OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS ENHANCED BY BONE ECM ANALOGOUS NANOMATRIX COMPOSITES

by

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BIOMEDICAL ENGINEERING

ABSTRACT

A growing trend in the area of bone tissue engineering is the development of materials that mimic the native bone extracellular matrix (ECM). This need is typically met through the creation of composites, which attempt to recapitulate both the organic and inorganic components of the native bone ECM. However, few composites have been created with organic ECM analogous components that are functionalized to direct cellular behaviors. Furthermore, a majority of these studies do not investigate how the ratio of the inorganic and organic components affects cellular behaviors. To address this issue, hydroxypatite nanoparticles (HANPs) were dispersed within peptide amphiphile (PA) nanofibers tailored with the RGDS cellular adhesion motif (PA-RGDS) to create bone ECM analogous nanomatrix composites and their ability to enhance the osteogenic differentiation of human mesenchymal stem cells (hMSCs) was assessed. These nanomatrix composites were prepared at various HANP to PA-RGDS ratios by weight. The successful synthesis of these composites was first confirmed via scanning electron microscope (SEM) and transmission electron microscope (TEM). The long term cellularity and osteogenic differentiation of hMSCs was then assessed in response to these nanomatrix composites. The timed expression of genes representing the early, middle, and late stages of osteogenic differentiation were quantified via RT-PCR. Increasing HANP to PA-RGDS ratios correlated with enhanced osteogenic differentiation for hMSCs as demonstrated by the timed up-regulation of key osteogenic markers and
decreased proliferative rates over time. Overall, these results demonstrate the importance of compositional modification for the regulation of cellular behaviors for bone tissue engineering applications.
ACKNOWLEDGEMENTS

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<td>Description</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>BSP</td>
<td>bone sailoprotein I</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamindino-2-phenyl-indole, dihydrochloride</td>
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<tr>
<td>DCM</td>
<td>methylene chloride</td>
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<tr>
<td>DGEA</td>
<td>Asp-Gly-Glu-Ala</td>
</tr>
<tr>
<td>DI</td>
<td>deionized</td>
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<tr>
<td>DiEA</td>
<td>diisopropylethlamine</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HA</td>
<td>hydroxyapatite</td>
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<td>HANP</td>
<td>hydroxyapatite nanoparticle</td>
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<tr>
<td>HBTu</td>
<td>o-benzotriazole-(N,N,N',N')-tetramethyluroniumhexafluorophosphate</td>
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<tr>
<td>hMSC</td>
<td>human mesenchymal stem cell</td>
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<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption ionization time of flight</td>
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<td>MMP-2</td>
<td>matrix metalloproteinase-2</td>
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<td>MSCBM</td>
<td>Mesenchymal Stem Cell Basal Medium</td>
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<tr>
<td>OCN</td>
<td>osteocalcin</td>
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<tr>
<td>OPN</td>
<td>osteopontin</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>OSM</td>
<td>osteogenic supplement medium</td>
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<td>PA</td>
<td>peptide amphiphile</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>RGDS</td>
<td>Arg-Gly-Asp-Ser</td>
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<td>runt related transcriptional factor 2</td>
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<td>scanning electron microscope</td>
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<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>TIPS</td>
<td>triisopropylsilane</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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INTRODUCTION

Specific Aims

In the area of orthopedics, there is currently a need for synthetic materials capable of recreating the structure and function of native bone by mimicking its ECM. Most current synthetic materials serve to simply restore the load-bearing functions of bone tissue; however, these materials are challenged in their ability to recapitulate the native ECM of bone. In addition, these materials are structurally mismatched with surrounding bone tissue, resulting in a phenomenon known as stress shielding, the mechanism through which most traditional implants weaken and eventually fail. To remedy these shortcomings, research is moving towards the development of bone scaffolds created with a biomimetic approach in mind. This biomimetic approach typically involves the use of materials capable of re-creating the native cellular environment, which achieves its hierarchal construction through bottom-up self-assembly at the nanoscale level. As opposed to simply providing a load bearing replacement, the aim of this strategy is to mimic the native ECM of bone such that graft incorporation and subsequent healing can be enhanced. These materials would ideally be combined with a degradable load-bearing element, with a focus on expedient regeneration and replacement of the scaffold with native tissue.

In regards to bone, it is a dense connective tissue comprised of a multi-component ECM. Bone ECM consists of organic (collagen, fibronectin, bone sialoprotein I (BSP), osteopontin (OPN), vitronectin, etc.), biological (osteoblasts, osteoclasts, etc.) and inorganic (HA nanocrystal) components. These components self-assemble to form the
complex hierarchal structure of bone. Utilizing a biomimetic approach, the goal of this study was to create a nanomatrix composite that mimics the self-assembling nature and composition of native bone ECM. This nanomatrix composite was developed utilizing a self-assembled PA nanomatrix to serve as the organic ECM analogous component with HANPs providing the inorganic aspect. Furthermore, these composites were fabricated at various HANP to PA-RGDS molecule ratios to assess the effects of HANP concentration on cellular behaviors. Specifically, the nanomatrix composites were seeded with hMSCs and evaluated based on their ability to promote osteogenic differentiation as shown in Figure 1.

Previous studies have shown that PAs tailored to mimic specific ECM proteins are capable of inducing osteogenic differentiation both with and without the presence of osteogenic supplement media\textsuperscript{5, 6}. It has also been proposed that both HA and HANPs are possibly capable of inducing osteogenic differentiation and facilitating mineralization\textsuperscript{7, 8}. However, the potential effects of combining these self-assembled PA nanofibers with HANPs have yet to be elucidated. It is hypothesized that the inclusion of HANPs into a PA nanofiber matrix enhances the osteogenic differentiation of hMSCs as compared to a PA nanofiber matrix alone. It is further hypothesized that enhanced effects on the osteogenic differentiation of hMSCs can be observed at higher HANP to PA nanofiber ratios. The hypotheses of this proposal were investigated utilizing the following specific aims.
Figure 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.
Specific Aim 1: To develop and characterize a composite, biomimetic nanomatrix by incorporating HANPs into a self-assembling PA nanofiber matrix

HANPs were incorporated into a PA-RGDS nanofiber matrix to create nanomatrix composites capable of recapitulating the ECM of native bone tissue. The effects of including HANPs on the self-assembly and structure of PA nanofibers had not yet been elucidated. To confirm the self-assembly of PA nanofibers around HANPs, composites were imaged under transmission electron microscope (TEM). Due to the fact that TEM imaging can only capture the local nanoscale environment, scanning electron microscopy (SEM) imaging was performed. This was necessary as SEM can determine the gross surface topography to assess HANP dispersion and aggregation.
Specific Aim 2: To evaluate the effects of embedding HANPs into a PA nanofiber matrix at various ratios on the in vitro viability and osteogenic differentiation of hMSCs.

PA molecules were tailored with a bioactive ligand isolated from native bone ECM proteins and incorporated with HANPs of various concentrations to create biomimetic, nanomatrix composites. These composites were assessed in terms of their ability to promote viability and enhance the osteogenic differentiation of hMSCs. The initial cellular response of the hMSCs was first examined in terms of proliferation. After which, a long term assessment designed to evaluate osteogenic differentiation was performed utilizing RT-PCR to assess the gene expression of common osteogenic markers. By assessing the osteogenic differentiation of hMSCs seeded on these composites, the efficacy of incorporating HANPs into an organic ECM mimicking nanomatrix in terms of its ability to elicit sufficient osteogenic responses was elucidated.
Clinical Significance

In 2005, there were roughly 1.6 million bone grafting procedures in the United States\(^9\). This amount is nearly five times the number of grafting procedures performed in 1990, reflecting a dramatic increase in demand. These grafting procedures are typically necessitated following the introduction of critical defects caused by tumor removal, treatment of infections, or severe bone trauma\(^{10}\). The prevalence of these treatments is demonstrated by the fact that in 2002 alone, orthopedic medical devices were responsible for roughly $14 billion dollars in sales worldwide\(^{11}\). Taking these statistics into account, it has been shown that there is a significant need for bone grafting therapies.

