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Hydroxyapatite nanoparticle reinforced peptide amphiphile nanomatrix enhances the osteogenic differentiation of mesenchymal stem cells by compositional ratios

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ABSTRACT

In the field of bone tissue engineering, there is a need for materials that mimic the native bone extracellular matrix (ECM). This need is met through the creation of biphasic composites intended to mimic both the organic and inorganic facets of the native bone ECM. However, few studies have created composites with organic ECM analogous components capable of directing cellular behaviors and many are not fabricated in the nanoscale. Furthermore, few attempts have been made at investigating how variations of organic and inorganic components affect the osteogenic differentiation of human mesenchymal stem cells (hMSCs). To address these issues, biphasic nanomatrix composites consisting of hydroxyapatite nanoparticles (HANPs) embedded within peptide amphiphile (PA) nanofibers tailored with the RGDS cellular adhesion motif (PA-RGDS) were created at various HANP to PA-RGDS ratios. Fabrication of these biphasic nanomatrix composites was confirmed via scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The long-term cellularity and osteogenic differentiation of hMSCs in response to the different compositional ratios were then assessed by quantifying the timed expression of genes indicative of osteogenic differentiation, alkaline phosphatase activity, and DNA content over time. Decreased cellularity and the expression of genes over time correlated with increasing compositional ratios between HANP and PA-RGDS. The highest HANP to PA-RGDS ratio (66% HANP) exhibited the greatest improvement to the osteogenic differentiation of hMSCs. Overall, these results demonstrate that the compositional ratio of biphasic nanomatrix composites plays an important role in influencing the osteogenic differentiation of hMSCs. Based on the observations presented within this study, these biphasic nanomatrix composites show promise for future usage in bone tissue engineering applications.

1. Introduction

Research for the development of biomaterials for orthopedic applications has rapidly shifted to consider a tissue engineering approach that focuses on mimicking the native bone extracellular matrix (ECM) as opposed to biomaterials designed for the sole purpose of providing a load-bearing replacement. This biomimetic approach involves the usage of materials capable of re-creating the native ECM microenvironment, which achieves its hierarchal construction through bottom-up self-assembly at the nanoscale level. The aim of this strategy is expedient regeneration and replacement of the scaffold with native bone tissue. By dispersing hydroxyapatite nanoparticles (HANPs) within a functionalized peptide amphiphile (PA) nanofiber matrix, we were able to successfully create a biphasic nanomatrix composite endowed with bioactivity derived from both the organic and inorganic facets of the native bone ECM. Supporting native osteogenic cells, the native bone ECM is a biphasic nanomatrix comprised of organic and inorganic components [1,2] where organic collagen nanofibers are mineralized with reinforcing HANPs [3].

The organic ECM of bone, representing ~20% of the native bone ECM by weight [2], is a network of proteins that directly regulates cellular behaviors through integrin-mediated binding mechanisms [4]. PAs were designed to elicit the cell–ECM interactions normally provided by an organic ECM. PAs are bipolar molecules consisting of a hydrophilic peptide group attached to a hydrophobic alkyl tail [5–7], self-assembling into cylindrical nanofibers ~8–10 nm in diameter under proper chemical conditions. The PA-driven material is versatile as the self-assembled nanofibers can form three-dimensional hydrogels through the addition of divalent ions, or two-dimensional multi-stacked layers through a solvent evaporation method [7–10]. These PAs have been modified through the incorporation of cellular adhesive ligand sequences isolated from common ECM proteins and peptide groups responsive to enzyme-mediated degradation, providing microenvironments that capture the

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signaling properties of native ECM proteins and enzyme-mediated degradation capability that does not lead to premature ECM erosion. Due to the customizable nature of the cellular adhesive ligand group, PAs have been utilized for a variety of tissue engineering fields such as cardiovascular, pancreatic, osteogenic, and drug delivery applications [8,9,11–13].

The native bone ECM serves to provide support for cells while also serving to regulate intercellular communication, influencing various cellular behaviors such as proliferation, migration, and differentiation [4,14]. In an attempt to capture some of these properties, many studies have proposed the development of materials that mimic either the organic or inorganic aspects of the native bone ECM [15]. However, mimicking only one aspect of the biphasic ECM of bone may have limited success in eliciting sufficient osteogenic responses. To overcome this potential drawback, several groups have proposed the development of composites that mimic both the organic and inorganic facets of the native bone ECM. However, a majority of these studies involve the creation of composites utilizing synthetic polymers to serve as the organic ECM analogous component with HA serving as the inorganic ECM analogous component [16–20]. These synthetic polymers do not recreate the instructive microenvironment provided by native ECM proteins such as collagen without further modification. Additionally, a majority of these studies rely on fixed ratios between materials intended to serve as the organic and inorganic analogous ECM components [16,21,22], providing little insight into how compositional variations between the two ECM analogous components affect cellular response.

To address these issues, both the structural and biological properties of the native bone ECM were imitated by creating bone ECM analogous nanomatrix composites consisting of HANPs dispersed within PA nanofibers functionalized with the RGDS cellular adhesion motif (PA-RGDS). Furthermore, these biphasic nanomatrix composites were fabricated at various HANP to PA-RGDS ratios by weight to gain insightful knowledge about how compositional variations within the inorganic and organic ECM analogous components affect the osteogenic differentiation of human mesenchymal stem cells (hMSCs).

