

## Regulating Myoblast Phenotype through Controlled Alginate Scaffold Degradation: A Delivery Vehicle for Skeletal Muscle Tissue Engineering

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**INTRODUCTION:** Skeletal muscle tissue engineering is a new therapeutic strategy for treatment of muscle loss or deficiency (e.g. muscular dystrophies). In general, skeletal muscle develops by proliferation and differentiation of mononucleated muscle precursor cells (myoblasts), followed by fusion of these cells into multinucleated myotubes, and finally alignment of tubes into fibers <sup>[1]</sup>. Tissue engineering aims to mimic this process by transplanting myoblasts using appropriate carriers.

A key issue in tissue engineering is to mimic specific functions of the native ECM to guide new tissue formation. Biomaterial vehicles are being designed to present signaling molecules, and regulate cell phenotype <sup>[2]</sup>. It is widely believed that these materials should degrade over time to provide new space for tissue development; however, the role of material degradation on cellular function has not been extensively investigated <sup>[3]</sup>.

This study addressed the hypothesis that the degradation rate of materials will control myoblast phenotype. Alginate, a naturally derived copolymer, has been utilized as a scaffold for skeletal muscle tissue engineering due to its biocompatibility and ability to form gels with a gentle gellation process. Coupling adhesive peptide sequence to alginate chains has provided a direct linkage between myoblasts and the alginate. This peptide-modified alginate has shown an ability to regulate myoblast proliferation and differentiation in 2-dimensional cell culture <sup>[4]</sup>. Recently, combining partial oxidation on alginate chains prior to gel formation with the utilization of a bimodal molecular weight distribution (binary system) has provided a means to control the degradation of alginate gels. We propose to utilize this material system to study the effect of alginate degradation on myoblast phenotype in 3-dimensional culture.

### METHODS

**Alginate modification:** Sodium alginate powder rich in GG-blocks ( $M_w = 2.7 \times 10^5$  g/mol,) was used as the high molecular weight component (HMW) to form gels. Low molecular weight

alginate ( $M_w = 5.3 \times 10^4$  g/mol, LMW) was obtained by gamma ( $\gamma$ )-irradiating HMW with a cobalt-60 source for 4 hours at a  $\gamma$ -dose of 5.0 Mrad. Alginates (both HMW and LMW) were partially oxidized to a theoretical extent of 1% of sugar residues with sodium periodate, and an oligopeptide (G<sub>4</sub>RGDSP) was subsequently coupled at an average density of 3.4 mM peptide/mole of alginate monomer, using carbodiimide chemistry.

**Cell culture and cell immobilization:** C2C12 mouse myoblasts were cultured in growth media (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) and maintained subconfluent prior to experiments. Myoblasts were then harvested and mixed with the 3 alginate gel types described below. Alginate/cell mixtures (at the density of  $20 \times 10^6$  cells/ml alginate) were mixed with CaSO<sub>4</sub> slurry, cast between two glass plates separated with 1 mm spacers, and allowed to gel 30 or 40 minutes for unary and binary systems, respectively. Following gelling, alginate/cell disks were punched out using a 4.5 mm puncher, and cultured with growth medium in spinner flasks.

**Assays:** Alginate disks were harvested and stained with a tetrazolium salt (MTT) to determine myoblast viability. Cell proliferation was assessed by incubating with <sup>3</sup>H-thymidine overnight, dissolving the gels with EDTA/PBS and counting the cell-associated radioactivity using a scintillation counter. Cell counts were performed by dissolving the gels with EDTA/PBS, incubating with trypsin, and counting cells using a Coulter counter. In addition, myoblasts were visualized by staining the actin cytoskeletal with Oregon Green Phalloidin. The alginate disks were mounted onto coverslips and imaged with a BioRad Radiance 2000 confocal system.

**RESULTS:** C2C12 myoblasts were encapsulated in three different gels: 1. partially oxidized binary gels (LMW: HMW = 1:1, fastest degradation rate), 2. partially oxidized unary gels (HMW, intermediate degradation rate), and 3. non-oxidized unary gels (HMW, no degradation) to investigate the effect of degradation on cell phenotype.

