

# 3D-printed Bi-layer Composite Scaffold Designed For Osteochondral Regeneration: A Preliminary Study In Porcine

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**Disclosures:** The authors have no disclosures.

**INTRODUCTION:** Scaffold-based tissue engineering strategies have emerged as promising alternatives to autograft, allograft, and prosthetics for treating musculoskeletal disease and injury [1]. Particularly for osteochondral (OC) tissue engineering, multilayer scaffolds aim to simultaneously repair both cartilage and bone. Our approach utilizes biomimetic hydrogels loaded with chondrogenic and osteogenic growth factors (e.g., TGF- $\beta$  & BMPs) to promote cartilage and bone regeneration, respectively. However, hydrogels are typically softer ( $E = \sim 10\text{--}100$  kPa) than articular cartilage ( $E = \sim 1\text{--}10$  MPa) and subchondral bone ( $E = \sim 20\text{--}3000$  MPa). Thus a structural support is needed to protect hydrogels and encapsulated cells from excessive strains that lead to failure, lack of tissue-implant integration [2], damage to neighboring tissues [3], and to deliver appropriate biomechanical cues for tissue-specific regeneration. We utilize advanced 3D-printing via a custom digital light processing (DLP) system to fabricate load-bearing (i.e., micro-truss) structures that recapitulate property gradients of tissues. We previously demonstrated a bi-layer scaffold infilled with biomimetic hydrogels that protected surrounding cartilage from degeneration in an *ex vivo* model [4]. Here, we sought to regenerate cartilage and bone tissue within appropriate regions of 3D-printed composite bi-layer scaffolds infilled with biomimetic hydrogels within OC defects in a porcine model.

**METHODS: Scaffold design and fabrication:** The design and fabrication of the mechanically-graded composite bilayer scaffold were described previously [6]. In-brief, a bilayer OC 3D-printed structure was designed by combining a micro-truss structure for the cartilage region and a vertical pillar-based design for the stiffer bone region, with elastic modulus of  $E = 0.51$  MPa and  $E = 6.5$  MPa, respectively (Fig. 1.a). Structures were fabricated using a custom DLP printer and a poly(ethylene glycol) diacrylate-based resin [4,5]. **Hydrogel:** Infilled around the structure was a degradable poly(ethylene glycol) hydrogel [5]. TGF- $\beta$ 3 and BMP-2 were tethered to cartilage layer and bone layer hydrogels, respectively, to encourage chondrogenesis and osteogenesis. **In vivo Study Design:** Four 6 mm diameter x 7 mm depth OC defects were made on the left femoral trochlear groove on 12-week-old Yorkshire pigs ( $n = 5$ ) that ranged from 30-35 kg in weight. Treatments (Fig. 1.b), including empty defect ( $n = 3$ ), osteochondral autograft transfer system (OATS) ( $n = 5$ ), cartilage mimetic gel only ( $n = 4$ ) and DLP 3D printed bi-layer scaffold with cartilage and bone mimetic hydrogel ( $n = 8$ ) were performed at random sites on the trochlear groove. All animal use was approved by the IACUC at Massachusetts General Hospital. **Imaging and micro-CT ( $\mu$ CT) analysis:** Following euthanasia 6 weeks post-surgery, each defect site was harvested and imaged for bone microarchitecture using  $\mu$ CT (SCANCO Medical AG) and subsequently processed for histology (H&E) (Fig. 1.c).  $\mu$ CT images were reconstructed in Dragonfly (2022.2) and evaluated for bone growth. 6 mm diameter x 4.5 mm depth region of interests (ROIs) represented the original bone region in the surgical defect were created for the empty, OATS and hydrogel-only groups. Due to observed implant subsidence, the ROIs for the composite scaffold group were created at the location of cartilage and bone scaffold layers (Fig. 1.d). Bone volume fraction (BV/TV) was calculated after segmentation using Otsu thresholding. Additionally, bone infiltration were measured at 25%, 50% and 75% z-plane of each ROI by averaging the distance of bone in-growth to the ROI boundary at x+, x-, y+, y- directions (aligned to the scaffold pores and pillar spaces) (Fig. 2.a).

