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# Enhancement of peptide coupling to hydroxyapatite and implant osseointegration through collagen mimetic peptide modified with a polyglutamate domain

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ABSTRACT

Hydroxyapatite (HA) is a widely-used biomaterial for bone repair due to its high degree of osteoconductivity. However, strategies for improving HA performance by functionalizing surfaces with bioactive factors are limited. In this study, we explored the use of a HA-binding domain (heptaglutamate, "E7") to facilitate coupling of the collagen mimetic peptide, DGEA, to two types of HA-containing materials, solid HA disks and electrospun polycaprolactone matrices incorporating nanoparticulate HA. We found that the E7 domain directed significantly more peptide to the surface of HA and enhanced peptide retention on both materials *in vitro*. Moreover, E7-modified peptides were retained *in vivo* for at least two months, highlighting the potential of this mechanism as a sustained delivery system for bioactive peptides. Most importantly, E7-DGEA-coupled HA, as compared with DGEA-HA, enhanced the adhesion and osteoblastic differentiation of mesenchymal stem cells, and also increased new bone formation and direct bone-implant contact on HA disks implanted into rat tibiae. Collectively, these results support the use of E7-DGEA peptides to promote osteogenesis on HA substrates, and further suggest that the E7 domain can serve as a universal tool for anchoring a wide variety of bone regenerative molecules to any type of HA-containing material.



## 1. Introduction

Hydroxyapatite (HA) is a calcium phosphate crystal that makes up the principal constituent of bone mineral. Synthetic HA has been used for decades as a coating for metal prosthetics and other devices, and it has been extensively reported that HA-coated implants promote more robust bone formation than uncoated metal implants [1,2]. More recently, calcium phosphates including HA have shown promise as a scaffolding material for synthetic bone grafts [2], and as a bone-mimetic component within composite degradable biomaterials [3,4]. HA is known to be highly osteoconductive; however, it has limited osteoinductive capacity. Thus, there is significant interest in developing methods for coupling osteogenic factors, including bioactive peptides, to the HA surface.

Covalent linkage of peptides to material surfaces is commonly employed to achieve tight peptide anchoring [5]. However, HA presents few functional groups that can be utilized for covalent bonding. As an alternative, Durrieu et al. immobilized peptides on HA by attaching silane-derived spacer arms to the HA surface [6]. In another approach, peptide immobilization was accomplished via chemisorptive-coupling techniques involving water-soluble carbodiimides to direct carboxyl-amine conjugation of peptides to HA [7]. While these protocols were successful in anchoring peptides to HA, one concern is that both of these methods significantly alter the chemistry and topography of the HA surface, which could affect the natural osteoconductivity of HA. HA is a highly adsorptive type of biomaterial, and it has been suggested that the adsorption of bioactive proteins from the bone microenvironment contributes to the favorable tissue responses observed with this substrate [8,9]. As an example, adhesion proteins such as fibronectin, vitronectin and fibrinogen, become rapidly adsorbed to HA following implantation into bone, and these adsorbed proteins can provide a provisional matrix for the attachment and survival of mesenchymal stem cells (MSCs) [9–11]. MSCs play a key role in bone regeneration because these cells migrate into sites of bone injury, differentiate along the osteoblastic lineage, and then lay down an osteoid matrix that is subsequently mineralized and remodeled to form bone [12–14].

As a substitute for covalent attachment, we and others have explored the use of an acidic amino acid domain to direct ionic coupling of peptides to HA [15–23]. This strategy recapitulates the

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mechanism employed by endogenous bone-matrix proteins to achieve bone localization. Many bone-matrix proteins, including osteocalcin (OCN) [24] and bone sialoprotein (BSP) [25], contain stretches of negatively-charged amino acids that bind to the calcium within the biologic HA of bone. Modeling this process, we previously engineered the integrin-binding adhesive peptide, RGD, with a heptaglutamate domain (E7), and found that this increased peptide anchoring to synthetic HA disks [21]. However, some studies have suggested that RGD peptides have limited efficacy *in vivo* [26,27], and in particular, RGD actually inhibits bone formation and bone-implant contact on implanted HA [11]. Therefore, there is a need to identify other peptides that can enhance tissue responses to HA.