Cell viability was first determined following cell encapsulation and culture. Alginate modification and gelling did not lead to cytotoxicity, and the chosen culture conditions provided sufficient oxygen and nutrient transportation up to 3 weeks in all three gel types, as shown by dark purple crystal produced by mitochondria of viable cells with the MTT assay (data not shown). Next,  $^3\text{H}$ -thymidine incorporation illustrated that cells could proliferate in all gel types (Fig. 1). The proliferation was highest in non-oxidized unary gels, followed by partially oxidized unary, and partially oxidized binary gels, respectively. The reliability of thymidine incorporation assay in this culturing system was confirmed by examining myoblasts in differentiation medium. As expected, the thymidine incorporation was arrested in all gel types (Fig. 1), as myoblasts typically withdraw from cell cycle when cultured in low serum containing medium. The thymidine incorporation data was consistent with cell count data (not shown), as the cell numbers increased in the order of non-oxidized unary gels, partially oxidized unary, and partially oxidized binary gels, respectively. In fact, the cell number in partially oxidized binary gels on day 5 decreased from the initial values.

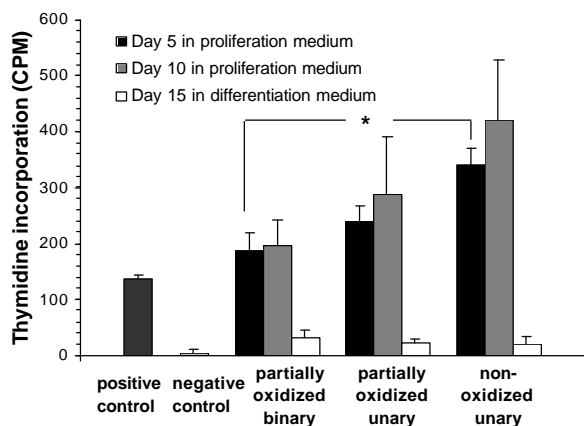


Fig. 1: Thymidine incorporation of myoblasts encapsulated in three different alginate gels. Positive and negative control represent proliferating myoblasts cultured on 2-dimensional alginate gels and blank alginate, respectively. Experimental values are reported as means  $\pm$  SD. \* denotes a statistically significant difference ( $p$ -value  $<$  0.05).

Myoblast morphology was visualized by staining the actin cytoskeleton to determine if cells differentiated by fusion into multinucleated myofibers. Myoblasts cultured in degradable gels (partially oxidized binary and unary gels) spread and fused on the top of the gel surface by day 7

(Fig.2). This result indicates the decrease in cell number at day 5 resulted from this cell fusion. On the contrary, the cells in non-oxidized gels maintained a round morphology throughout the gel volume. No spread or fused cells were observed in these non-degradable gels.

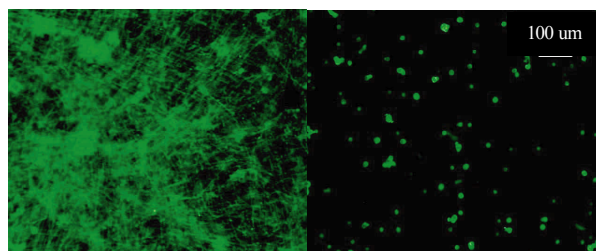


Fig. 2: Actin visualization (green) highlights fusing myoblasts on partially oxidized binary and unary gels (left) and round-shaped myoblasts in non-oxidized gels (right).

**DISCUSSION & CONCLUSIONS:** The results of this study indicate the degradation rate of the encapsulation material dramatically influenced myoblast phenotype. Cells immobilized in degradable gels (partially oxidized binary and unary gels) illustrated lower proliferation than those in non-degradable gels (Fig.1). Cells in these degradable gels exited the cell cycle, committed to the differentiation pathway, and fused (Fig. 2). This response may be due to the mechanical stiffness reduction upon gel degradation, which allows the cells to spread, migrate, and fuse. In contrast, the non-degradable gels maintained a high stiffness over time, and this likely prevented cell spreading and cell-cell interaction. The cells still proliferated in these gels, likely due to the high proliferative nature of these C2C12 cells.

This study provides a cell-delivery vehicle for skeletal muscle tissue engineering, and emphasizes the critical role of material degradation in regulating cellular behaviour. This latter result may be applied to a broad range of other tissue/cell types as well.

**REFERENCES:** <sup>1</sup> M.D Grounds, J.D White, N. Rosenthal et al (2002) *J Histochem Cytochem* **50**: 589-610 <sup>2</sup> M.P Lutolf, F.E Weber, H.G Schmoeker, et al (2003) *Nat Biotechnol* **21**:513-8 <sup>3</sup> E. Alsberg, H.J Kong, Y. Hirano, et al (2003) *J Dent Res* **82**: 903-8 <sup>4</sup> J.A Rowley, D.J Mooney (2002) *J Biomed Mat Res*, **60**: 217-23

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