline phosphatase (ALP) expression showed that supramolecular nanofibers of the glycopeptide PA enhanced signaling of wild type BMP-2, but not of a mutant BMP-2 lacking its heparin-binding domain[165].

The glycopeptide PA supramolecular polymers were tested for *in vivo* bioactivity by absorbing them into porous collagen sponges, and interestingly these biomaterials were found to reduce by a factor of 100 the necessary therapeutic dose to achieve rat spinal fusion. This extremely low dose was sufficient to achieve a fusion rate of 100% when pre-loaded with only 0.1 μg BMP-2/rat[165] (Figure 6C). High-resolution microCT (computerized tomography) revealed the robust formation of bone throughout the entire fusion bed[165] (Figure 6D). PA nanofibers bearing a non-sulfated version of the same monosaccharide achieved minimal fusion (10%) at the sub-therapeutic dose of 0.1 μg rhBMP-2/rat[165] (Figure 6C). Similar to the heparin-binding PA supramolecular polymers, the glycopeptide PA ones potentiate BMP-2 signal, but the bioactivity derives from the PA assemblies themselves and not from bound heparan sulfate. Given the experiment mentioned above about the system with a mutated heparin-binding domain, it is reasonable to conclude that the glycopeptide PA supramolecular nanofibers orient BMP-2 protein molecules in the correct spatial orientation to signal their receptor. This may be the basis of the biomaterial's remarkable bioactivity toward bone regeneration in spinal fusion[165].

Motivated by the clinical translation of BMP-2 to promote bone regeneration, a PA supramolecular polymer with capacity to directly bind this protein was developed in the authors' laboratory[164, 178]. The monomer of this supramolecular system is known as the BMP-2- binding PA which includes a bioactive peptide sequence discovered by phage display[178] (Figure 7A–B). While the glycopeptide PA utilizes a tri-sulfated monosaccharide to emulate sequences in heparan sulfate that would recognize BMP-2's heparin binding domain, the BMP-2 binding PA is functionalized instead with a short peptide sequence capable of binding the protein. The BMP-2 binding PA was tested in a pre-clinical rat model of spinal fusion, where the established positive control—rhBMP-2 delivered using a collagen sponge (similar to the clinical product Infuse™)—requires a dose of 10 μg/rat in order to achieve a fusion rate of 100%[164]. When deployed in this model, the BMP-2-binding PA reduced the therapeutic BMP-2 requirement 10-fold, achieving successful fusion in 100% of the animals, using a dose of 1 μg BMP-2/rat (0.5 μg/implant)[164] (Figure 7C–D). Interestingly, when this PA was delivered alone (i.e. *without* any rhBMP-2), successful fusion was noted in 42% of animals, which was assumed to be a result of the capacity for the supramolecular PA assembly to bind endogenous BMP-2 and potentiate signaling (Figure 7C–D)[164].

In summary, all three of the PA-based bone regenerative materials described here have demonstrated ability to lower the therapeutic BMP dose in animal models. Osteoinductive signals were incorporated into these PA supramolecular polymers not only by encapsulating

BMP-2, but also by rational chemical design to potentiate its signal. All three PA systems formed nanofibrous structures reminiscent of natural ECM, which likely contributed to the ability of cells to infiltrate the scaffolds containing PA assemblies. In surgical settings, the periosteum and bone marrow space are typically exposed and serve as a source of osteoprogenitor cells that can infiltrate the scaffold. Although obviating the need to source live cells is ideal, given that PA nanofibers can present bioactive signals, PA-based materials for cell delivery may also lead to promising therapies. In particular, bone marrow aspirate contains self-renewing cells with the capacity to synthesize growth factors such as BMPs and VEGF[179, 180]. Thus, if these cells were delivered with a PA vehicle, the PA scaffold may increase cell survival as well as potentiate the signal of the synthesized growth factors.