It is estimated that there are roughly 6 million bone fractures in the United States yearly\(^{12}\). Of these fractures, around 5-10\% results in either delayed union, or non-union healing. A non-union is a fracture that does not heal on its own, and a delayed union is a fracture that does not heal in a normal time frame as defined by the fracture healing process\(^{13, 14}\). Physiologically, when bone is fractured, the surrounding tissue is ruptured, causing the tissues to bleed. The blood will then coagulate to form a large clot around the fracture within the time frame of a week. This large clot is known as a fracture hematoma. Anywhere from two to three weeks following the fracture, blood vessels will penetrate the matrix of the hematoma, recruiting phagocytes to remove non-viable material; furthermore, recruited fibroblasts will remodel the ECM and form collagen fibers, resulting in a rubbery callus consisting of hyaline cartilage. Around the beginning of week four, osteoblasts are recruited to mineralize this collagen matrix with HA nanocrystals, leading to the formation of immature bone within 16 weeks. Over time, this
immature bone matrix is remodeled to create mature, lamellar bone. The normal fracture healing process is shown in Figure 215-19.

Figure 2: Illustration of Normal Bone Fracture Healing. (A) Following fracture, surrounding tissues hemorrhage leading to the eventual formation of a hematoma. (B) The hematoma is vascularized and a collagen matrix is deposited to form a rubbery callus. (C) The collagen matrix is then mineralized, forming a hard, bony callus. (D) The callus is then remodeled over time due to applied stress according to Wolff’s law


Unfortunately, depending on the mechanics of the fracture, the bone may not be capable of healing properly without some form of clinical assistance. High energy fractures, such as those created by gunshot wounds and motor vehicle accidents typically
fragment the bone tissue into small pieces, leaving large, gaping defects\textsuperscript{20, 21}. The scale of
these defects may prevent the proper coagulation of blood around the defect since there is
no tissue by which to bind, preventing blood vessel formation and halting the normal
healing process. When fractures fail to heal in this manner, it is known as an atrophic
non-union\textsuperscript{13}. To treat these defects, a synthetic or native filler material must be provided
to bridge the gap between the two disjoined bone regions, thereby allowing the formation
of a fracture hematoma that facilitates functional replacement of the bone to its normal,
load-bearing state. The procedure by which bone is replaced surgically is known as bone
grafting\textsuperscript{22}.

The most common materials used for bone grafting applications are autografts,
allografts, and synthetic materials\textsuperscript{22}. Autografts are bone tissue harvested from a donor
site within the same individual (typically from the iliac crest of the pelvis) and
transplanted to the fracture site. The innately autologous nature of these scaffolds
combined with their highly osteoinductive properties has led them to be considered the
“gold standard”. However, while they are useful for providing a filler material that is
conducive to bone regeneration without any fear of rejection, material is limited and the
removal of bone from the donor site often results in residual pain and morbidity\textsuperscript{23}. Should
there be an insufficient amount of material at the donor site, an alternative treatment must
be adopted. In allografting, bone is harvested from a cadaver, and sterilized before
implantation into the individual. Unfortunately, sterilizing the bone causes it to lose much
of its biological signaling capabilities. Furthermore, these sterilization measures are not
always successful and rejection can still occur\textsuperscript{24}. 
To bypass the difficulties associated with allografts and autografts, synthetic materials are typically utilized instead. Synthetic materials are designed with one of two intended design parameters; to either provide an inert, structurally rigid conduit that enables load-bearing functionality or a biologically active material intended for the temporary replacement and eventual regeneration of native bone tissue.

Traditional synthetic materials such as titanium or cobalt chromium, are tailored to replace the structural support provided by native bone. These materials are designed to remain inert in order to minimize undesired interactions with surrounding tissue. In addition, these materials are mechanically mismatched with native bone tissues. The structural remodeling of bone is a result of the constant deposition and re-adsorption of bone matrix by osteoblasts and osteoclasts, respectively, in response to weight bearing loads applied through daily routines such as walking or exercising; this phenomenon is known as Wolff’s Law. Most traditional synthetic materials are mechanically stronger than bone, lessening the load on endogenous bone and resulting in a loss of bone density. This is often referred to as stress shielding. This loss in bone density makes surrounding bone susceptible to further bone fracture, leading to the eventual failure of the implant.

Due to the inherent problems associated with the usage of inert materials, research has moved towards the development of bioactive synthetic materials. The goal of these materials is to provide a temporary, functional replacement for native bone tissue, while providing an osteoconducive and osteoinductive environment intended to encourage the eventual remodeling of the synthetic scaffold into native bone tissue.
Current Clinical Strategy

Due to the inherent problems associated with allografts, autografts, and inert synthetic materials, research has moved towards the development of synthetic bioactive materials that mimic the natural structure and function of native bone tissue. In order to develop an ideal synthetic bone scaffold, the following attributes must be considered (Fig. 3):

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<tr>
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<th>General remarks</th>
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<tr>
<td>Biocompatible</td>
<td>Biologically compatible to host tissue i.e. should not provoke any rejection, inflammation, and immune responses.</td>
</tr>
<tr>
<td>Bioactive</td>
<td>To facilitate a direct biochemical bonding to host tissue.</td>
</tr>
<tr>
<td>Biodegradable</td>
<td>The rate of biodegradation has to be adjusted to match the rate of bone tissue formation.</td>
</tr>
<tr>
<td>Mode of degradation</td>
<td>Bulk or surface erosion.</td>
</tr>
<tr>
<td>Osteoconductive</td>
<td>Capable of supporting in-growth of sprouting capillaries, perivascular mesenchymal tissues, and osteoprogenitor cells from the recipient host into the three dimensional structure of a graft that act as a scaffold.</td>
</tr>
<tr>
<td>Vascular supportive</td>
<td>Should provide channels for blood supply for fast and healthy bone regeneration.</td>
</tr>
<tr>
<td>Porous structure</td>
<td>To maximize the space for cellular adhesion, growth, ECM secretion, revascularization, adequate nutrition and oxygen supply.</td>
</tr>
<tr>
<td>Three dimensional structure</td>
<td>For the assistance of cellular in-growth and transportation of nutrition and oxygen.</td>
</tr>
<tr>
<td>Adequate mechanical strength Sterilizable</td>
<td>To withstand in-vivo stimuli during bone formation. To avoid toxic contamination.</td>
</tr>
<tr>
<td>Cost-effective</td>
<td>Affordable to all.</td>
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Figure 3: Desired Properties of an Ideal Synthetic Scaffold.

Currently, the only materials considered to be capable of meeting these criteria are autografts. This is because they are harvested from viable, native bone. However, as mentioned earlier, they are not always an acceptable choice due to the lack of material availability. As a result, research has moved towards the development of synthetic materials that recreate the structural and functional qualities of bone ECM as a means by which to meet as many of the above criteria as possible.
In the development of synthetic scaffolds that recreate the structural and functional qualities of bone, a biomimetic approach is utilized. The embodiment of a biomimetic approach involves recapitulating native tissue ECM. This project proposes to develop a biomimetic, nanomatrix composite by capturing the composite structure of native bone ECM. For this reason, it is necessary to understand the composition of native bone ECM.

Bone is a highly versatile tissue responsible for providing support, movement, and protection\(^2\). Supporting native osteogenic cells, bone ECM is comprised of organic (collagen, BSP, OPN, vitronectin, etc.), biological (osteoblasts, osteoclasts, etc.) and inorganic (HA) components\(^2\)\(^4\). Bone nanostructure is made bottom-up from the mineralization of collagen fibrils with HA nanocrystals. These mineralized fibrils then bundle together into structural units to make lamellar bone as displayed in Figure 4.

The organic component of ECM (~20% of bone by weight\(^2\)) is a network of proteins and polysaccharides that directly regulates cellular behaviors through cell-ECM interactions\(^2\)\(^9\). Cells interact with the ECM via various integrin and non-integrin binding mechanisms in which different cell types will recognize and bind to cellular adhesive ligands transcribed into organic ECM proteins such as collagen, fibronectin, vitronectin,
and laminin$^{30,31}$. Cellular adhesive ligands are signaling mechanisms built into various ECM proteins and are designed to bind to trans-membrane receptors known as integrins to induce conformational changes in the receptor, leading to various cellular responses$^{32}$. Depending on the type of integrin signaling molecules present within the ECM, cellular behaviors such as proliferation, growth, migration, and differentiation will be modified.

