Enthesis-inspired transitional mineral layers for collagen hydrogel constructs

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Introduction

- Collagen hydrogels are increasingly used in tissue-engineered constructs to replace damaged joints[1]. The native tissues that they replace typically osseointegrate with bone via a fibrocartilage transitional region that has a gradient of mineral content[2].
- A tissue-engineered analogue of this transitional structure is advantageous for the osseointegration of collagen hydrogels to bone (*e.g.* synthetic intervertebral disc (IVD) and meniscus constructs).
- Synthetic constraints represent key technical challenges to the integration of biologically-relevant mineralized gradients within cell-containing collagen hydrogel constructs and/or the surface deposition of a conformal mineral layer.

Objective

Engineering of a multi-layer mineral gradient structure in tissue-engineered collagen hydrogels under incubation conditions (humidified, 37 °C, and 5% CO_2).

Methods

- **Collagen:** Type 1 collagen was extracted from Sprague-Dawley rat tails, lyophilized, and reconstituted in 0.1% (v/v) acetic acid at 20 mg/mL[3]. The reconstituted collagen was mixed with a working solution (WS) comprised of 1N NaOH, 1× PBS, and 10× PBS to initiate gelation[3].
- **Treatment 1 (Fig. 1):** Collagen hydrogels (2mm) were formed by extruding the collagen solution onto a glass plate and incubated for 10 min (37 °C and 5% CO_2).

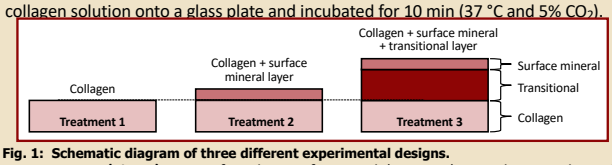

Fig. 1: Schematic diagram of three different experimental designs.

- **Treatment 2 (Fig. 1):** A surface layer of mineral (<10 µm) was deposited on the collagen hydrogel by drop-casting (DC) a suspension of $CaCO_3$ nanoparticles ($CaCO_3$-NP, 2.5 mg/mL in 10× PBS) for 20 min in an incubator (37 °C and 5% CO_2).
- **Treatment 3 (Fig. 1):** A transitional layer of collagen + mineral (2 mm) was formed by incorporating $CaCO_3$-NPs in the WS (final conc. of 2.5 mg/mL). The collagen + mineral solution was extruded onto the collagen hydrogel and the DC steps described for Treatment 2 were repeated to deposit a surface mineral layer.
- **Cellular Constructs:** Cells (ovine bone marrow stromal cells (OvMSC), P3) were seeded in the collagen layer at a density of 8×10^6 cells/mL.
- **Incubation/Culturing Conditions:** Samples were transferred to a new well-plate and cultured in DMEM for 3 days and 7 days.
- **Scanning Electron Microscopy (SEM)** was performed to assess the morphology and structure. Samples were fixed/stained with formalin/OsO_4, dehydrated with ethanol/hexamethyldisilazane (HDMS) series dilutions, and sputter-coated (Au-Pd).
- **Fourier Transform Infrared Spectroscopy (FTIR)** was performed to determine the chemical composition of the mineral particles after treatment in WS and DC.
- **Live/Dead Assay** (calcein, ethidium homodimer-1) was performed to determine cell viability and to assess biocompatibility. Cells (ovine bone marrow stromal cells (OvMSC), P3) were seeded in the collagen layer at a density of 8×10^6 cells/mL.
- **Related preliminary studies:** SEM micrographs of OvMSCs seeded in the transitional layer ($CaCO_3$-NP, 2.5 mg/mL) and cultured for 7 days and 23 days.

Results and Discussion

- SEM micrographs of $CaCO_3$-NPs following WS treatment indicate that the nanoparticles agglomerated into clusters (**Fig. 2b**), while those following DC treatment exhibit significant intergrowth (**Fig. 2c**).
- SEM micrographs of the surface DC layer indicate that the mineral particles exhibit a rosette-like morphology characteristic of synthetically formed carbonate apatite (**Fig. 3a,d**). The transitional layer is comprised of mineral clusters entangled within a collagen fibril network (**Fig. 3b,e**). Day 3 and Day 7 results are consistent (**Fig. 2d**).
- Structures resembling mineralized collagen are observed in SEM samples from related studies where cells are seeded in the transitional layer (**Fig. 4**); this may be attributed to cell remodeling of apatite/collagen to mineralized collagen.
- FTIR spectra indicate that precursor $CaCO_3$-NP particles remained as calcite following WS treatment, but phosphate peaks characteristic of carbonate apatite were observed for particles following DC treatment (**Fig. 5**). The phase change is probably driven by dissolution-reprecipitation processes[4].
- Cell viability is high (**Fig. 6, 7**), indicating biocompatibility of constructs.