In addition to BMP delivery, the PA platform offers many other advantages for bone regeneration applications. Although PA nanofibers can support cell adhesion without added biological functionality[160, 181, 182], cell adhesion to PA nanofibers can be improved by adding the fibronectin cell adhesion motif, RGD[183], and the chemical structure of RGD-bearing PAs can be tuned to optimize biological response[173]. Furthermore, PA nanofibers can support mineral deposition, a critical process in new bone formation. In particular, PAs with phosphoserine residues show the ability to nucleate hydroxyapatite crystallization[157], as well as the ability to promote the expression of early osteogenic markers *in vitro*[184]. In one report, a composite of RGD-bearing PA and phosphoserine-bearing PA was implanted in a rat femoral defect model, where the combination of both PAs led to more bone growth than either PA alone[162]. Furthermore, the morphology of PA assemblies can be tuned to template macroscopic alignment of hydroxyapatite across length scales, thus orienting hydroxyapatite in ways that emulate the structure of bone.[185]

Beyond direct BMP-2 delivery, PAs can also potentiate BMP-2 signaling via modulation of lipid raft mobility[186]. A PA molecule lacking a bioactive epitope was shown to significantly enhance both BMP-2 and Wnt signaling *in vitro*; this was attributed to the ability of PA assemblies to associate with the cell membrane and affect lipid raft structures[186]. More specifically, this PA had positive charge, opposite to negatively charged cell membranes, and its supramolecular assemblies also had weak internal cohesion[186]. This allowed the PA molecules to interact with the cell membrane and increase diffusion within its lipid rafts, which interestingly led to an increase in signaling[186]. An analogous PA with similar charge but strong internal cohesion in fact did not enhance BMP-2 signaling[186]. While many studies have examined how PAs can influence BMP-2 signaling, other signaling pathways are of course integral in the bone healing process. PAs with biomimetic epitopes for VEGF[168] and FGF-2[187] have been developed, which could promote angiogenic, mitogenic, and chemotactic activities that are all required for successful bone regeneration.

Comprised of the same amino acids that build natural proteins, self-assembling peptides are uniquely qualified as biomaterials with cell-signaling capabilities. Compared to covalent polymers, supramolecular polymers based upon dynamic non-covalent bonds more closely mimic the nature of biological tissues and are therefore often more biocompatible and biodegradable. With the potential for further modification of peptides, particularly through conjugation of an aliphatic tail or monosaccharides, the PA platform can achieve physical and biological properties not possible with self-assembling peptides alone. The ability of

PAs to regenerate bone with low BMP-2 doses was first observed in rodents and the next challenge is to establish their safety and efficacy in large animal models, which will clearly be necessary for these systems to advance toward clinical translation. Large animals will require higher BMP doses to promote bone regenearation, so the ability of PAs to potentiate BMP signaling safely on scales closer to the ones required in humans will be investigated in these models. At the same time, in large animals greater amounts of PA will be required to fill defects or achieve spinal fusion. On these larger scales, the kinetics of biodegradation, overall biocompatibility, as well as mechanical and rheological properties will have to be tested as well. PA nanofibers have a high charge density by design in order to promote solubility in aqueous media[159], and interactions of these charges with cells and proteins might be different in large versus small animals. In this regard it is encouraging that there have been reports where self-assembling peptides were used effectively in in large animals such as rabbits[166] and pigs[188]. Also, we are greatly encouraged by the fact that preliminary work in the authors' laboratory and elsewhere has demonstrated great efficacy and safety in rabbit models of spinal fusion with low dose BMP-2 and PAs[189].