As described in our previous studies, it has been shown that these PAs, when functionalized with the RGDS cellular adhesion motif, are capable of inducing the osteogenic differentiation of hMSCs both with and without the presence of osteogenic supplements [10,23]. The RGD sequence has been shown to be one of the key cell binding domains present within the ECM, being found in multiple proteins such as fibronectin, vitronectin, fibrinogen, and osteopontin [24–26]. RGDS was chosen for this study as its incorporation into PAs has been shown to be the most conducive to the osteogenic differentiation of hMSCs relative to other cellular adhesive ligand sequences such as DGEA, which is derived from collagen, and KRSR, which originates from various proteoglycans [10,23].

While PA nanofiber matrices can promote and enhance the osteogenic differentiation of hMSCs, they only mimic the organic component of native bone ECM. To create a biphasic environment similar to that seen within the native bone ECM, HANPs were supplemented to serve as the inorganic ECM component. The inorganic phase of the native bone ECM is predominately made up of HA, representing ~70% of the ECM by weight [1,2]. HA has seen increasing usage in bone graft therapies, both by itself and in combination with other bulk materials due to its improved bioactivity over traditional synthetic materials, and previous studies have even proposed that HA has osteoinductive potential, although this fact is contested [27–29]. However, while capable of providing bioactivity and potentially inducing osteogenic differentiation, HA, when utilized as a scaffold by itself, is slowly invaded by host tissues, demonstrating limited bioactive capacity when compared to native bone [30–32]. Therefore, to enhance bioactive capacity, HANPs were encapsulated within an organic ECM mimicking PA nanofiber matrix to create biphasic nanomatrix composites.

In order to assess the bioactive properties of these biphasic nanomatrix composites, hMSCs were introduced and long-term differentiation was measured. hMSCs are multi-potent cells capable of undergoing osteoblastic differentiation under various circumstances, usually with the aid of osteogenic supplement medium (OSM) (i.e. dexamethasone, β-glycerol phosphate). However, in a PA-guided microenvironment, hMSCs have been shown to undergo osteogenic differentiation in response to extracellular matrix-mimicking ligands and without the aid of OSM [10,23].

In this study, the biphasic nanomatrix composite comprising HANPs and PA-RGDS nanofibers was created to provide a bone analogous microenvironment. It was hypothesized that the osteogenic differentiation of hMSCs would be affected based on compositional ratios of HANP and PA-RGDS (Fig. 1). These nanomatrix composites were fabricated and characterized using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Following this, the long-term cellularity and osteogenic differentiation of hMSCs were assessed in vitro, utilizing quantitative real-time PCR (RT-PCR), alkaline phosphatase, picogreen dsDNA, and Live/Dead assays. This study is thus one of the first to assess the osteogenic differentiation of hMSCs in relation to variations in the ratio of organic and inorganic analogous ECM components capable of capturing both the nanoscale structure and bioactivity of the native bone ECM.

2. Methods and materials

2.1. Peptide amphiphile synthesis

Peptide amphiphiles (PAs) were synthesized utilizing previously described methods [10,23,33]. Briefly, a peptide sequence consisting of a matrix metalloproteinase (MMP-2) enzyme-mediated degradation sequence (GTAGLIGQ) and a cell adhesive ligand sequence (RGDS) was prepared using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry in an Advanced Chemtech Apex 396 peptide synthesizer (Aapptec, Louisville, KY). Following this, a long alkyl chain was attached to the peptide sequence by reacting the *N*-termini with two equivalents of palmitic acid, two equivalents of *o*-benzotriazole-*N*,*N*,*N*′,*N*′ tetramethyluroniumhexafluorophosphate (HBTU), and four equivalents of diisopropylethylamine (DiEA). The resin was cleaved through the addition of trifluoroacetic

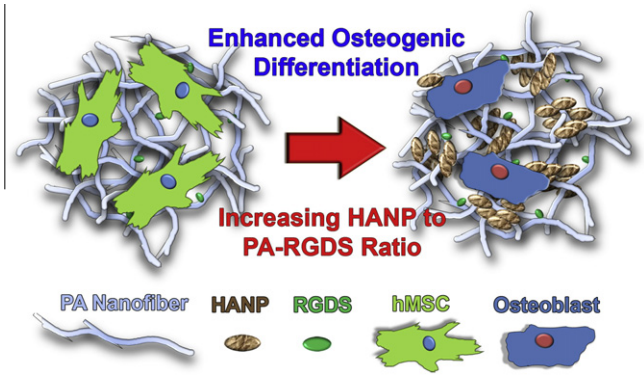

Fig. 1. General scheme. Bone analogous nanomatrix composites in which HANPs are encapsulated by PA nanofibers tailored with the RGDS binding motif to mimic the native ECM of bone were fabricated. These composites were fabricated at various HANP to PA-RGDS ratios to assess how variations in the nanomatrix composition influence the osteogenic differentiation of hMSCs. It was hypothesized that the composites with the highest HANP to PA-RGDS ratio are the most conducive to osteogenic differentiation.

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