Results SEM – Morphological Structure

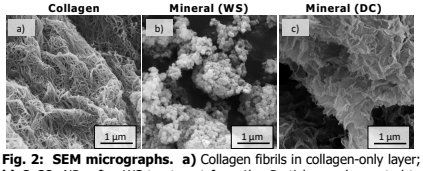

Fig. 2: SEM micrographs. a) Collagen fibrils in collagen-only layer; **b)** $CaCO_3$-NPs after WS treatment for ~1h. Particles agglomerated to form clusters of ~1 µm; **c)** $CaCO_3$-NPs after treatment with DC solution of 10X PBS for ~1h. *All samples imaged in secondary electron (SE) mode.*

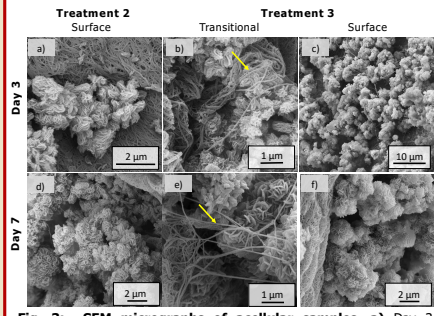

Fig. 3: SEM micrographs of acellular samples. a) Day 3, Treatment 2: rosette-like morphology of the particles are characteristic of synthetically-formed carbonate apatite; **b)** Day 3, Treatment 3: mineral clusters entangled in network of collagen fibrils (yellow arrow); **c)** Day 3, Treatment 3: discontinuous conformal deposition of mineral particles; **d)** Day 7, Treatment 2: see Fig. 3a; **e)** Day 7, Treatment 3: see Fig. 3b; **f)** Day 7, Treatment 3: see Fig. 3c. *All samples imaged in SE mode.*

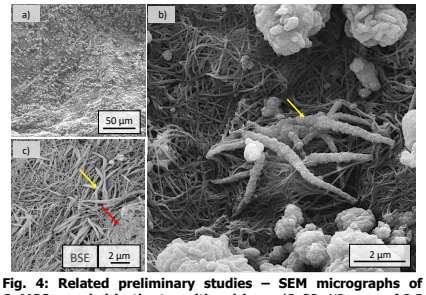

Fig. 4: Related preliminary studies – SEM micrographs of OvMSCs seeded in the transitional layer ($CaCO_3$-NP conc. of 2.5 mg/mL)**. a)** Day 23 (planar): discontinuous conformal mineral layer by DC; **b)** Day 23 (planar): structures that resemble mineralized collagen observed (yellow arrow); **c)** Day 7 (planar): see Fig. 3b). Structures resembling mineralized collagen (yellow arrow) are observed near cells (red arrow). *Fig. 3a and 3b imaged in SE mode, Fig. 3c imaged in backscattered electron (BSE) mode.*

FTIR – Chemical Composition

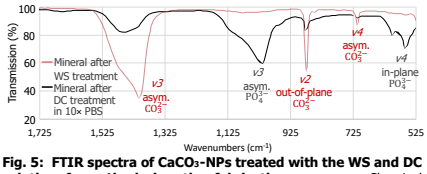

Fig. 5: FTIR spectra of $CaCO_3$-NPs treated with the WS and DC solution for ~1h during the fabrication process. Chemical composition of the mineral after WS treatment is predominantly calcite, whereas phosphate peaks characteristic of carbonate apatite are observed in mineral particles after immersion in 10X PBS during the DC process.

Live/Dead Assay

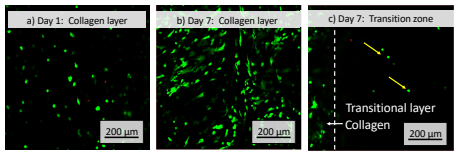

Fig. 6: Live/dead assay for Treatment 3 (collagen hydrogel construct with transitional mineral layer and DC surface layer). a) Day 1: Collagen layer with high cell viability; b) Day 7: Collagen layer, where high cell viability persists and cell spreading behavior is observed; c) Day 7: Cell migration is observed in the interface region of the collagen layer and transitional layer.

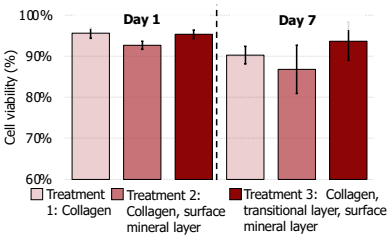

Fig. 7: Cell viability after 1 day and 7 days. Cell viability was >90% for all treatments after 1 day and >85% after 7 days, indicating biocompatibility; n=3, error bars denote standard error.

Conclusions and Future Work

- A multi-layer mineral gradient structure in tissue-engineered collagen hydrogels was achieved.
- Long term (Day 14, 21, 28) cultures of acellular and cell-seeded samples will be performed (including SEM and FTIR to to determine changes in fiber/mineral composition, organization, and integration).
- Histology/immunohistochemistry will be performed to determine protein expression (e.g., collagen, osteopontin, osteocalcin) as a function of culture time and construct design. Gene expression analysis to determine the differentiation pathway of ovine bone marrow stromal cells. Target time points are Day 0, 3, 7, 14, 21, 28.
- Transmission electron microscopy (TEM) may be performed to confirm the presence of mineralized collagen.
- FTIR, Raman mapping, and/or SEM/Energy Dispersive X-ray spectroscopy (EDS) of hydrogel constructs to determine chemical composition of mineral gradients. Target time points are Day 0, 3, 7, 14, 21, 28.

Significance

- **Potential to enable the osseointegration of cell-seeded collagen hydrogels directly to bone**
- **Potential cell remodeling of apatite/collagen to mineralized collagen without synthetic mediators**

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