CONCLUSIONS AND OUTLOOK

Given the many requirements for successful bone healing, the ideal synthetic biomaterial to support the regeneration of bone will likely require the integration of multiple components. Although recombinant BMPs provide a highly effective osteoinductive signal, this family of growth factors cannot be used effectively without a self-supporting, implantable carrier. Absorbable collagen sponges have been used as carriers for recombinant human BMP-2 and BMP-7 with clinical efficacy, but not without side effects, which are attributed to the supraphysiological BMP dosages required. Thus, the development of carriers that can reduce the effective BMP dose by slowing BMP release and/or potentiating BMP signal would be beneficial. In this review, we have discussed the use of self-assembling peptides to fulfill these important functional features. Bio-inspired strategies based upon our understanding of the composition and function of natural extracellular matrices, as well as osteoinductive growth factor signaling, have led to the design of PA structures which not only slow BMP-2 release, but may also to recruit and potentiate endogenous growth factor activity. Beyond BMP, we discussed the notion of multipotent protein activation by heparan sulfate-mimetic PA systems, which are capable of binding a multitude of growth factors involved in bone regeneration, including VEGF, and FGF. In addition to binding and delivering growth factors, the filamentous structures that result from PA self-assembly are highly mimetic of the natural extracellular matrix, which can support cell adhesion as well as mineralization.

Self-assembling peptides such as PA-based supramolecular polymers have demonstrated potential to become effective BMP carriers, and can complement the large variety of existing materials in orthopedic surgery. In this respect, since PAs are relatively soft materials, the combination of PAs with materials that have load-bearing capacity would be beneficial. The heparin-binding PA and glycopeptide PA discussed in this review were applied to collagen sponges, similar to how recombinant BMP-2 is currently used. PA liquid solutions may also be loaded onto metal, ceramic, or 3D-printed scaffolds, creating completely synthetic composites that have both load-bearing capabilities and bioactive functions. A composite of PA and demineralized bone matrix (DBM) represents an alternative strategy to PA-based

recombinant growth factor delivery. Furthermore, cells can be suspended in a PA solution, which could then be used to coat a scaffold. With the capacity to safely harness growth factor-based bioactivity, PA-functionalized composite materials could potentially overcome the limitations associated with many currently available bone regenerative products. We anticipate that self-assembling peptides combined with BMPs as well as load-bearing materials for structural integrity could generate highly effective systems for bone regeneration and completely novel clinical opportunities.

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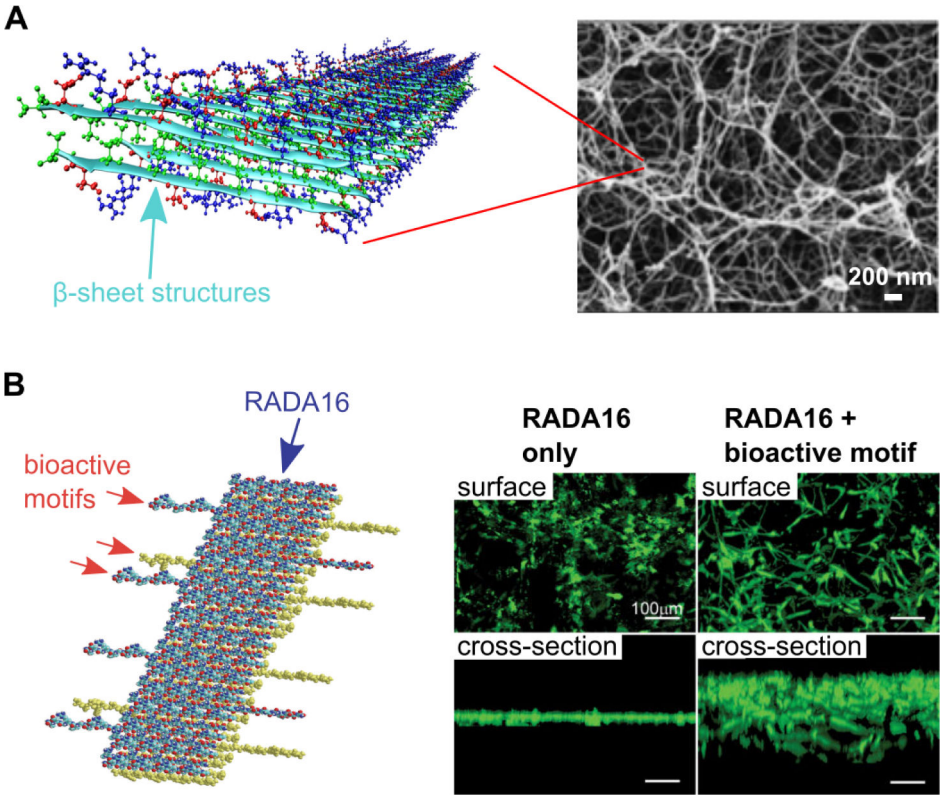

Figure 1.

