# Osteoblast cell differentiation and matrix mineralization in response to Hyaluronic Acid Hydrogels

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Statement of Purpose: Hyaluronic acid (HA, Hyaluronan) is a naturally occurring, biocompatible, and biodegradable linear polysaccharide. HA plays a prominent role in various wound healing and bone regeneration processes, as it is naturally angiogenic when degraded to small fragments upon degradation.[1] This evidence suggests that HA is an ideal candidate material for wound healing and tissues regeneration. Modification of non-adhesive hydrogels with integrin binding-RGD peptides promotes adhesion, spreading, and proliferation of cells. The objective of this study is to investigate *in vitro* differentiation and matrix mineralization of MC3T3 osteoblast cells in the presence of HAGM and HAGM with RGD peptide. The clinical goal is to develop HA scaffolds that deliver biologically active components to enhance healing of bone fractures.

**Methods**

Photopolymerizable methacrylate groups were added to HA to yield HA-glycidyl methacrylate (HAGM) conjugates. Briefly, HAGM was prepared by treating 0.5% w/v solution of HA (~ 1.6 MDa) in phosphate buffer saline, and dimethylformamide with 50-fold molar excess of GM in the presence of triethylamine. A 5% by mass fraction of HAGM was mixed in deionized water and combined with ACRL-PEG-GRGDS to a final concentration of 4 µmol/mL. The hydrogels were made by exposing the aqueous solution of HAGM to UV-light for 10 min in the presence of the photoinitiator Irgacure 2959 (0.1% by mass fraction).

HAGM scaffolds were sterilized in 70% ethanol and seeded with $5x10^4$ cell and cultured in a 24-well tissue culture plate for 1, 7, and 28 days. For osteoblast differentiation and mineralization, MC3T3 and MG63 cells were cultured in osteogenic media (OS+) containing 50 mg/mL of ascorbic acid, 10 mM β-glycerophosphate. MG63 cells received $10^5$ M dexamethasone and the controls were treated with plain media (OS-). To detect osteoblast mineralization, 8 µg/mL of Tetracycline- HCl was added 2 days prior to harvest. Media samples and cell lysate were collected and assayed by ELISA for calcium, phosphorous, alkaline phosphatase, and osteocalcin after 1, 4, 7, 14, 21 and 28 days. Polymer gels seeded with cells were fixed in 2% paraformaldehyde for histology or 2.5% glutaraldehyde for SEM-EDAX and FT-IR analysis. Immunofluorescent staining was performed on the gels seeded with cells for OSX, Runx2, and ALP osteoblast differentiation markers. Mineralization staining for phosphorous (Von Kossa) and calcium (alizarin red) was performed on the polymer samples at 28 days.

**Results/Discussion:**

Facile synthesis of HA with GM yields polymerizable HAGM macromers. $^1$H-NMR spectroscopy was used to determine the percent of methacrylation (~ 32%) and verify the degree of purity on the modified HA. The resulting macromers were photocross-linked in water to form hydrogels.

The HA gel is biocompatible and promote cell attachment and proliferation, as evidenced from the phase contrast images after Day 1 and Day 4 (**Figure 1.**). Both the osteogenic (OS+) and non-osteogenic (OS-) cultures showed good cell attachment on the HAGM with RGD peptide and very poor cell attachment on the unmodified gel, suggesting the importance of the oligomeric RGD ligand for cell attachment and proliferation. HAGM with RGD surface, favored early differentiation of osteoblast cells as early as Day 4, as observed from the immunofluorescence staining for ALP, Type-I collagen, OSX, and RUNX-2, which showed increased expression in the presence of OS+ media when compared to OS- controls. Tetracycline labeling of the HAGM with RGD gels (**Figure 2**) showed mineralized extracellular matrix by 21 days in the presence of osteogenic media, while no mineralization was observed in controls (OS-). HAGM alone did not show any mineralization in OS+ and Os- media even after 28 days, suggesting incorporation of RGD peptide influenced cell attachment, proliferation and matrix mineralization. Furthermore, Von Kossa, Alizarin red staining, FT-IR, and SEM-EDAX confirmed greater cell/matrix mineralization with HAGM with RGD peptide in OS+ media when compared to OS- media. Unmodified HAGM did not support any mineralization.

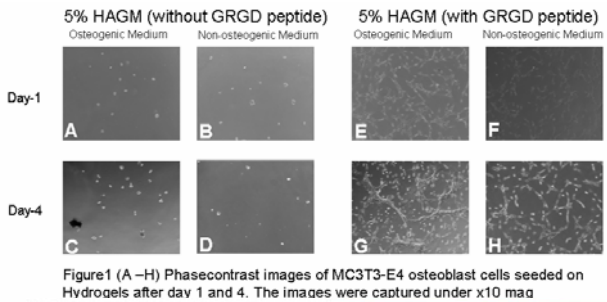


Figure1 (A –H) Phasecontrast images of MC3T3-E4 osteoblast cells seeded on Hydrogels after day 1 and 4. The images were captured under x10 mag

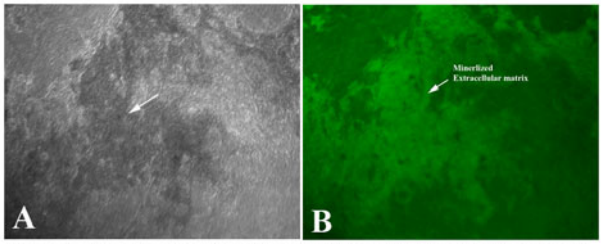


**Figure 2. show phase contrast (A) and tetracycline labeled (B) image of mineralized extracellular matrix after 21 days on HAGM with RGD surface**

**Conclusions:**

Incorporating RGD peptides promote osteoblast cell spreading, proliferation and matrix mineralization. HAGM gel with RGD seems to be good candidate for bone tissue engineering and can be potentially used for protein and DNA delivery.

**References**

1. Chen WY., Abatangelo G. Wound Repair Regen. 1999; 7: 79-89.