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# Glycosaminoglycan mimetic peptide nanofibers promote mineralization by osteogenic cells

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ABSTRACT

Bone tissue regeneration is accomplished by concerted regulation of protein-based extracellular matrix components, glycosaminoglycans (GAGs) and inductive growth factors. GAGs constitute a significant portion of the extracellular matrix and have a significant impact on regulating cellular behavior, either directly or through encapsulation and presentation of growth factors to the cells. In this study we utilized a supramolecular peptide nanofiber system that can emulate both the nanofibrous architecture of collagenous extracellular matrix and the major chemical composition found on GAGs. GAGs and collagen mimetic peptide nanofibers were designed and synthesized with sulfonate and carboxylate groups on the peptide scaffold. The GAG mimetic peptide nanofibers interact with bone morphogenetic protein-2 (BMP-2), which is a critical growth factor for osteogenic activity. The GAG mimicking ability of the peptide nanofibers and their interaction with BMP-2 promoted osteogenic activity and mineralization by osteoblastic cells. Alkaline phosphatase activity, Alizarin red staining and energy dispersive X-ray analysis spectroscopy indicated the efficacy of the peptide nanofibers in inducing mineralization. The multifunctional and bioactive microenvironment presented here provides osteoblastic cells with osteogenic stimuli similar to those observed in native bone tissue.



## 1. Introduction

Regeneration of damaged bone tissue is a significant health problem in aging populations. Fractures, degenerative diseases and infections cause bone loss. Inducing bone formation and regeneration using allografts, natural extracellular matrix (ECM) components or collagen-derived materials may cause immunological responses or disease transmission. For this reason synthetic scaffold materials have been developed to induce tissue regeneration [1]. Peptide amphiphile molecules can be decorated with various bioactive sequences in high density while self-assembling into nanofibrillar networks similar to natural ECM [2]. These characteristics of peptide amphiphile molecules provide considerable opportunity to design ECM mimetic structures, which can be utilized to direct cellular behavior and guide proper restoration of damaged tissues by creating functional microenvironments [3–5].

The ECM plays an indispensible role in bone regeneration and mineralization. It regulates cell-specific interactions via collagenous and non-collagenous molecules, which guide cellular behaviors such as adhesion, migration, proliferation and differentiation [6–8]. In bone tissue the ECM is composed of approximately 50–70% inorganic calcium and phosphate minerals and 20–40% organic components, which mainly consist of collagen type I surrounded by proteoglycans, glycosaminoglycans (GAGs) and other proteins [9]. GAGs have important roles in bone remodeling, such as stabilizing growth factors and enhancing growth factor–receptor interactions [10]. Bone ECM contains a variety of sulfated and non-sulfated GAGs, including chondroitin sulfate, dermatan sulfate and hyaluronan, while heparin and heparan sulfate can be found in bone marrow [11–13]. These GAGs can trigger bone remodeling by affecting cellular proliferation and differentiation via binding growth factors and through direct cell surface receptor activation [14]. While sulfated GAGs, including heparin and heparan sulfate, induce binding of growth factors and facilitate growth factor-mediatedsignaling, several non-sulfated GAGs, such as hyaluronan, are able to interact with cell surface molecules such as CD44, CD168, intercellular adhesion molecule (ICAM) and hyaluronan-mediated motility receptor (RHAMM) to initiate cellular responses such as differentiation and migration [15–18]. In previous studies over-sulfated chondroitin was shown to promote collagen deposition, alkaline phosphatase (ALP) activity and mineral accumulation in osteoblasts [19]. Moreover, synthetic materials composed of sulfated hyaluronan increased tissue non-specific ALP activity and formation of osteoblastic cell aggregates [20].

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In addition to the interaction between GAGs and cells, the types of growth factors interacting with GAGs are also crucial determinants for bone regeneration. A large number of bone regulating proteins and cytokines, such as bone morphogenetic proteins, tumor necrosis factor (TNF)-α, osteoprotegerin, receptor activator of nuclear factor κB ligand (RANKL) and other members of transforming growth factor-β family were previously shown to interact with GAGs [21–24]. Among them, BMP-2 is one of the most osteoinductive growth factors, inducing osteogenic differentiation of multipotent mesenchymal stem cells and directing bone formation [25–28]. BMP-2 binding GAGs found in the ECM synergistically enhance the osteogenic activity of cells. When used with highly sulfated heparin the osteogenic activity increases approximately 5-fold compared with BMP-2 alone [29]. Besides increasing the biological activity of BMP-2, GAGs also serve as delivery agents by capturing and increasing the local concentration of proteins [30]. Therefore, utilizing a GAG mimetic system with osteoinductive properties is a promising technique for bone regeneration applications. We have previously shown that GAG mimetic peptide nanofibers can interact with several growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and hepatocyte growth factor (HGF) [31], and can induce differentiation of cells involved in angiogenesis and neural differentiation [32–33]. In this study we show that GAG mimetic peptide nanofibers with sulfonate and carboxylate groups can provide a suitable microenvironment for bone regeneration and mineralization. We also demonstrate that these peptide nanofibers bind to BMP-2 and increase the viability, proliferation and mineralization of osteogenic cells.

## 2. Materials and methods

### 2.1. Materials

All protected amino acids, lauric acid, [4-[α-(2′,4′-dimethoxyphenyl) Fmoc-aminomethylphenoxyacetomidonorleucyl-MBHA resin (Rink amide MBHA resin), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and diisopropylethylamine (DIEA) were purchased from Nova-Biochem, ABCR, or Sigma–Aldrich. rhBMP-2 (PHC7141), Calcein-AM and other cell culture materials were purchased from Invitrogen. Anti-BMP-2 antibodies and reagents for ELISA assay were purchased from R&D. All other chemicals and materials used in this study were purchased from Fisher, Merck, Alfa-Aesar and/or Sigma Aldrich.