(A) Molecular graphics representation of RADA16 nanofibrils, and scanning electron micrograph (SEM) of a RADA16 hydrogel. Adapted with permission.[190, 191] Copyright 2013, American Chemical Society; Copyright 2011, Royal Society of Chemistry **(B)** Molecular graphics representation of RADA16 nanofibrils with a bioactive motif incorporated, and calcein-stained cells cultured on RADA16 hydrogels with and without bioactive motifs (in this case, a cell adhesion ligand). The bioactive cell adhesion motif improves cell spreading on and infiltration into RADA16 hydrogels. Adapted with permission under the terms of the Creative Commons Attribution License.[152] Copyright 2007, S. Zhang, published by PLOS.

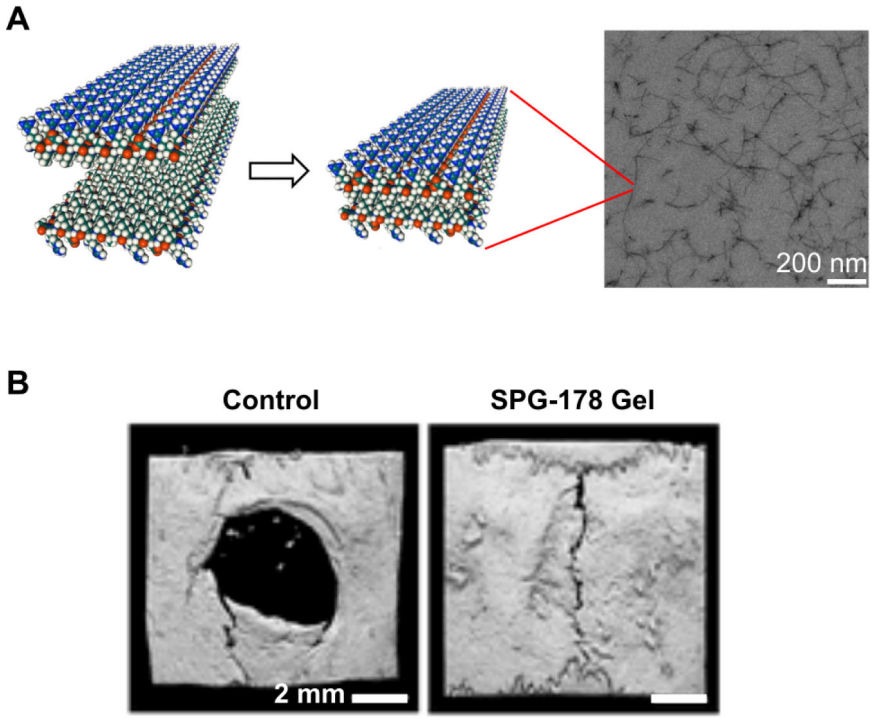

Figure 2.

(A) Molecular graphics representation of SPG-178 peptide nanofiber assemblies, and transmission electron micrograph (TEM) of a SPG-178 hydrogel. Adapted with permission.[153] Copyright 2012, Elsevier **(B)** Representative microCT (computerized tomography) reconstructions of rat calvarial defects, showing the degree of bone healing after 3 weeks when untreated (Control) or treated with SPG-178 hydrogels. Reproduced with permission.[155] Copyright 2017, Mary Ann Liebert, Inc.