Figure 4: Hierarchal Structure and Composition of Bone. Hydroxyapatite crystals and collagen fibrils combine on the nanoscale level to form functional units called osteons which are responsible for structural bone.

The inorganic ECM component is made up of HA \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\). HA is the main component of bone, making up \(~70\%\) of the native ECM \(^{2-4}\) and serves as its load bearing constituent, providing structural support that is comparable to steel by weight\(^{33}\). In order to properly recapitulate the native structure of bone for a biomimetic approach, it is necessary to incorporate both organic and inorganic ECM analogous components.
Development of Biomimetic Nanomatrix Composites

Addressing the need for synthetic materials capable of recapitulating the structural and functional qualities of natural bone, this study proposes to create a novel, composite nanomatrix that is mimetic of native bone tissue by combining HANPs with an organic ECM mimicking nanofiber matrix created from the self-assembly of PAs. hMSCs were seeded on this composite to assess their capacity to induce osteogenic differentiation, as this design is intended for the eventual regeneration of natural bone tissue.

The structural components of bone are fabricated through bottom-up self-assembly at the nanoscale level. Thus, to be effective in the development of a biomimetic composite, it must recreate the nanoscale environment. Scaffolds developed with nanoscale architectures have demonstrated enhanced performance as compared to similar scaffolds fabricated with microscale architectures. The nanoscale approach has been shown to be better suited in promoting enhanced cell-ECM interactions through more natural cell adhesion and spreading (Fig. 5). In the case of HA, working at the nanoscale results in enhanced strength, hardness, and super plasticity34-36. In addition, scaffolds containing nanoscale HA are also known to enhance the attachment and osteogenic differentiation of hMSCs as compared to HA fabricated with microscale architecture37.
Figure 5: *Scaffold Architecture Versus Cell Binding*: The inherent benefit of utilizing a nanofibrous scaffold architecture (C) is increased surface area for protein absorption as well as enhanced cell-ECM interactions via cell-membrane receptors. Microscale architecture (A,B) is not as effective in this sense.


The ideal biomimetic material would consist of a bottom-up fabrication method that recreates the nanostructure of bone by merging an organically mimetic ECM component with an inorganic ECM component. The material would then be supplemented with a biological component to facilitate ECM remodeling and bone regeneration. The goal is to provide a osteoconducive and osteoinductive environment similar to that found in native bone tissue as a means by which to encourage subsequent bone tissue regeneration. As mentioned earlier, this was accomplished by combining a
PA nanofiber matrix with embedded HANPs to create a biphasic environment. hMSCs were then introduced to this biphasic environment to assess its ability to induce osteogenic differentiation. The relative extent to which hMSCs achieve terminal differentiation provides insight into the efficiency of the scaffolds in promoting bone tissue regeneration.
Peptide Amphiphiles

PAs were designed as a material capable of mimicking organic ECM proteins at the nanoscale level, providing cell to organic ECM interactions via integrin and non-integrin mediated binding mechanisms. A PA is a bipolar molecule with a hydrophilic peptide portion consisting of 6-15 amino acid groups coupled by an amide bond to a hydrophobic alkyl chain that contains roughly 10 to 22 carbon atoms\textsuperscript{38-40}. Under the proper chemical conditions, these molecules can self-assemble into higher ordered structures such as spheres, rod-like fibers, disks, and channels\textsuperscript{41}. The material is readily available as it can be manufactured on demand via standard solid phase chemistry. The peptide structure of the molecule allows for great versatility, as our research group has adapted PAs to include various cell-adhesive ligands in the head group and an enzyme-mediated degradable sequence within the internal peptide structure (Fig. 6). Owing to the fact that cellular mediated degradation is a necessity for cell invasion and subsequent remodeling\textsuperscript{42}, our PAs have been endowed with and demonstrated to have the capability of cellular mediated enzymatic degradation through the incorporation of a matrix metalloproteinase-2 (MMP-2) enzyme mediated degradable peptide sequence, Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln (GTAGLIGQ).

Under proper conditions, these PAs self-assemble into nanofibers of around 9-10 nanometers in diameter and several microns in length, exposing the cell adhesive ligands to the outside for cell attachment and signaling\textsuperscript{38,40,43}. The material is versatile since it can be used to form either 2D surface coatings or 3D hydrogels depending on the needed application. 2D surface coatings are fabricated via a solvent evaporation method\textsuperscript{44,45} in which self-assembly is achieved by evaporating the solvent out of the solution while 3D
hydrogels are formed by adding polyvalent ions (i.e. Ca\(^{2+}\)) to PA solutions in order to trigger self-assembly\(^{40}\).

In this study, 2D coatings of biomimetic, nanomatrix composites were fabricated through the solvent evaporation of solutions containing PA-RGDS molecules and HANPs to induce PA self-assembly. The assessment of hMSCs seeded on these 2D composites is an important step as it establishes which nanomatrix composite is the most conducive to osteogenic differentiation such that the most optimal condition can be selected for further studies involving 3D hydrogels.
Figure 6: Peptide Amphiphile Structure and Self-Assembly. (A) Under the proper chemical conditions, PA molecules can self-assemble to form nanofiber constructs. (B) The Peptide Amphiphiles in this study consist of a hydrophobic tail, MMP-2 enzyme degradation site, and cell adhesive ligand sequence.


(b) From Joel M. Anderson. Master’s Thesis: Biomimetic self-assembled nanomatrix for bone tissue regeneration. Submitted to the graduate faculty of the University of Alabama at Birmingham (2008).”
Modification of Cellular Adhesive Ligands

As mentioned previously, PA molecules can be tailored to mimic natural, organic ECM proteins through the inclusion of various cellular adhesive ligands isolated from naturally occurring ECM proteins. Previous work has shown that PA nanofiber matrices tailored to mimic various bone ECM proteins such as fibronectin, vitronectin, BSP, OPN and collagen are capable of inducing osteogenic differentiation of hMSCs in an in-vitro environment based only on the specific ligand sequences inscribed and without the aid of osteogenic supplemental factors (i.e. dexamethasone, β-glycerol phosphate)\(^5\).

According to these previous studies, two specific cellular adhesive ligand sequences initiated substantial osteogenic differentiation through integrin mediated binding mechanisms. These peptide sequences were: (1) Arg-Gly-Asp-Ser (RGDS) and (2) Asp-Gly-Glu-Ala (DGEA).

The RGD cellular adhesive ligand sequence is common to multiple bone ECM proteins such as fibronectin, vitronectin, BSP, and OPN. It is well established that the RGD peptide sequence is an important cue for cell attachment\(^46, 47\). RGD interacts with cells via the integrins alpha5-beta1, alpha V-beta3, and alphaIIb-beta3\(^48\). In addition, RGD-binding integrins have been shown to mediate cell morphology, differentiation, proliferation, and gene expression\(^49\). The sequence is ubiquitous in fibronectin, whose combination with various HA surfaces in previous studies has led to enhanced levels of osteoblastic differentiation, adhesion, and proliferation\(^50, 51\). DGEA is a cellular adhesive ligand sequence isolated specifically from Collagen type I. This sequence interacts with
the alpha2-beta1 integrin receptor which has been shown to be important in triggering the osteogenic differentiation of bone marrow cells\textsuperscript{52}.

When these cellular adhesive ligand peptide sequences are included in PA molecules, they become known as PA-RGDS and PA-DGEA respectively. In terms of enhancing osteogenic differentiation, PA-RGDS has been shown to better promote the osteogenic differentiation of hMSCs than that of PA-DGEA\textsuperscript{5,6}. In addition to this, collagen, from which the DGEA sequence is isolated, has shown less osteoinductive potential than fibronectin when deposited on HA\textsuperscript{51}. For these reasons PA-DGEA was not included in this study.
Hydroxyapatite

While PA nanofiber matrices can increase proliferation as well as promote and enhance the osteoblastic differentiation of hMSCs, they do not mimic the biphasic nature of the native bone ECM, and are thus, limited in their ability to fully enhance osteogenic differentiation. Most current, synthetic scaffolds are made of materials that mimic the inorganic ECM component of bone. The material of choice for this application tends to be HA, which has a calcium phosphate composition similar to native bone. In addition, studies have proposed that HA has osteoinductive potential and can enhance osteogenic differentiation. However, this fact is contentious, and the exact mechanism(s) pertaining to how HA might induce osteogenic differentiation has yet to be elucidated.

