Injectable Chitosan-Collagen Composite Hydrogels for Tissue Engineering Applications

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Purpose: In this study, β-glycerophosphate (β-GP), a weak base and an osteogenic supplement, was used to initiate both chitosan and collagen gelling at physiological temperature and pH, in the presence of adult human bone marrow-derived stromal cells (hBMSC). The composite hydrogel was further chemically crosslinked using glyoxal, a small dialdehyde with relatively low cytotoxicity. Most methods to fabricate chitosan-collagen composite materials involve freeze drying to create stable porous scaffolds, which subsequently can be seeded with cells. In contrast, the goal of this work was to create a chitosan-collagen composite hydrogel that contains embedded cells, and which supports cell adhesion, proliferation and directed differentiation.

Methods: Chitosan and collagen were dissolved in cold acetic acid and then supplemented with β-GP. Isolated hBMSC were added at a concentration of 2.0×10^6 cells/ml. The temperature of the solution was increased to 37 °C for 30 min to create a chitosan-collagen composite hydrogel. The effects of different chitosan:collagen ratios (100:0, 35:65, 75:25, 0:100) and a range of β-GP concentrations (2.5-20.0 wt%) on gel formation and cellular response were evaluated. Changes in pH were monitored during gelling, energy-dispersive X-ray spectroscopy (EDS) was used to examine chemical composition of gels, and scanning electron microscopy (SEM) was used to observe gel morphology. Cell viability and osteogenic differentiation triggered by dexamethasone and β-GP were evaluated *in vitro* over 3 weeks. Cytotoxicity of glyoxal crosslinking of composite hydrogels also was evaluated.

Results: Thermosensitive chitosan-collagen hydrogels were successfully created using a range of β-GP concentrations from 5-12.5 wt%, while the chitosan-collagen solution failed to gel properly when the β-GP concentration was out of this range. Final pH values ranged from 7.0-7.5 when increasing the temperature to 37 °C. X-ray microanalysis demonstrated that Na and P, the two characteristic elements in β-GP, diminished after washing with either DI water or PBS. SEM analysis (Fig. 1) showed that pure chitosan materials had a porous structure, while pure collagen gels exhibited the expected fibrous network morphology. Mixed chitosan-collagen materials exhibited a composite structure with collagen fibrils interspersed with chitosan matrix.

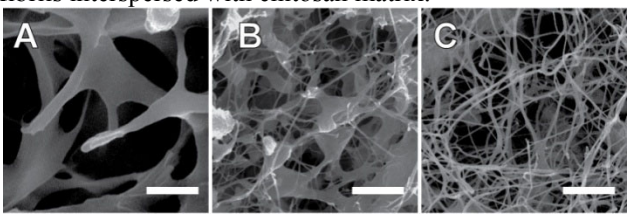

Fig. 1: *SEM analysis. Pure chitosan (A). chitosan-collagen composite (B). Pure collagen (C). Scale bar is 3 μm.*

hBMSC embedded in chitosan-collagen materials demonstrated high cell viability (>90%) after gel formation and continued to proliferate with time (Fig. 2A-B). hBMSC exposed to osteogenic supplements differentiated along the bone lineage as evidenced by the upregulation of osterix and bone sialoprotein genes, an increase in ALP activity, and increased calcium deposition. Higher chitosan content promoted osteogenic differentiation with higher osteogenic gene expression and mineralization (Fig. 3C).

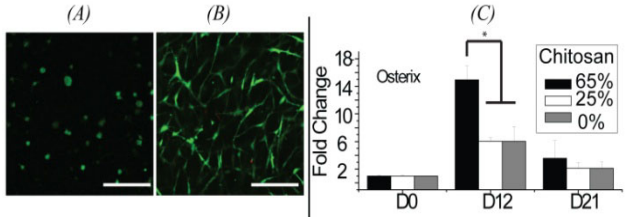

Fig. 2: *Live/dead staining immediately after gel formation (A) and after 1 wk in culture (B). Osteogenic gene expression over a 3-wk period (C). Scale bar is 100 μm.*

Glyoxal exposure at concentrations below 1.0 mM resulted in low cytotoxicity in monolayer culture with exposures up to 15 h (Fig. 3A). In 3D chitosan-collagen composites, glyoxal concentrations less than 0.3 mM exhibited low cytotoxicity (Fig. 3B) when examined at 1 week after gel formation.

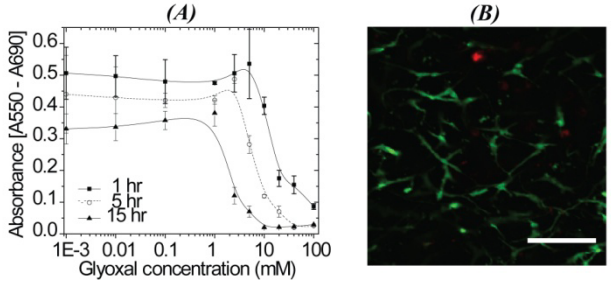

Fig. 3 *Cytotoxicity of glyoxal. MTT assay with hBMSCs on the monolayer (A). Live/dead staining with hBMSCs treated with 0.3 mM glyoxal after 1 week in chitosan-collagen composite hydrogel (B). Scale bar is 100 μm.*

Conclusions: Pure chitosan, pure collagen, and chitosan-collagen composite materials can be fabricated by thermal gelation in combination with addition of 5-12.5 wt% β-GP. Gel formation occurred at physiological temperature and pH, thus allowing embedded cells to maintain high viability. β-GP initiated the chitosan gelling process and subsequently diffused out of the hydrogel matrix. Further stabilization of the matrix could be achieved by chemical crosslinking with glyoxal, which was shown to have low cytotoxity in both monolayer and 3D cultures. Chitosan-collagen composite gels demonstrated better cell adhesion and proliferation than pure chitosan gels, and more robust osteogenic differentiation potential than both pure chitosan and collagen gels. These composite hydrogel materials have potential application in regenerative medicine, including as injectable cell carriers to fill irregularly-shaped defects.