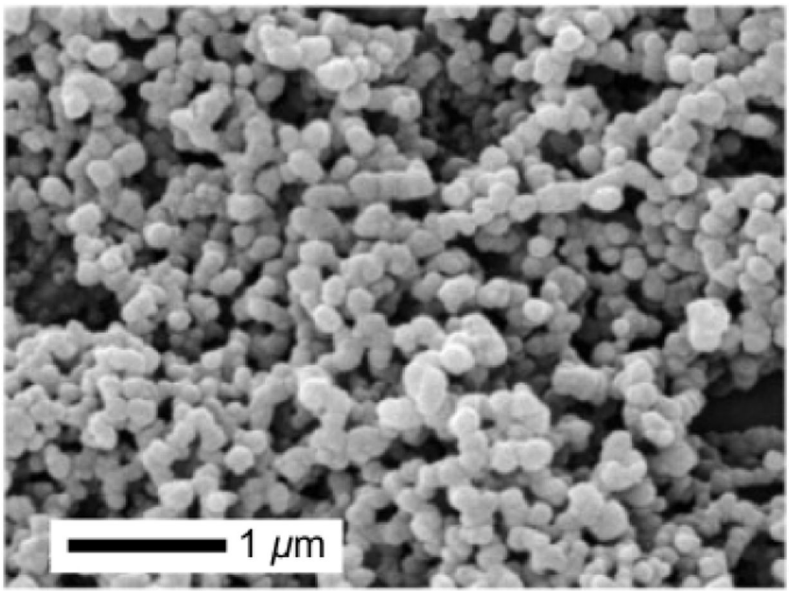

Figure 3.
Scanning electron micrograph (SEM) of elastin-like Poly(VPAVG)$_{220}$ supramolecular assemblies. Reproduced with permission.[156] Copyright 2010 Elsevier.