Unfortunately, while capable of providing structural support and potentially inducing osteogenic differentiation, HA, when utilized as a bioactive scaffold by itself, is slowly invaded by host tissues and its mechanical properties are still not completely comparable to native, vascularized bone. By combining HANPs with an organic ECM mimicking PA nanofiber matrix, osteogenic differentiation should be improved, eliminating the weaknesses associated with utilizing HA alone as a scaffold. By creating a composite that incorporates the organic and inorganic components of bone ECM, cellular behaviors beneficial to bone regeneration may be exhibited.
Human Mesenchymal Stem Cells

To assess the ability of the composite, biomimetic nanomatrix to stimulate osteogenic differentiation, hMSCs isolated from human bone marrow were seeded on 2D coatings of the composite nanomatrix in vitro and assessed. In general, stem cells have specific advantages over mature cell lineages. Embryonic stem cells, unlike their more mature counterparts, have greater self-renewal potential and are capable of differentiating into various mature cell types\textsuperscript{56, 57}. Unfortunately, these cells are surrounded by controversy and technical limitations prevent them from achieving clinical practicality in the short term\textsuperscript{56, 58}. Induced pluripotent stem cells (iPSCs) have been developed to overcome the controversy and certain technical limitations associated with embryonic stem cells\textsuperscript{58}. However, this technology is still in its infancy and the process of reprogramming through the introduction of retroviral and lentiviral vectors creates genetic mutations selective for cancer\textsuperscript{59}. Unlike embryonic stem cells and iPSCs, hMSCs are only capable of differentiating into a small, defined set of cell types. Currently, hMSCs are known to differentiate into adipocytes, fibroblasts, myoblasts, hepatocytes, osteoblasts, chondrocytes, and tenocytes (Fig. 7)\textsuperscript{60-62}. 

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Figure 7: **Multi-lineage Potential of MSCs**: MSCs are capable of forming multiple cell types such as adipocytes, osteoblasts, myoblasts, chondrocytes, etc.


hMSCs have been shown to exhibit osteoblastic differentiation under various circumstances, typically with the aid of osteogenic supplement medium (i.e. dexamethasone, β-glycerol phosphate). However, as mentioned earlier, hMSCs have also been shown to exhibit osteoblastic differentiation based solely on integrin mediated cellular adhesive binding mechanisms and without the aid of osteogenic supplement medium⁵,⁶. Osteoblastic differentiation of hMSCs has also been shown to occur on various HA surfaces without the aid of supplemental stimulatory factors⁸.

hMSCs cultured within standard, in-vitro conditions exhibit an elongated, spindle shaped morphology (Fig. 8). However, during the process of osteogenic differentiation
and subsequent phenotype commitment, the cells should exhibit a morphological change in which they will exhibit a cuboidal shape\textsuperscript{63}.

Figure 8: *Human Mesenchymal Stem Cell Morphology*: hMSCs imaged under fluorescence following staining with Rhodamine Phalloidin and DAPI. Cells exhibit an elongated, spindle shaped morphology.

The goal of this study was to assess the osteoblastic differentiation of hMSCs when seeded on nanomatrix composites relative to varying ratios between HANP and PA-RGDS.
Evaluating Osteogenic Differentiation

As hMSCs come in contact with ECM proteins and interact via integrin mediated binding mechanisms or receive signaling cues from various soluble factors, they begin to activate various signaling pathways such as MAPK and JNK, affecting transcriptional processes and changing which genes are expressed\(^64\). Depending on which signaling mechanisms are present and cell membrane receptors activated, gene expression will change, resulting in the differentiation of hMSCs from one cell type to another\(^65-67\). This differentiation manifests itself as changes in cell morphology, rate of proliferation, and protein production. By introducing hMSCs to a composite nanomatrix similar to that of native bone, they should undergo enhanced osteogenic differentiation as a result of the cues present within the ECM.

In order to determine the state of osteogenic differentiation of hMSCs, they must be assessed for changes in morphology, gene expression, and proliferation over time. At the onset of differentiation, hMSCs slowly up-regulate genes indicative of an osteogenic phenotype such as ALP, OCN, and runt related transcriptional factor 2 (Runx2). One of the earliest indications of osteoblastic differentiation is the up-regulation of Runx2\(^68\), which is important in initiating and driving osteogenic differentiation. Runx2 has been demonstrated to be an essential mediator of cell phenotype commitment and osteogenesis\(^69\). As hMSCs differentiate into osteoblasts, their rate of proliferation should slow\(^70,71\) as a result of Runx2 up-regulation\(^71,72\), further highlighting its importance in the modulation of osteogenic differentiation.
ALP plays an important role in the mineralization of bone tissue. As differentiation proceeds, ALP activity rises and cleaves organic phosphates to produce free, inorganic phosphates\textsuperscript{52, 73}. Because of this, ALP activity has become an important indication of osteogenic differentiation. ALP is slowly up-regulated over time with significant expression typically being detected around days 14-28\textsuperscript{68, 70}.

As a late term marker of osteogenic differentiation, osteocalcein is up-regulated during the process of mineralization. If osteocalcein is up-regulated significantly, the cell has likely achieved terminal differentiation into an osteoblast\textsuperscript{68, 70}. A graphical representation of cellular behavior and gene up-regulation over time is shown in Figure 9.
Figure 9: Gene Expression and Behavior Over Time. Notice that proliferative rate decreases at the onset of differentiation.

Cell Response to Differing Surface Topographies

Considering that HANP concentration can affect scaffold surface topography, it is important to discuss how surface characteristics can affect the cellular behaviors of hMSCs. Multiple research groups have fabricated composite scaffold materials out of various polymers and HANPs. Unfortunately, in the fabrication of these composites, aggregation of HANPs is a persistent problem. HANPs are typically shipped as a white powder containing particulates of varying sizes consisting of aggregated HANPs. Most studies attempt to eliminate HANP aggregation as a means to improve the composites from a mechanical strength standpoint. However, few studies have correlated HANP particle size and aggregation to changes in cellular behavior.

To address how the surface characteristics of nanoscale HA can affect the attachment and proliferation of hMSCs, Dulgar-Tulloch, et. al. fabricated Titanium, Aluminum, and HA ceramics with grain sizes of 1500, 200, and 50 nm. hMSCs were then seeded on these constructs and assessed for attachment at 1 and 4 hours as well as proliferation at days 1, 3, and 7. Results showed that attachment was greatest on the 1500 nm grain sized constructs and the lowest on the 50 nm grain size constructs. However, cellular growth proceeded the quickest on the 200 nm constructs and the slowest on the 50 nm constructs. Taking these results in account, it was demonstrated that surface characteristics can affect the short term behavior of hMSCs. Therefore, the surface characteristics of our nanomatrix composite must considered when assessing cellular behaviors.
Hydroxyapatite and Osteogenic Differentiation

Considering that one of the central tenets of this study is to assess the osteogenic differentiation of hMSCs in response to nanomatrix composites of various HANP to PARGD5 molecule ratios, it is important to consider the contribution of HA in affecting the differentiation of hMSCs.

In a study designed to address this question, Jiawei He, et. Al, fabricated HA / poly(lactide-co-glycolide) (PLG) scaffolds at various ratios and seeded them with hMSCs to determine how scaffold composition affected osteogenic differentiation7. They determined that the osteogenic differentiation of hMSCs was enhanced with increasing HA-PLG ratios as shown by ALP activity and OPN secretion. Additionally, cellular proliferation decreased substantially in accordance with increasing HA-PLG ratios.

In another study evaluating HANP concentration in relation to osteogenic differentiation, Yukan Liu, et. Al., supplemented hMSCs with HANPs of 20 nm diameter at concentrations of 0, 2, 4, 20, 40 and 200 μg/10^4 cells seeded75. It was found that at concentrations above 20 μg/10^4 cells, proliferative rate decreased in a stepwise manner. Furthermore, as HANP concentration is increased, genes characteristic of osteogenic activity such as Collagen I, OCN, and OPN are up-regulated.