Native bone consists primarily of collagen I and HA; therefore, a biomaterial that incorporates both of these components would closely model the environment MSCs encounter *in vivo*. Studies from our group have shown that biomimetic peptides derived from collagen I, including DGEA and P15, stimulate MSC attachment to HA disks and induce osteoblastic differentiation of MSCs adherent to HA [11]. Furthermore, these peptides enhanced bone formation on HA disks implanted into rat tibiae [11]. Accordingly, in the current study we engineered the DGEA peptide with an E7 domain and compared the tethering capacity of DGEA and E7-DGEA on two HA-containing materials, sintered HA disks and electrospun degradable polycaprolactone matrices incorporating nano-HA particles (PCL/HA). Additionally, the bioactivity of DGEA-coated and E7-DGEA-coated HA disks was evaluated by monitoring MSC adhesion, spreading and osteoblastic differentiation *in vitro*, and bone formation *in vivo*.

## 2. Materials and methods

### 2.1. Peptide preparation

Collagen mimetic peptide DGEA (390.3 g/mol) was obtained from American Peptide Co., Inc. (Sunnyvale, CA). Synthesized modifications of the above peptide sequence were also prepared by American Peptide Co., Inc and included E7-DGEA (EEEEEEEDGEA, 1294.2 g/mol), DGEA-FITC (DGEAK-FITC, 906.9 g/mol), and E7-DGEA-FITC (EEEEEEEDGEAK-FITC, 1811.7 g/mol). All peptides were reconstituted in $ddH_2O$ at 1 mg/ml, aliquoted and stored at −20 °C. FITC-tagged peptides were used for studies of peptide retention, whereas untagged peptides were used for the studies of MSC adhesion, cell spreading, osteoblastic differentiation and bone formation.

### 2.2. Disk preparation

For both *in vitro* and *in vivo* studies of peptide retention and bioactivity, HA powder (MP Biomedicals, Solon, Ohio) was pressed into disks using a 15.875 mm die, under 3000 psi. For *in vivo* bone formation studies, HA powder (MP Biomedicals, Solon, Ohio) was pressed into disks using a 3 mm die at 1000 psi. Both disk sizes were sintered at 1000 °C for 3 h and allowed to cool in a furnace at decreasing intervals. Disks were stored under sterile conditions.

### 2.3. Scaffold preparation

Polycaprolactone (PCL) (MW = 110,000 Da) was purchased from Birmingham Polymers (Birmingham, AL), and HA nanoparticles (20–70 nm particle size) from Berkeley Advanced Biomaterials (Berkeley, CA). Two types of scaffolds, (1) 100% PCL, (2) 80wt% PCL + 20wt% HA, were produced by electrospinning as described previously [28]. Briefly, solutions were made with hexafluoroisopropanol (HFP, Sigma–Aldrich, St. Louis, MO), such that the solid weight was 7.5% of the total solution weight. Solutions were magnetically stirred at room temperature for 1h before being electrospun using 15 kV voltage (Gamma High Voltage Research, Ormond Beach, FL). The scaffold was collected on a grounded aluminum foil located 12 cm from the tip of the electrically charged 27- gauge needle. A syringe pump was used to feed polymer solution into the needle at a rate of 2 mL/h. No chemical or radiation-induced cross-linking of PCL was performed. Scaffolds were sterilized with ethanol, rinsed in sterile phosphate buffered saline (PBS), and stored under sterile conditions.

### 2.4. Peptide coating

Peptides were coated onto materials overnight at 4 °C at concentrations of 50 μM, 100 μM, or 200 μM. For differentiation studies, after overnight coating with appropriate peptide, disks were coated for 2 h with fetal bovine serum (FBS) at 37°. The disks and scaffolds were washed with PBS to remove unbound peptides and proteins, and warmed in PBS to 37 °C prior to cell seeding or *in vivo* implantation.