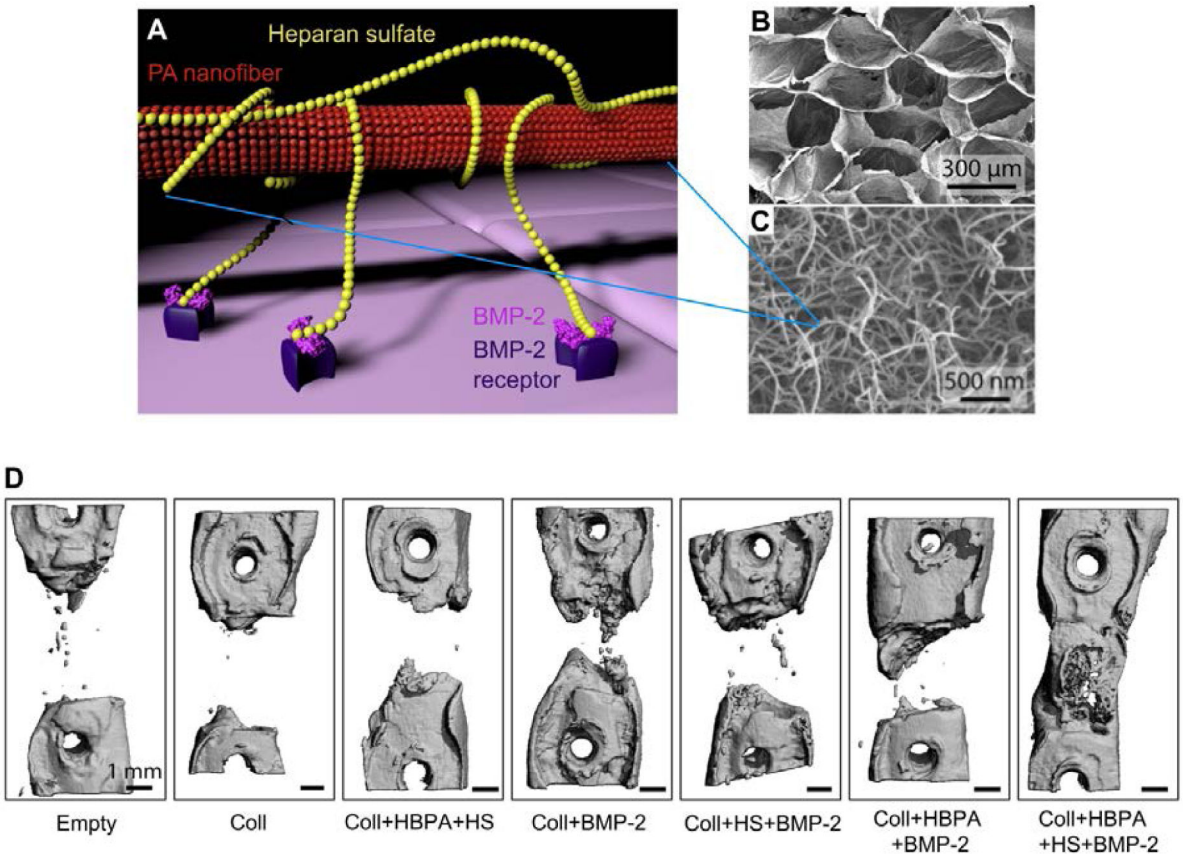

Figure 4.

(A) Molecular graphics representation of heparin-binding PA structure and function. The heparin-binding PA nanofiber binds heparan sulfate, which is a lengthy polysaccharide. The heparan sulfate in turn binds BMP-2 growth factor and presents it to receptors on the cell membrane, thus potentiating the signal of BMP-2. **(B)** Empty porous collagen sponge, which heparin-binding PA nanofibers were loaded onto to improve surgical handling properties. **(C)** Scanning electron micrograph (SEM) of heparin-binding PA nanofibers, mixed with heparan sulfate and loaded onto a porous collagen sponge. **(D)** Representative microCT (computerized tomography) reconstructions of rat femur defects, showing the degree of bone healing after 6 weeks when treated with the indicated materials. Abbreviations: Coll – collagen sponge; HBPA – heparin-binding PA; HS – heparan sulfate. A) Adapted B-D) Reproduced with permission.[163] Copyright 2013, Elsevier.

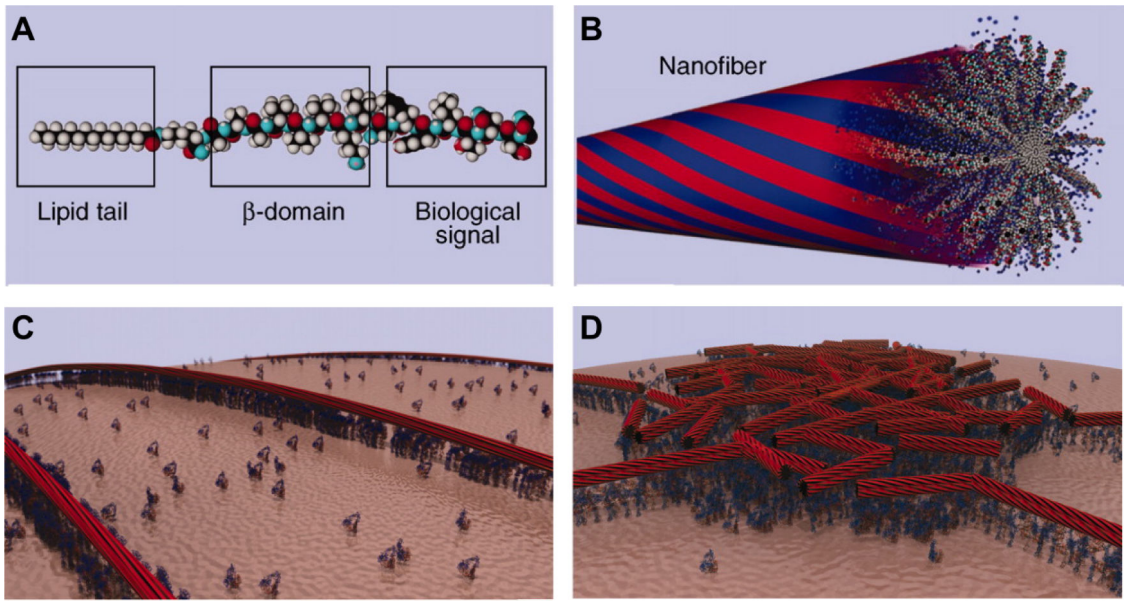

Figure 5.