Taking these results into consideration, there are two prevailing themes:

1) Increasing HA concentration enhances osteogenic differentiation

2) Increasing HA concentration causes a decrease in the proliferative rate of hMSCs
These observations establish a clear indication that increasing HANP content may contribute to enhanced osteogenic differentiation. It is my understanding that decreased proliferative rates are a direct consequence of the initiation of the differentiation process as explained earlier (see *Evaluating Osteogenic Differentiation*).
EXPERIMENTAL APPROACH

In this study, we assessed the effects of combining PA-RGDS matrices with HANPs of 100 nm diameter at varying concentrations to determine if we can enhance the rate of osteogenic differentiation of hMSCs. The ECM of bone is roughly 20% organic and 70% inorganic by weight\(^2-^4\). However, it is undetermined as to what ratios of organic to inorganic ECM are the most conducive to osteogenic differentiation and subsequent mineralization. In order to determine the condition most conducive to osteogenic differentiation, HANPs were suspended in a solution of 0.1% PA-RGDS at concentrations of 16%, 33%, 50% and 66% of PA by weight. A control consisting solely of PA-RGDS was included to contrast the differentiation of hMSCs on the PA-RGDS nanofiber matrix both with and without HANP supplementation. 2D nanomatrix composites were formed on tissue culture plates utilizing a solvent evaporation method to trigger self-assembly of the PAs around the HANPs, effectively trapping the HANPs within the PA nanomatrices and, creating a biphasic environment.
Specific Aim 1

To develop and characterize a composite, biomimetic nanomatrix by incorporating HANPs into a self-assembling PA nanofiber matrix

Rationale

HANPs were suspended in an aqueous solution of PA-RGDS at various HANP to PA-RGDS ratios. Following which, PA self-assembly was induced via solvent evaporation, creating a composite, biomimetic nanomatrix in which HANPs are embedded within PA nanofibers. However, the effects of including HANPs on the self-assembly and structure of PA nanofibers had yet to be elucidated. To confirm nanofiber assembly around suspended HANPs they were imaged under transmission electron microscope (TEM).

Research groups have attempted to fabricate composite scaffolds containing HA particles and various polymers such as PLLA, PLGA, PGA, etc. However, the hydrophobic nature of HA leads to the formation of aggregates. To determine if PA nanofiber assembly could exacerbate HANP aggregation, a HANP only control was imaged via TEM. The aggregation of HANPs within the PA nanomatrix will modify the composite surface characteristics. Therefore, to qualitatively determine the gross surface topology and assess the degree of aggregation present, all composites were imaged utilizing scanning electron microscopy (SEM).
Specific Aim 2

To evaluate the effects of embedding HANPs into a PA nanofiber matrix at various ratios on the in vitro viability and osteogenic differentiation of hMSCs

Rationale

By introducing hMSCs to a composite matrix consisting of PA-RGDS and HANPs, osteogenic differentiation is expected to be enhanced as compared to the PA-RGDS alone. Previous research has shown the osteoinductive potential of PA nanofiber matrices tailored with the RGDS motif. This previous study showed that cell-ECM interactions can induce the osteogenic differentiation of hMSCs without the aid of osteogenic supplement medium. Increasing the amount of HA available to hMSCs may also enhance osteogenic differentiation. Results show that increasing HA content can result in increased ALP activity, OPN secretion, and up-regulation of genes specific to osteoblastic activity. Furthermore, research has shown enhanced osteogenic differentiation when organic ECM of both native and synthetic origin is combined with inorganic HA. As a result, it was reasonable to expect that the combination of a PA-RGDS nanofiber matrix with HANPs of increasing concentrations would yield higher levels of osteogenic differentiation than a PA-RGDS nanofiber matrix alone. To assess the osteogenic differentiation of hMSCs on this composite nanomatrix, long-term RT-PCR gene expression and proliferation was assessed. In addition, cellular viability was confirmed via live/dead imaging. Based on previous studies, it is hypothesized that the inclusion of HANPs into PA-RGDS nanofiber matrices results in enhanced osteogenic
differentiation with decreased proliferation. These effects should be enhanced as the HANP to PA-RGDS ratio increases.
METHODS AND MATERIALS

Peptide Amphiphile Synthesis

PAs were fabricated utilizing previously described methods\textsuperscript{5,6,78}. An amino acid sequence consisting of a metalloproteinase (MMP-2) enzyme mediated degradation group (GTAGLIGQ) and a cell adhesive ligand sequence isolated from common ECM proteins (RGDS) was fabricated utilizing standard Fmoc-chemistry in an Advanced Chemtech Apex 396 peptide synthesizer. Following this, a 16-carbon chain was attached to the peptide sequence by reacting the $N$-termini with 2 equivalents of palmitic acid, 2 equivalents of $o$-benzotriazole-$N,N,N',N'$ tetramethyluroniumhexafluorophosphate (HBTU), and 4 equivalents of diisopropylethylamine (DiEA) in dimethylformamide (DMF) for two 12 hour intervals at room temperature. The resin was cleaved through the addition of TFA. Excess TFA was then removed through rotary evaporation, and the solution the precipitated in cold ether. Lastly, samples were lyophilized for 2-3 days, resulting in purified PA molecules. Proper fabrication of the PA was then confirmed via matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. This Procedure is outlined in Figure 10.
Figure 10: Peptide Amphiphile Synthesis Procedure.

From: “Joel M. Anderson. Master’s Thesis: Biomimetic self-assembled nanomatrix for bone tissue regeneration. Submitted to the graduate faculty of the University of Alabama at Birmingham (2008).”

Preparation of Nanomatrix Composites

PAs were first dissolved in filter sterilized DI water at 0.1% by weight, followed by pH neutralization through the addition of NaOH. Autoclaved HANPs (Berkeley Advanced Biomaterials, Berkeley, CA) were then suspended within these solutions at various HANP to PA-RGDS ratios (16%, 33%, 50%, and 66%) based on the amount of PA present per mL of solution (Table 1). Solutions were initially sonicated and vortexed to ensure the complete dispersion of HANPs within each PA solution. Under continuous
stirring, 200 μL aliquots of each solution were placed into corresponding wells of a 48-well tissue culture plate (TCP; BD Biosciences, Sparks, MD). TCPs were placed into a non-humidifying incubator for 2 days to allow the formation of PA nanofibers incorporating HANPs through solvent evaporation, resulting in nanomatrix composite coatings. Plates were then placed under a UV hood for 1 hour, followed by well rinsing using sterilized DI water 12 hours prior to cell seeding.

<table>
<thead>
<tr>
<th>Table 1. Experimental Conditions</th>
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<tr>
<td><strong>Control</strong></td>
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<tr>
<td>CH$_3$(CH$<em>2$)$</em>{14}$CONH–GTAGLIGQ–RGDS (PA-RGDS)</td>
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<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td>PA-RGDS (16% HANP)</td>
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<td>PA-RGDS (33% HANP)</td>
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<td>PA-RGDS (50% HANP)</td>
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<td>PA-RGDS (66% HANP)</td>
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Transmission Electron Microscopy

HANPs and a 16% HANP nanomatrix composite were prepared on a carbon coated formvar copper grid (400 mesh) and dried for at least 4 hours in a chemical fume hood. The grids were then negatively stained with 2% phosphotungstic acid (PTA) buffered to pH 7 for 30 seconds. Excess solution was then wicked off the grid. Samples were imaged on a Tecnai T12 microscope (FEI, Hillsboro, OR) operated at 80 kV accelerating voltage.

Scanning Electron Microscopy

Nanomatrix composites were fabricated onto aluminum stubs utilizing solvent evaporation and characterized by a scanning electron microscope (SEM). Aluminum stubs were first sealed to create a well in which solution could be held and placed into a non-humidifying incubator. The amount of solution aliquoted to these stubs was scaled based on surface area relative to those formed on tissue culture plates. The composite coatings were then sputter coated with gold/palladium, and morphology was observed under Philips SEM 510 at an accelerating voltage of 20 kV.