### 2.5. Visualization of immobilized peptides in vitro

The fluorescein conjugate, FITC, allows direct visualization of immobilized peptide using fluorescent microscopy. Materials coated with 50 μM, 100 μM or 200 μM DGEA-FITC or E7-DGEA-FITC peptides were washed in PBS with agitation for 7 days, changing PBS every 1–2 days. Upon completion of washes, relative retention of peptides was visualized using a fluorescent Nikon microscope. Additionally 100 μM E7-DGEA-FITC was coated onto materials and washed for 1 h with agitation. Using a fluorescent microscope, side by side analysis of peptide retention was performed on HA disks, titanium alloy (Ti-64), and stainless steel (SS).

### 2.6. Peptide quantification using enzyme- linked immunosorbent assay (ELISA)

Conjugation of peptides to a FITC tag also allows detection of immobilized peptides using a modified ELISA assay, described previously [21]. HA disks were placed in 24-well low attachment plates and coated with 100 μM peptide, as described above. Disks were then washed for 1 or 7 days, before blocking with 2% heat-denatured BSA for 2 h at 4 °C. Additionally, non-coated disks were blocked to provide a control for non-specific adsorption of ELISA reagents. After peptide-coating and blocking, disks were exposed to an anti-FITC antibody conjugated to horseradish peroxidase (1:5000 dilution) (A21253, Molecular Probes, Carlsbard, CA) for 1 h at 37 °C. The disks were subsequently washed and incubated with a horseradish peroxidase chromogenic substrate (Biosource International, Camarillo, CA) for 30 min at room temperature, with the reaction being stopped by incubation with the stop solution (SS02, Invitrogen, Carlsbad, CA) for 15 min at room temperature. Enzyme activity in the solution was quantified by absorbance spectroscopy (450 nm). The absorbance of uncoated disks was also measured to account for relative amounts of background fluorescence.

### 2.7. Depletion of fluorescence from solution

Equimolar peptide solutions were made (100 μM DGEA-FITC or E7-DGEA-FITC) and used to coat HA disks. These solutions were confirmed to have equivalent fluorescent values (3533 ± 226 for DGEA-FITC, and 3262 ± 54 for E7-DGEA-FITC). Samples were taken from the supernatant initially, after 2 h, and after 24 h of incubation with HA disks, diluted with PBS, and solution fluorescence measured using a fluorometer. These fluorescence values were plotted to show depletion of fluorescence from solution (indicating amount of peptide directed to the surface of scaffolds or disks).

### 2.8. In vivo peptide retention

HA disks or scaffolds were coated with 100 μM DGEA-FITC or E7-DGEA-FITC as described above and implanted into dorsal subcutaneous sites of male Sprague–Dawley rats. After 7–60 days, animals were sacrificed and implants were retrieved. Qualitative assessments of relative peptide retention were made by fluorescently visualizing the explanted materials in side-by-side comparisons under a Nikon Fluorescent microscope.

### 2.9. Cell isolation and culture

Isolation and culture of MSCs was performed as previously described [9]. Human bone marrow cells were centrifuged at low speeds and resuspended in Dulbecco's modified Eagle's Medium (DMEM). A density gradient was established by applying the cell suspension to a histopaque-1077 column. The MSCs were extracted from the column and grown in DMEM with 10% FBS (growth media).

Osteogenic media (OS media) was used for all differentiation studies and consisting of DMEM supplemented with PenStrep, Amphotericin B, 10% FBS, 100 nM dexamethasone, 10 mM sodium β–glycerolphosphate, and 0.05 mM l-ascorbic acid-2-phosphate, a well known formulation for differentiation media [29]. For all experiments, cells used were between passages 3 and 10. All cell culture work was performed with the prior approval of the University of Alabama at Birmingham Institutional Review Board.

### 2.10. Cell adhesion

Adhesion of MSCs to peptide-coated disks was evaluated using a standard fluorescence based assay adopted from a previous source [30]. Briefly, MSCs were incubated with 2 μM CMFDA ("Cell Tracker Green" Molecular Probes) in serum free/thiol free media to label cells. Cells were detached from the tissue culture flasks by trypsinization and the reaction stopped using trypsin inhibitor (Sigma Aldrich).