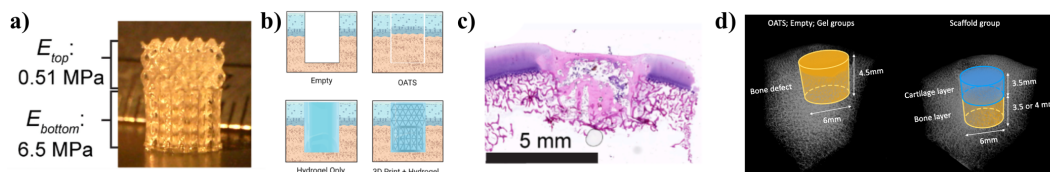
**RESULTS:** Structures were fabricated using DLP 3D-printing and the printing accuracy was established using  $\mu$ CT of dry and iodine-stained (for X-ray contrast) scaffolds [6]. Histology of the implant region (Fig. 1.c) following 6 weeks *in vivo* verified the location of each composite scaffold, with visible 'pillars' and new bone within the bone layer and fibrous cartilaginous tissue was observed in the cartilage layer of composite scaffolds.  $\mu$ CT images confirmed bone tissue surrounding the 3D-printed pillars in the bone layer with minor intrusion into the cartilage layer (Fig. 2.a). Bone tissue was also present in the empty defect and gel-only groups. BV/TV of the gel-only group and scaffold bone layer were significantly lower than those of the OATS group (Fig. 2.b). Bone infiltration measurements showed that bone penetrated much deeper into the OC scaffold bone layer than the cartilage layer. Bone was also present and increased with depth in defects filled with cartilage mimetic hydrogel (Fig. 2.c).

**DISCUSSION:** Our structure design supported loads at high (>5%) strains without buckling [6] and was far stiffer in the cartilage-interfacing region (0.51 MPa) than typical hydrogels. After 6 weeks *in vivo*, histological assessment showed fibrocartilage-like tissue within the articular cartilage region of each defect; mineralized bone tissue was only observed in the bony defect regions for all treatments, suggesting that our system promotes layer-specific tissue regeneration. As expected, OATS treatment showed higher BV/TV and osteointegration due to extant healthy bone tissue from the donor sites. Implant subsidence was observed for all scaffold (but not OATS) groups with mean displacement of 5.1 mm into the bone tissue, which was sufficiently deep for the scaffold cartilage layer to locate within the subchondral bone. This finding may explain why bone was also present in the scaffold cartilage layer. However, the use of cartilage mimetic hydrogel was associated with lower bone penetration into the scaffolds. Overall, our bilayer composite scaffold showed promising results where soft tissue largely formed in the cartilage region while promoting bone growth in the bone region in an *in vivo* large animal model. Further investigations are needed to address the implant subsidence and improve strategies to regenerate hyaline-like cartilage *in vivo*.

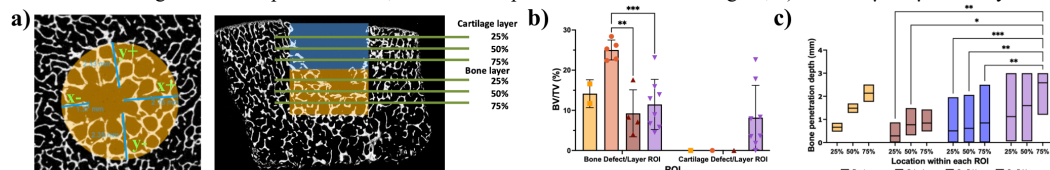
**SIGNIFICANCE/CLINICAL RELEVANCE:** This study addresses a critical need in OC tissue engineering using hydrogels that are stiffened by 3D-printed micro-trusses to deliver region-specific mechanical properties to support the surrounding native tissue while regenerate both cartilage and bone tissue. If successful, this strategy may improve long-term outcomes for OC regenerative treatments.

**REFERENCES:** [1] Smith *et al.*, *Nat Rev Rheumatol* **11** (2015) [2] Ahsan, T. & Sah, R. L., *Osteoarthritis Cartilage* **7** (1999) [3] Khan *et al.*, *Eur. Cell. Mater.* **16** (2008) [4] Uzcategui *et al. Adv. Eng. Mater.* **20** (2018) [5] Sarah *et al.*, *Biofabrication* **13** (2021) [6] Eckstein *et al.*, *SB3C2023-517* (2023)

**ACKNOWLEDGEMENTS:** Funded by NIH 1R01AR069060, NIH 4R33HD090696-03



**Figure 1:** a) Osteochondral 3D-printed structure, with regional stiffnesses; b) A schematic of four treatment groups of the in-vivo porcine model; c) Histology image of the scaffold treatment region show implant location, with visible 'pillar' features in bone region; d) ROIs of  $\mu$ CT  $\mu$ CT analysis.



**Figure 2:**  $\mu$ CT analysis of bone growth. a) A schematic of bone infiltration measurements into a bilayer composite scaffold; b) BV/TV calculation revealed bone forming in the osteochondral defect bone region of all treatments and in the cartilage layer of composite scaffolds; c) Bone infiltration was significantly higher in the bone layer of composite scaffolds.