Mineralization and Bone Regeneration of Injectable, Dual-Gelling Hydrogel-Cell Constructs

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Statement of Purpose: Tissue engineering strategies involving hydrogels that can be administered minimally invasively, crosslinked *in situ*, and remodeled locally have been investigated as alternatives to current treatments for complex craniofacial defects. To this end, we developed injectable, dual-gelling hydrogels capable of physical gelation through copolymerization of thermosensitive poly(*N*-isopropylacrylamide) (PNiPAAm) with a hydrolyzable lactone ring and epoxy pendant groups, and chemical gelation through crosslinking with diamine polyamidoamine (PAMAM) crosslinkers. We have previously shown that these hydrogels, with and without gelatin microparticle (GMPs) as sites for cell attachment, can support and direct the osteogenic differentiation of mesenchymal stem cells (MSCs) *in vitro*. Additionally, the hydrogels exhibited controllable mineralization based on the degree of polymer wt %. Therefore, the aims of this study were to elucidate the mechanism for hydrogel mineralization, determine the composition of the mineral deposits, and ultimately, evaluate the regenerative potential of the hydrogels *in vivo*. We hypothesized that the hydrophobicity of the hydrogels would promote mineralization, and that the mineral composition would be similar to amorphous hydroxyapatite. We further hypothesized that the mineralizing gels with encapsulated MSCs and GMPs would lead to enhanced bone regeneration in an orthotopic defect.

Methods: Thermogelling macromers (TGMs) with PNiPAAm, glycidyl methacrylate, acrylic acid and dimethyl-γ-butyrolactone acrylate hydrolyzable ring and PAMAM crosslinkers were synthesized following previously described protocols [1]. 10 mM glutaraldehyde crosslinked 50-100 μm diameter GMPs were prepared as previously described [2]. MSCs were harvested from the long bones of 6-week-old Fischer 344 rats in accordance with IACUC-approved animal protocols. 15 and 20 wt % (w/w) acellular hydrogels were fabricated and immersed in phosphate buffered saline (PBS), 1X simulated body fluid (SBF), complete osteogenic media without serum (NS) and complete osteogenic media with serum (S) for 0, 7, 14, 21, and 28 days (n=6). Quantity and composition of mineralization will be evaluated via calcium and phosphate biochemical assays, X-ray diffraction analysis (XRD) and scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDX). *In vivo* evaluation of the more hydrophobic hydrogels with and without cells and/or GMPs (n=8), as listed in Table 1, was performed in an 8 mm rat calvarial critical size defect following established protocols [4]. After harvest at 4 and 12 weeks, samples will be analyzed with microcomputed tomography, histology and histomorphometry for bony union, bone volume, mineralization and tissue response.

Results: Two injectable, dual-gelling hydrogel formulations of varying polymer wt %, and thus hydrophobicity, were fabricated. Acellular hydrogels exhibited a significantly higher mineralization capacity in serum-containing media over 28 days (Figure 1). The more hydrophobic 20 wt % hydrogels also showed significantly increased calcium binding at later timepoints for the NS and SBF groups compared to the 15 wt % groups. Ongoing studies are currently being performed to detect the presence of surface-deposited minerals with SEM/EDX, and characterize the composition with XRD. *In vivo* studies with MSC-laden hydrogel constructs in the rat cranial defect are also in progress.

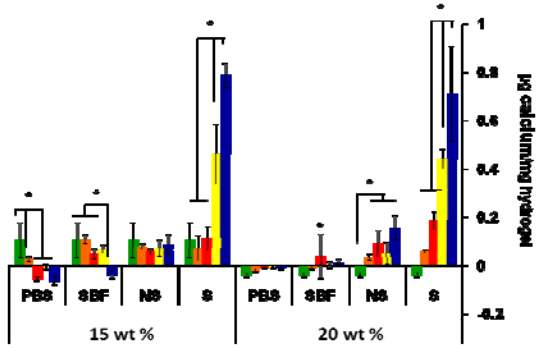

Figure 1. Calcium content of acellular 15 and 20 wt % hydrogels over 28 days in four different solutions. (*) refers to significant difference across timepoints within group and media ($p < 0.05$).

Table 1. Groups for *In Vivo* Encapsulation Study

Group	Polymer wt %	GMP loading %	Encapsulation density (cells/mL)
1	20	20	0
2	20	20	15 million
3	20	0	15 million

Conclusions: The results demonstrate that the hydrophobicity of an injectable, dual-gelling hydrogel can be successfully modulated to promote hydrogel mineralization in the absence of incorporated minerals, factors or cells. Higher hydrogel hydrophobicity in a protein-rich environment was found to mediate mineralization, and ongoing studies are aimed to characterize the composition of the mineral deposits. The hydrogels also provide a suitable hydrated environment for the survival and directed osteogenesis of encapsulated MSCs, which is now being evaluated *in vivo*. The data suggest that these *in situ* forming hydrogels may be a promising minimally invasive cell delivery vehicle for craniofacial bone regeneration.

References: 1) Vo TN et al. *Biomacromolecules.* 2014; 15:132-142. 2) Holland TA et al. *J Control Release.* 2003;91:299-313. 3) Klouda L et al. *Acta Biomaterialia*. 2011;7:1460-1467. 4) Spicer P et al. *Nature Protocols*. 2012;7:1918-1929.

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