

# Engineering Composite Tissues: Coupling Angiogenesis and Osteogenesis via Material and Chemical Signals

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**INTRODUCTION:** Tissue engineers have recognized for some time that the development of healthy vascularized bone is contingent on the activation of both endothelial cells and osteoblasts. To this end, many studies have examined the effects of independently targeting osteogenesis or angiogenesis using biomaterial systems to deliver VEGF or BMP2 to promote bone formation [1]. Deferoxamine (DFO), however, an FDA-approved small molecule and HIF-1 $\alpha$  stabilizer, couples osteogenesis and angiogenesis upstream of VEGF and BMP2 [2] and has been utilized extensively in fracture healing experiments [3]. The use of HIF-1 $\alpha$  stabilizers in tandem with other tissue engineering tools is limited, and no studies have combined these approaches for composite tissue engineered constructs that require spatially-controlled bone deposition. Here, we develop a drug delivery system that could be used to discretely deliver DFO directly before in vivo scaffold implantation without affecting chondrogenic maturation of an adjacent engineered disc construct. We then evaluate the ability for bone to form on osteoconductive hydroxyapatite-coated polycaprolactone (PCL) scaffolds with or without the delivery of deferoxamine.

**METHODS:** DFO-loaded microspheres were prepared using an oil-in-water double emulsion method [4]. Microspheres were imaged via SEM, and DFO encapsulation was evaluated using an iron chloride assay [5]. Sterile chitosan hydrogels were prepared as previously described [6]. Gel viscosity was assessed via timed inversion of 200  $\mu$ l hydrogel. To assess DFO release over 7 days, 0.25 mg DFO/ml or an equivalent mass of microspheres was dissolved/suspended in 3% chitosan + PBS or PBS alone and incubated at 37  $^{\circ}$ C with constant mechanical agitation. Salt-leached PCL scaffolds 16 mm in diameter and 2 mm in height were fabricated using PDMS molds with channel geometries, as previously described [4,7]. To characterize the handleability and retention of these microsphere-laden-hydrogels, the material (72.67 mg of microspheres in 1 ml of gel, yielding a deferoxamine dose of 400 $\mu$ M) was injected into the channels of hydrated scaffolds. Injected scaffolds were imaged immediately following injection using confocal microscopy and after 30 minutes of inversion using SEM. To assess the combined effect of scaffold implantation and drug delivery on new bone deposition, a rat subcutaneous model of semi-orthotopic bone formation was used [8]. Rings of bovine bone (10 mm diameter x 5 mm height) were harvested from the tibias of three 1 month old animals. Bone rings were divided into six experimental groups and either (1) left empty (negative control) or filled with (2) bone chips (positive control), (3) PCL scaffolds, (4) hydroxyapatite (HA)-coated PCL scaffolds, (5) PCL scaffolds + DFO delivery system, or (6) HA-coated PCL scaffolds + DFO delivery system. Scaffolds were acellular, measured 5 mm in diameter and 5 mm in height, and were HA-coated using an established biomimetic method [9]. Animals were euthanized 8 weeks post-implantation, at which point implants were removed, and utilized for  $\mu$ CT and histologic visualization (Mallory-heidenhain trichrome and RGB stain) of bone tissue deposition. Sections also underwent immunofluorescence using antibodies for HIF-1 $\alpha$ , VEGF, CD31 (PECAM-1), and CD66 (CEACAM) to visualize vessel formation.

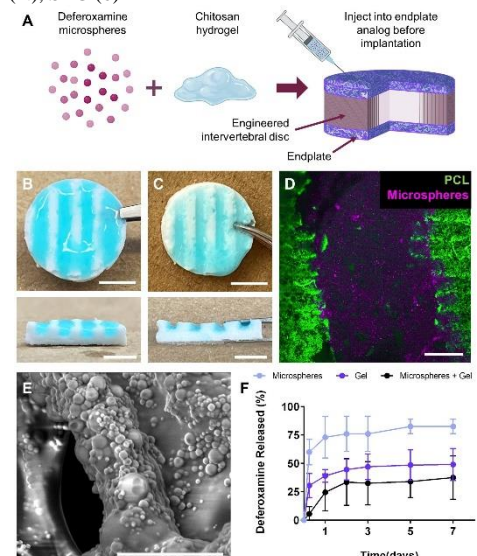
**RESULTS:** Average DFO encapsulation per microsphere batch was 0.313  $\pm$  0.154 mg DFO or 0.00344  $\pm$  0.00159 mg DFO encapsulated per mg of microspheres (n = 6). 98% of microspheres were 0.25 – 2.5  $\mu$ m in diameter. A 3% chitosan hydrogel was chosen for the injectable delivery of DFO microspheres due to its significant increase in viscosity when compared to lower wt% gels. Deferoxamine was successfully delivered to scaffolds in situ via injection of the microsphere-laden-hydrogel into the scaffolds' custom-molded channels and dispersed homogeneously (**Figure 1B&D**). The drug delivery system was retained in the scaffold even after 30 minutes of inversion (**Figure 1C&E**). Encapsulation of DFO-loaded microspheres in a 3% chitosan gel reduced the burst release of DFO over 7 days (**Figure 1F**). PCL and HA-Coated PCL scaffolds performed similarly in the subcutaneous semi-orthotopic bone formation model (**Figure 2**). No differences were observed when quantifying microCT bone volume/total volume (**Figure 2A**) or Mallory-Heidenhain staining area (**Figure 2B**). Representative histology showed similar levels of collagen deposition in red (**Figure 2C-D**) and unmineralized and mineralized bone in pink and blue-violet, respectively (**Figure 2E**). Negative controls showed no signs of mineralization and positive controls demonstrated robust bone deposition over 8 weeks. Analysis of angiogenic markers and of PCL + DFO and HA-Coated PCL + DFO experimental groups is ongoing.