Cell Culture

hMSCs were purchased from Lonza, Inc. (Walkersville, MD), and only cells from passage numbers 4-6 were used. Cells were grown in Mesenchymal Stem Cell Basal Medium (MSCBM; Lonza, MD) supplemented with MSCBM SingleQuots (Lonza, MD). At confluency, cells were lifted from the culture surface and deactivated by adding a matching volume of Dulbecco’s Modified Eagle’s Medium (DMEM; Mediatech, VA) supplemented with 10% Fetal Bovine Serum (FBS; HyClone, UT), 1% Amphotericin B,
1% penicillin, 1% streptomycin (Mediatech, VA), and 1% L-glutamine. Cells were then isolated through centrifugation at 1,000G for 5 minutes and re-suspended at a concentration of 15,000 cells per 400 μL of osteogenic supplement medium (OSM) made by supplementing DMEM with dexamethasone, L-ascorbic acid, and β-glycerol phosphate. 400 μL aliquots of this solution were then transferred to the coated 48-well tissue culture plates and cultured in 400 μL of OSM. Cell cultures were maintained under standard culture conditions (37°C, 95% relative humidity, 5% CO2). Media was replaced with fresh media every 3-4 days.

**Analysis of Cellularity**

Cells were cultured and harvested through trypsinization at 1 hour and days 7, 14, and 28 for analysis of cellularity. Cells were stored within eppendorf tubes at -80°C until analysis of cellularity. For analysis, 100 μL cell extracts were placed in 96-well plates. A Picogreen assay (Molecular Probes, Eugene, OR) was used to determine the double stranded DNA content according to manufacturer specifications. Picogreen Dye from the kit was added into the sample preparations and allowed to incubate for 15 minutes in the dark. The double-stranded DNA content was then measured on a fluorescent microplate reader (Synergy HT, BIO-TEK Instruments, Winooski, VT) filtered at 485/528 (EX/EM) and compared to a standard curve, correlating known DNA content. To determine the amount of cells per sample, hMSCs were determined to have 7.88 x 10⁻⁶ μg DNA per cell⁷⁹.
Quantitative Real-Time PCR (RT-PCR)

Cells were first collected and lysed with Trizol (Invitrogen, Carlsbad, CA). RNA was then isolated according to the Trizol protocol. Following isolation, the dried RNA pellet was re-suspended in nuclease free water. To determine RNA concentration, a ND-1000 UV spectrophotometer (Nanodrop, Wilmington, DE) was used. 1 μg of RNA was then transferred to 0.2 mL eppendorf tubes for cDNA synthesis and reverse transcribed utilizing the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) per manufacturer’s instructions. RT-PCR reactions were completed utilizing iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in an iCycler iQ Real-Time PCR machine (Bio-Rad, Hercules, CA). Reactions were cycled under the following parameters: 95°C for 3 min, 40 cycles at 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. The following primers (Runx2, ALP, OCN, and 18S) were used to evaluate gene expression (Table 2). To ensure primer specificity and check for genomic DNA contamination, melt-curve analysis was performed. The $2^{-\Delta\Delta CT}$ method was used to determine gene expression. Data was normalized by 18S and shown as a fold ratio relative to cells seeded on TCP cultured in OSM.
Cell Viability

Cells were cultured on matrices for 7, 14, and 28 days. At the respective time points, cells were rinsed with PBS and stained using a Live/Dead assay kit (Invitrogen, Carlsbad, CA) consisting of calcein AM and ethidium homodimer-1. The stained cells were imaged under fluorescence microscopy (Nikon TE2000-S; Nikon, Japan)

Statistical Analysis

The results are presented as a mean ± standard error of measurement (n=12) for each condition at each time point. One-way analysis of variance (ANOVA) was performed using SPSS software (SPSS, Chicago, IL) to assess significant differences between conditions at each time point. Tukey multiple comparison tests were further conducted to determine significant differences between different conditions in the same time point. For all tests, p<0.05 was considered significant. The statistics were performed as shown in Table 3. An outline of the full experimental procedure is shown in Figure 11.
<table>
<thead>
<tr>
<th>Time Point</th>
<th>Condition</th>
<th>Samples</th>
<th>Analysis</th>
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<tr>
<td>Day 7</td>
<td>PA-RGDS</td>
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<td></td>
<td>PA-RGDS (16% HANP)</td>
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<td>PA-RGDS (33% HANP)</td>
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<td></td>
<td>TCP</td>
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<td>One-Way ANOVA Analysis</td>
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<td>PA-RGDS (50% HANP)</td>
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<td>PA-RGDS (66% HANP)</td>
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<td>Day 14</td>
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<td>PA-RGDS (16% HANP)</td>
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<td>PA-RGDS (33% HANP)</td>
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<td>Day 28</td>
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<td>PA-RGDS (50% HANP)</td>
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<td>PA-RGDS (66% HANP)</td>
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To clarify, results are reflective of one experiment in which cells were cultured, passaged, and seeded on two plates (6 samples for each plate) for each condition for each time point. One-way ANOVA was performed to determine statistical differences between experimental groups at each time point.
Figure 11: *Experimental Methodology*

(A) Macroscopic appearance of biphasic coatings following assembly via solvent evaporation.

(B) Flow chart depicting the mechanics of the study. First, coatings were formed via solvent evaporation. hMSCs were then seeded on the coatings and allowed to culture until they were collected at their specified time points. hMSCs were then assessed for proliferation, and osteogenic differentiation.

(B) From: “Joel M. Anderson. *Master’s Thesis: Biomimetic self-assembled nanomatrix for bone tissue regeneration.* Submitted to the graduate faculty of the University of Alabama at Birmingham (2008).”
RESULTS

Scaffold Characterization

Bone analogous nanomatrix composites were synthesized onto the surface of tissue culture plates utilizing a solvent evaporation method in which PA-RGDS molecules were induced into self-assembly, forming cylindrical nanofibers around HANPs. These composites were fabricated at various HANP to PA-RGDS ratios to assess how compositional variations would change the cellular behavior of hMSCs. In order to confirm the self-assembly of PA-RGDS nanofibers around HANPs, TEM imaging was performed.

HANPs of around 100nm diameter (Fig 12.A) were first suspended in a solution of PA-RGDS molecules at various concentrations and aliquoted to tissue culture wells. Following this, the self-assembly of PAs into cylindrical nanofibers was induced via solvent evaporation, enmeshing HANPs within the nanomatrix. TEM confirms the formation of cylindrical nanofibers 8-10 nm in diameter and several microns in length around small HANP aggregates 100-200 nm in diameter, forming nanomatrix, composite coatings (Fig 12.B). The dark blots within the nanomatrix composite condition are likely a result from the pooling of PTA on HANP aggregates and is a testament to the highly absorptive properties of HA, as has been demonstrated thoroughly in numerous studies 50, 80-84.

SEM demonstrates a dose-wise increase in the HANP content between the 16% (Fig. 12.C), 33% (Fig. 12.D), 50% (Fig. 12.E) and 66% (Fig. 12.F) nanomatrix composite conditions. Due to the hydrophobic nature of HA, HANPs tend aggregate when dispersed
in water. Variations in surface topography and slight increases in mean aggregate sizes were exhibited based on increases in the HANP to PA-RGDS ratios. SEM and TEM confirmed that biphasic nanomatrix composites could be successfully fabricated at various HANP to PA-RGDS ratios through the self-assembly of PA nanofibers around included HANPs.
Figure 12. Characterization of Nanomatrix Composites. TEM images of (A) HANPs and (B) PA-RGDS (16% HANP) at 42000x. SEM Images of (C) PA-RGDS (16% HANP) (D) PA-RGDS (33% HANP) (E) PA-RGDS (50% HANP) and (F) PA-RGDS (66% HANP) at 625x. Scale bars represent 100nm for TEM and 50μM for SEM respectively. Considered together, TEM and SEM images demonstrate aggregates of varying size and distribution made up of HANPs enmeshed within PA-RGDS nanofibers.
Analysis of Cellularity