Molecular graphics representations showing **(A)** a PA molecule incorporating a bioactive peptide epitope and **(B)** the supramolecular nanofiber formed by these PA molecules. Due to hydrophobic collapse of the lipid tail in aqueous environments, the bioactive signals are displayed at high density of the surface of the nanofiber (idealized in red portions). The blue portions represent idealized water domains. **(C)** Molecular graphics representation of PA nanofibers concentrating and presenting biological signals to cell membranes. **(D)** Due to their non-covalent nature, PA nanofibers may dynamically rearrange over time as they interact with cells. In this molecular graphics representation, PA nanofibers have concentrated around a lipid raft structure where cell signaling activity is centered. Reproduced with permission.[146] Copyright 2012, AAAS.

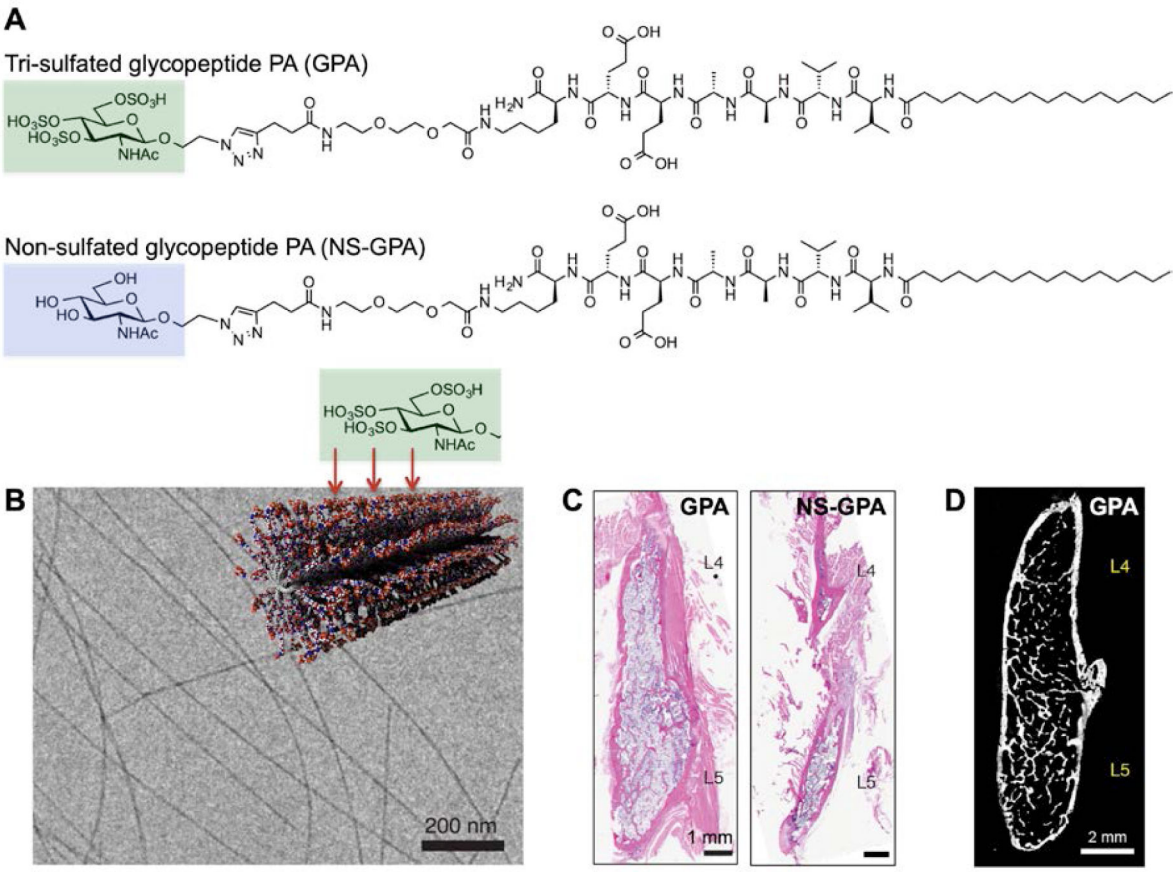

Figure 6.