### 2.2. Synthesis and characterization of peptide amphiphile molecules

Peptide amphiphile (PA) molecules were synthesized on Rink amide MBHA resin or Fmoc-Glu(OtBu)-Wang resin using a standard Fmoc solid phase peptide synthesis method. 0.25 mmol resin and 0.5 mmol amino acids were used in the synthesis. Amino acid coupling was performed with 2 equivalents of amino acids activated with 1.95 equivalents of HBTU and 3 equivalents of DIEA for 2 h. Fmoc removal was performed with 20% piperidine–dimethylformamide (DMF) solution for 20 min. 10% acetic anhydride–DMF solution was used to permanently acetylate unreacted amine groups after each coupling step. DMF and dichloromethane (DCM) were used as washing solvents after each step. To synthesize sulfonated PAs a p-sulfobenzoic acid was added to the side-chain of lysine, which has 4-methytrityl (Mtt) side-chain protection as used for selective deprotection of amine groups (see Scheme S1). Mtt removal was performed by shaking the resin for 5 min with trifluoroacetic acid (TFA):triisopropylsilane (TIS):$H_2O$:DCM at a ratio of 5:2.5:2.5:90. Cleavage of the PAs and protection groups from the resin was carried out with a mixture of TFA:TIS:$H_2O$ at a ratio of 95:2.5:2.5 for 3 h. Excess TFA was removed by rotary evaporation. The PAs in the remaining solution were precipitated in ice-cold diethyl ether overnight. The precipitate was collected by centrifugation next day and dissolved in ultrapure water. This solution was frozen at −80 °C for 4 h and then lyophilized for 4–5 days. The peptides were identified using a quadruple time of flight mass spectrometer with electrospray ionization source equipped with a reverse phase analytical high performance liquid chromatography (HPLC). The purification of PAs was performed using a preparative HPLC system (Agilent 1200). In order to remove residual TFA positively charged peptide amphiphiles were treated with 0.1 M HCl solution and lyophilized. The yield of PAs after purification was ~70% for negatively charged PAs and 90% for lauryl-VVAGK-Am (K-PA). All peptide batches were freeze-dried and reconstituted in ultrapure water at pH 7.4 before use.

### 2.3. Preparation and characterization of self-assembled PA nanofibers

PA stock solutions were prepared in distilled water and adjusted to pH 7.4 before self-assembly. For nanofiber formation lauryl-VVAGEGD-K (*p*-sulfobenzoyl)-S-Am ($SO_3$-PA) and K-PA were mixed at a 1:3 ratio in order to stabilize all net charges. In the same way lauryl-VVAGE (E-PA) and K-PA were mixed at a 1:2 ratio. For surface coatings used in cellular experiments 1 mM PA mixture was coated on tissue culture plate (TCP) surfaces and then placed in a fume hood to dry. To measure the thickness of the PA coatings the coated surfaces were investigated by scanning electron microscopy (SEM) after drying (3.64 ± 0.19 μm for $SO_3$-PA/K-PA and 4.27 ± 0.25 μm for E-PA/K-PA) (Fig. S9). SEM and transmission electron microscopy (TEM) imaging techniques were used to visualize gel formation. For SEM imaging 1 wt.% PA solutions were mixed at 1:2 and 1:3 ratios and then dehydrated sequentially in 20%, 40%, 60%, 80%, and 100% ethanol. Samples were critical point dried with an Autosamdri®-815B Tousimis and coated with 5 nm Au/Pd before imaging. A FEI Quanta 200 FEG scanning electron microscope with an electron-transfer dissociation (ETD) detector in high vacuum mode at 10 keV beam energy was used. For TEM imaging the samples were prepared by mixing 1 mM PA solutions at 1:2 and 1:3 ratios on a 200 mesh carbon TEM grid. After 10 min incubation the unbound peptide nanofibers were rinsed off with water and the remaining peptide nanofibers were air-dried in a fume hood. TEM imaging was performed with a FEI Tecnai G2 F30 transmission electron microscope at 300 kV. Circular dichroism (Jasco J-815) samples were prepared using $1 \times 10^{-5}$ M $SO_3$-PA/$3 \times 10^{-5}$ M K-PA and $1 \times 10^{-5}$ M $SO_3$-PA/$2 \times 10^{-5}$ M K-PA mixtures. Measurements were performed from 300 to 190 nm with three acquisition points. For the Fourier transform infrared spectroscopy (FTIR) analysis 1 wt.% PA mixtures ($SO_3$-PA/K-PA and E-PA/K-PA) were prepared, lyophilized, and pellets obtained after mixing with KBr. A Vortex70 Fourier transform infrared spectrometer was used to identify the FTIR spectrum of the peptide nanofibers in the spectrum range 4000–400 $cm^{-1}$. An Anton Paar Physica RM301 rheometer was used to reveal the viscoelastic properties of the nanofiber network with a 25 mm plate configuration and a gap distance of 0.5 mm at 25 °C. 1 mM PA concentrations at the abovementioned ratios were allowed to gel for 15 min prior to measurement. Frequency sweep rheology measurements were performed at 0.1% constant strain with logarithmic ramping from 0.1 to 100 rad $s^{-1}$.

### 2.4. Cell culture and maintenance

Saos-2 cells (human osteosarcoma cell line, ATCCR HTB-85TM) were used in all cell culture experiments including viability, proliferation, ALP activity and calcium deposition. Cells were cultured in 75 $cm^2$ flasks at 37 °C in a humidified incubator and supplied with