Withdrawal from the cell growth cycle is an important indication of the onset of differentiation towards an osteogenic lineage from an undifferentiated state\textsuperscript{71, 85}. To determine their proliferative rate over time, hMSCs were cultured on nanomatrix composites of increasing HANP to PA-RGDS ratios and their long term cellularity was assessed over 28 days \textit{in vitro} (Fig. 13). By day 7, cells had not demonstrated significant differences in attachment. However, by day 14, cellular proliferation had progressed in a fashion highlighting a decrease in proliferative rate based on increases in the HANP to PA-RGDS ratios. Proliferation proceeded the quickest on TCP, with PA-RGDS displaying slightly lower levels of attachment. The inclusion of HANPs into the PA nanofiber matrix further slowed proliferation based on increases in the HANP to PA-RGDS ratios, with the 50\% and 66\% HANP nanomatrix composites showing little increase in cellularity between days 7 and 14. This trend continued up to day 28, with cells cultured on TCP and PA-RGDS exhibiting the greatest proliferation, approaching a density of \(\text{~85,000 cells/cm}^2\). In contrast, hMSCs cultured on the 66\% HANP nanomatrix composite only approached a density of \(\text{~25,000 cells/cm}^2\), demonstrating little proliferation over the course of the 28 day experiment. These results indicate modifications to proliferation inversely related to increases within the HANP to PA-RGDS ratios. Considering that proliferation is known to plateau at the onset of differentiation, it is possible that increases in the HANP to PA-RGDS ratio enhances osteogenic differentiation.
Figure 13. *Cellular Proliferation Over 28 Days*. Over the 28 day time frame, there were step-wise decreases in proliferation, with differences first manifesting at the 14 day time point. The 50% and 66% conditions demonstrated the slowest proliferation over the course of the experiment. Values are expressed as a mean ± standard error of measurement (*p<0.05, **p<0.01).

**Cellular Viability**

To ensure viability across all conditions, hMSCS were cultured for 28 days and stained with calcein AM and ethidium homodimer (Fig. 14). No clear differences in terms of viability were found between the nanomatrix composites and PA-RGDS, implying that HANP incorporation did not have a detrimental effect on the viability of hMSCs.
Figure 14. *Cellular Viability Over 28 Days.* Cells on all culture conditions were imaged utilizing a Live/Dead assay kit over 28 days. Overall, there were no differences in terms of viability between the nanomatrix composites and PA-RGDS. Images are taken at 20x. Scale bars represent 100 μM.
Gene Expression Utilizing RT-PCR

To confirm phenotypic commitment towards an osteogenic lineage, hMSCs were assessed for the up-regulation of genes specific to the temporal progression of osteogenic differentiation. hMSCs were cultured on the nanomatrix composites over 28 days, following which, they were assessed for early, middle, and late term markers of osteogenic differentiation. At the onset of differentiation, hMSCs slowly up-regulate genes indicative of an osteogenic phenotype, such as alkaline phosphatase (ALP), osteocalcin (OCN), and runt related transcriptional factor 2 (Runx2). One of the earliest indications of osteoblastic differentiation is the up-regulation of Runx2, which has been demonstrated to be an essential mediator of cell phenotype commitment and osteogenesis. To determine the onset of osteogenic commitment, Runx2 gene expression was assessed (Fig. 15)

At day 7, Runx2 is relatively down-regulated on all surfaces relative to TCP with the exception of the 66% HANP composite condition, signifying that hMSCs had not begun their phenotypic commitment towards an osteogenic lineage. However, by day 14, Runx2 had up-regulated in a step-wise fashion based on nanomatrix composition, signaling that early phenotypic commitment had begun to occur by day 14, with expression increasing based on increases to the HANP to PA-RGDS ratio. By day 28, Runx2 was slightly less expressed on all surfaces relative to TCP.
Figure 15. Gene Expression Profile for Runx2 Over 28 Days. Runx2 gene expression peaked at the day 14 time point on all conditions, with enhancements linearly increasing based on increasing HANP to PA-RGDS ratios. The early up-regulation for 66% at day 7 demonstrates its improved capability for osteoinduction relative to other conditions. Values are expressed as a mean ± standard error of measurement relative to TCP (dashed line) for all incubation periods. (p < 0.05, **p<0.01).

ALP plays an important role in the mineralization of bone tissue. As differentiation proceeds, ALP activity increases, cleaving organic phosphates to produce free, inorganic phosphates in order to prepare the organic ECM for mineralization\textsuperscript{52,73}. Because of this, ALP activity has become an important indicator of osteogenic differentiation. ALP is slowly up-regulated over time with significant expression...
occurring during mid-term differentiation\textsuperscript{68, 70}. ALP gene expression appears to coincide with that of Runx2, as all but the 66% HANP condition demonstrated down-regulation by the first week (Fig. 16). However, by day 14, ALP expression was considerably higher than TCP, correlating with increases in the HANP to PA-RGDS ratio. Once again, TCP catches up at the day 28 time point, demonstrating considerable delay in osteogenic differentiation relative to the other conditions.

Figure 16. \textit{Gene Expression Profile for ALP Over 28 Days}. At day 7, the 66% HANP containing composite had the greatest expression. For all conditions, ALP peaked at day 14 with greater expression exhibited in conditions with higher HANP to PA-RGDS ratios. Values are expressed as a mean ± standard error of measurement relative to TCP (dashed line) for all incubation periods. (*p < 0.05, **p<0.01).
As a late term marker of osteogenic differentiation, OCN is up-regulated during the process of mineralization. OCN plays an important role in the mineralization process, serving as a calcium binding protein. OCN up-regulated typically indicates a shift from mid to late term osteogenic differentiation\textsuperscript{68, 70}. Throughout the time frame of the 28 day experiment, OCN expression is slowly up-regulated in a dose dependent fashion, with the greatest expression manifesting at the four weeks time point, indicating terminal differentiation relative to the other time points (Fig. 17).

![Gene Expression Profile for OCN Over 28 Days](image)

**Figure 17.** Gene Expression Profile for OCN Over 28 Days. For all conditions, OCN activity is most pronounced at the 14 and 28 day time points with expression increasing in a step-wise fashion based on increasing HANP to PA-RGDS ratios. OCN expression is greatest at day 28, with the 66% HANP containing composite demonstrating the greatest increase. Values are expressed as a mean ± standard error of measurement relative to TCP (dashed line) for all incubation periods. (*p < 0.05, **p<0.01).
DISCUSSION

In this study, we synthesized bone analogous nanomatrix composites that recreate the biphasic ECM of bone through the incorporation of organic ECM mimicking PA nanofibers tailored with the RGDS cellular adhesion motif and HANPs; the combination of these two components were intended to capture both the nanoscale characteristics and bioactivity provided by the native bone ECM. Few studies have systematically investigated how variations in the ratio of inorganic to organic analogous ECM components affects the differentiation of osteogenic precursors as a majority of studies assessing composite materials either do so at fixed ratios or with an organic ECM analogous component that is not functionalized to provide signaling. Therefore, to assess changes to cellular behaviors in response to nanomatrix composition, we synthesized these composites with increasing HANP to PA-RGDS ratios and compared them to our PA-RGDS nanomatrix alone.

These composites were first prepared utilizing a solvent evaporation method and characterized via SEM and TEM. TEM confirmed the successful self-assembly of PA nanofibers 8-10 nm in diameter around HANPs roughly 100 nm in diameter that appeared to form small aggregates. SEM demonstrated increasing HANP content between the 16%, 33%, 50%, and 66% nanomatrix composite conditions. By considering both the SEM and TEM images together, it appears that PA nanofibers are sandwiched in-between smaller HANP aggregates. These smaller aggregates are stacked on top of each other to form larger aggregates. As the HANP to PA-RGDS ratio was increased there were slight increases to the size of the HANP/PA-RGDS aggregates, resulting in variations to surface topography. HA is hydrophobic by nature, thus, increasing the
concentration of HANPs suspended within hydrophilic solutions of PA-RGDS resulted in larger HANP/PA-RGDS aggregates. The capability of self-assembly for the fabrication of composites provided by PAs may offer certain advantages over traditional fabrication methods. This is because fabricating composites in such a manner does not require sintering and applied pressure to fixate HANPs through particle unification, thus potentially conserving grain size and improving nanoscale characteristics.