(A) Chemical structure of PA containing a tri-sulfated monosaccharide, highlighted in green, and the same PA bearing a non-sulfated version of the monosaccharide, highlighted in blue. **(B)** Cryogenic transmission electron micrograph (cryoTEM) of the tri-sulfated glycopeptide PA and molecular graphics representation of the PA nanofibers. The tri-sulfated monosaccharide moities are displayed on the surface of supramolecular nanofibers. **(C)** Representative sagittal cross-sectional images of the fusion bed in rat spines, visualized with hematoxylin and eosin (H&E) staining. Rats received 0.1 μg of BMP-2 and the indicated PAs. **(D)** High-resolution microCT (computerized tomography) reconstruction from a fused rat treated with tri-sulfated glycopeptide PA and 0.1 μg BMP-2. A) Adapted B-D) Reproduced with permission.[165] Copyright 2017, Springer Nature.

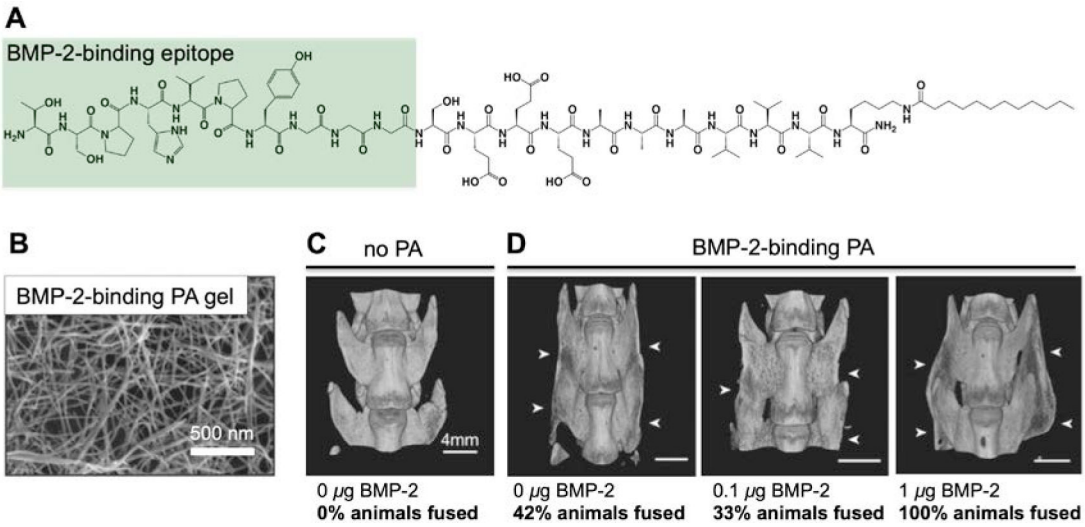

Figure 7.

(A) Chemical structure of a PA containing a short peptide sequence identified to bind BMP-2 via phage display. The bioactive peptide portion is highlighted in green. **(B)** Scanning electron micrograph (SEM) of a hydrogel containing the BMP-2 binding PA. **(C)** MicroCT (computerized tomography) reconstruction of an unfused animal treated with no PA and no BMP-2, included for comparison with **(D)** microCT (computerized tomography) reconstructions of fused animals treated with BMP-2 bindign PA and indicated BMP-2 dosages. The images are specifically of fused animals in the groups; the overall fusion rates[164] are indicated. The white arrows indicate the fusion bed. Adapted with permission. [164] Copyright 2015, Wiley VCH.