**DISCUSSION:** This work demonstrates the ability to deliver deferoxamine discretely to a tissue-engineered construct before implantation. The microsphere-laden-hydrogel had excellent handleability and was retained in the scaffold after injection, even after inversion for 30 minutes. The use of a positively charged hydrogel not only facilitated microsphere injection and localization, but also further slowed the release of deferoxamine, which is negatively charged. In this study we have also utilized a recently proposed subcutaneous implantation model of semi-orthotopic bone formation to screen scaffold designs conducive to the formation of vascularized bone. Our data demonstrate that PCL and HA-coated PCL scaffolds performed similarly in this model, producing more collagenous and mineralizing matrix than negative controls. Previously, more bone formation was observed on HA-coated PCL scaffolds than PCL-only scaffolds at 15 weeks when implanted into the rat caudal disc space [9]. These differences may be due to the lack of mechanical loading in the subcutaneous space compared to the caudal disc space. Ongoing work will determine whether deferoxamine delivery will enhance bone and blood vessel formation. The optimal scaffold formulation will be employed in a large animal model to accelerate functional integration of a tissue engineered disc replacement.

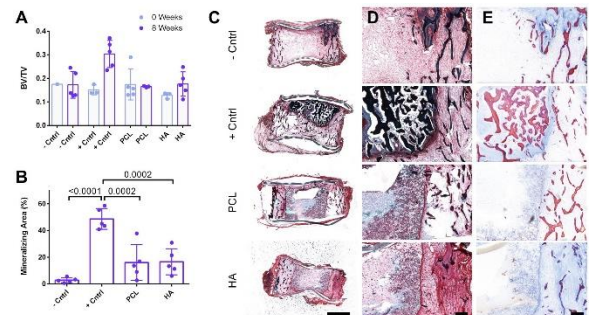
**SIGNIFICANCE:** Enabling the controlled delivery of cost-effective small molecules is critical to the success of many composite tissue engineered therapies, such as those that seek to promote discrete, regionalized vascularization, including a multi-compartment tissue-engineered total disc replacement.

**REFERENCES:** [1] Rather+ *Materials Science & Engineering C*, 2019. [2] Schipani+ *JBMR*, 2009. [3] Lang+ *Bone*, 2022. [5] Fainor+ *ORS 2023, Paper #1245*. [5] Jia+ *Society for Biomaterials*, 2016. [6] San Juan+ *IOP Conf. Ser.: Mater. Sci. Eng.*, 2012. [7] Kim+ *Acta Biomater.*, 2020. [8] Sastre+ *Biomaterials*, 2021. [9] Fainor+ *Cells Tissues Organs*, 2023.

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**Figure 1: Delivering Deferoxamine In Situ:** (A) Schematic of drug delivery system. Injected deferoxamine microsphere-laden-hydrogel (dyed blue) into hydrated PCL scaffolds at (B) 0 minutes and (C) 30 minutes post injection (scale = 5 mm). (D) Confocal image of microsphere distribution immediately following gel injection into scaffold channels (scale = 500  $\mu$ m). (E) SEM image of channels 30 minutes after injection with microsphere-laden hydrogels (scale = 15  $\mu$ m). (F) DFO release over 7 days (n = 2).



**Figure 2: In Vivo Subcutaneous Model.** After 8 weeks in vivo: (A) BV/TV of bone rings and their implants, (B) quantification of mineralizing bone area within implant from Mallory-heidenhain-stained sections, (C) macroscopic RGB stain (scale = 3 mm), and magnification (scale = 500  $\mu$ m) of (D) RGB stain and (E) Mallory-heidenhain stain at implant-bone ring boundary (Left: implant; Right: bone ring).