PA-RGDS nanofibers were chosen to serve as the organic ECM analogous component as our previous research has shown its potential in eliciting the osteogenic differentiation of hMSCs based solely on the inscribed RGDS cellular adhesive ligand sequence. The RGD cellular adhesive ligand was incorporated into our PAs as it has been shown to mediate cellular behaviors such as proliferation, and differentiation. This sequence is also common to multiple organic ECM proteins such as collagen and interacts with cells via the integrins alpha5-beta1, alpha V-beta3, and alphaIIb-beta3. The combination of a PA-RGDS nanofiber matrix with HANPs in increasing HANP to PA-RGDS ratios was hypothesized to yield higher levels of osteogenic differentiation than a PA-RGDS nanofiber matrix alone. This reasoning was established as studies have demonstrated enhanced osteogenic differentiation when organic ECM of both native and synthetic origin is combined with HA. Furthermore, it has been shown that increasing extracellular HA concentrations can result in increased ALP activity, OPN secretion, and up-regulation of genes specific to osteoblastic activity.

Because few studies how the cellular behavior of osteoprogenitors change in relation to the ratio between organic and inorganic analogous ECM components, hMSCs were cultured on nanomatrix composites of varying HANP to PA-RGDS ratios and
assessed for long term differentiation. The first step in assessing the differentiation of hMSCs was to investigate proliferation over time. By seeding hMSCs on these nanomatrix composites and assessing their proliferation over time, it was possible to correlate cellular senescence in relation to the onset of osteogenic differentiation. Long term phenomena such as proliferation and osteogenic gene expression appeared to correlate with changes in the HANP to PA-RGDS ratios. As noted earlier, proliferation was modified in a manner inversely related to the HANP to PA-RGDS ratio of each nanomatrix composite. To confirm biocompatibility and rule out the possibility that differences in cell density over time are a result of cell death, viability imaging was performed. From assessing the images, it was determined that there was no difference in terms of viability between PA-RGDS and the nanomatrix composites. These findings are supported by past studies showing that HA does not negatively affect biocompatibility with osteogenic cell types\textsuperscript{86-88}.

Differences in proliferation were reflective of the initial timeframe upon which genes indicative of early osteogenic differentiation were expressed. The process of osteogenic differentiation occurs in three distinct stages. These stages proceed in the following manner: cellular senescence, followed by matrix maturation, and finally, mineralization\textsuperscript{85}. Runx2 is an early indicator of osteogenic differentiation, serving as an essential mediator for cell phenotype commitment and osteogenesis\textsuperscript{69}. Runx2 had the greatest expression at the day 14 time point with expression increasing based on the HANP to PA-RGDS ratios. This expression pattern was inversely correlated with proliferation, where increases to the HANP to PA-RGDS ratio led to decreasing proliferative rates at the same time point. As cellular senescence is an early event in the
process of osteogenic differentiation, the fact that it corresponded with the up-regulation of genes indicative of early cellular commitment was expected. In this respect, multiple groups have demonstrated that Runx2 inhibits the proliferation of osteoprogenitor cells\textsuperscript{72, 89}. Some studies investigating osteogenic differentiation in relation to HA concentration demonstrate similar patterns with respect to proliferation as well\textsuperscript{75, 90}. In addition, PA-RGDS showed a statistically significant decrease in proliferative rate relative to TCP, demonstrating that the cell to ligand interactions provided by the functionalized PA nanofibers serve to modify proliferative rate through osteogenic induction as well, confirming our results from previous studies\textsuperscript{5, 6, 91}.

Further gene expression analysis of ALP and OCN displayed similar patterns in which the timed expression of middle and late term markers of osteogenic differentiation were enhanced by increasing the HANP to PA-RGDS ratios. Based on these results, it is implicated that our nanomatrix composites regulate proliferation and osteogenic differentiation based on the ratio of the inorganic to organic analogous ECM components. These results further imply that our nanomatrix composites could potentially be optimized to time the differentiation of osteoprogenitors based on their initial compositional makeup in terms of their inorganic to organic analogous ECM component ratios.

In conclusion, bone analogous nanomatrix composites consisting of HANPs encapsulated within PA-RGDS nanofibers were fabricated, mimicking both the inorganic and organic facets of the native bone ECM. These composites were synthesized at various HANP to PA-RGDS ratios with the intention of gaining insight about how modifications to nanomatrix composition influence osteogenic differentiation. The
osteogenic differentiation of hMSCs in response to these composites was then assessed via long term proliferation and gene expression assays. Results showed that osteogenic differentiation was enhanced as the HANP to PA-RGDS ratio was increased, confirming our hypothesis. Overall, these nanomatrix composites were successful in enhancing the osteogenic differentiation of hMSCs relative to an organic ECM analogous PA-RGDS nanomatrix alone. However, HANPs could not be fixated to tissue culture wells without the inclusion of a PA-RGDS nanofiber matrix to encapsulate them, meaning that we could not determine how the combination of HANPs and PA-RGDS enhances osteogenic differentiation relative to HANPs alone. This would have been helpful as there is conflicting evidence as to whether osteogenic differentiation is enhanced, or delayed by the incorporation of organic ECM proteins. This is because multiple groups have previously demonstrated that the combination of various organic ECM proteins such as collagen with HA can enhance osteogenic differentiation relative to HA alone $^{51, 77}$, contrasting other such studies where the incorporation of organic ECM proteins enhances proliferation over time $^{50}$, a behavior indicating delayed differentiation. To elucidate this issue in the future, studies can be performed in which composites consisting of functionalized PAs are compared to composites with non-functionalized PAs to determine the role that the cellular adhesive ligand sequences play with respect to osteogenic differentiation.

While this study determined that increases in the HANP to PA-RGDS ratios enhanced the osteogenic differentiation of hMSCs, it did not elucidate the exact mechanism(s) by which this occurs. It is unknown as to the exact mechanism(s) by which HANP inclusion enhances osteogenic differentiation. Some proposed mechanisms by
which HA may enhance osteogenic differentiation include variations in surface
topography, modification of surrounding media conditions with respect to calcium
and phosphate, and enhanced absorption of serum proteins. Therefore, it is noted
that changes to cellular behavior could potentially be in response to variations
to conditions such as surface topography, as increasing the HANP to PA-RGDS ratio also
increased HANP aggregate size, thereby changing gross surface characteristics.
Additionally, due to time constraints, multiple experiments of the same long term assays
could not be performed to confirm reproducibility. This issue will be addressed in the
future by performing repeat experiments.
CONCLUSION

In this study, we developed a bone analogous nanomatrix composite capable of mimicking both the nanoscale structure and bioactivity of native bone ECM. This was achieved by encapsulating HANPs within a self-assembled PA-RGDS nanomatrix. The resulting composites were fabricated at increasing HANP to PA-RGDS ratios to assess how nanomatrix composition influences the cellular behaviors of hMSCs. Long term observation of cellular response showed that the osteogenic differentiation of hMSCs seeded on these nanomatrix composites was improved based on the ratio of inorganic to organic analogous ECM components, with the greatest enhancement occurring on the composites with the highest HANP to PA-RGDS ratios. Therefore, following the growing need for materials capable of recapitulating the native ECM of bone, a bone analogous nanomatrix composite was successfully fabricated and shown to enhance osteogenic differentiation. Furthermore, due to the lack of studies addressing how modifications to nanomatrix composition can influence cellular behaviors, the osteogenic differentiation of hMSCs in response to various HANP to PA-RGDS ratios was demonstrated.
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APPENDIX

IRB Approval

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

August 11, 2011

MEMORANDUM

TO: Jeremy Benton Vines
    Principal Investigator

FROM: Nancy Stansfield RN, MSN
      Assistant Director, Institutional Review Board

RE: Application for Request for Documentation Regarding Use of Established Human Cell Lines Not Requiring IRB Review

The Office of the IRB has reviewed and approved your Human Cell Lines Application for “Osteogenic Differentiation of Human Mesenchymal Stem Cells Enhanced by Bone ECM Analogous Nanomatrix Composites.” The IRB has determined that this activity is not research involving human subjects and does not require IRB approval or continuing review.
August 11, 2011

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TO: Jeremy Benton Vines
   Principal Investigator

FROM: Nancy Stansfield RN, MSN
       Assistant Director, Institutional Review Board

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