Surface Texture Alterations Change Mesenchymal Cell Proliferation on Additively Manufactured Zinc Implants

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INTRODUCTION: Advances in biomaterials and additive manufacturing (3D printing) are rapidly changing the way orthopedic implants are designed and implemented. Our lab has recently developed a method to 3D print medical grade Zinc, a biocompatible and biodegradable material, and a key micronutrient involved in human health. Zinc is a promising orthopedic biomaterial because it has been shown to stimulate the expression of a transcription factor related to the differentiation of stem cells to pre-osteoblast cells (precursor cells that become osteoblasts) [1]. Recent studies have shown that Zinc promotes osteoblast proliferation and increases mineralized matrix deposition via the cAMP-PKA-CREB signaling pathway [2]. Positive effects of Zinc on osteoblast activity occur over a defined dose range [3]. We believe that rates of zinc elution are related to the surface texture of an implant, which can be easily manipulated with laser additive manufacturing techniques. However, we still do not fully understand the relationships between Zinc implant surface texture and mesenchymal stem cell (MSC) behavior. In this bovine cell culture experiment, we sought to quantify survival of MSCs exposed to substrates of different textures and in the presence/absence of fibronectin. We hypothesized that, in comparison to smooth untreated surfaces, textured surfaces with a fibronectin coating result in increases in cell fixation and metabolic activity.

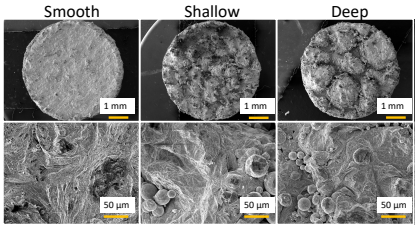
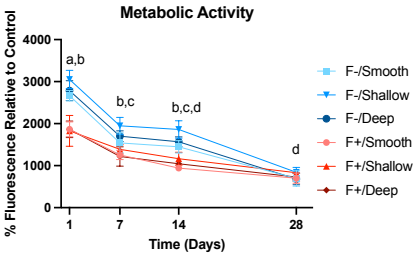


Figure 1: *Example SEM images for the three surface textures tested. Images shown are at 40x (top row) and 1000x (bottom row).*

METHODS: We manufactured 36 cylindrical test coupons (99.99% Zn, 5 mm diameter, 2.5 mm height **Fig 1**.) using laser powder bed fusion (LPBF) with 3 different textures: Smooth (as-built), Shallow, and Deep (n=6) that were seeded with cells with and without fibronectin coating (F+/-). The 3D printing parameters included: layer thickness = 0.06mm, laser power = 99W, speed = 350 mm/s, hatch spacing = 0.11 mm, and offset = 0.128 mm. Surface characterization of the as-built surface textures were conducted using scanning electron microscopy (TESCAN MIRA3). Images were taken at a working distance of 15mm, 10kV working voltage, and magnifications from 40x up to 5000x (**Fig 1**). For the cell culture model, zinc coupons were autoclaved and immersed in either sterile PBS (F- group) or 20 microgram/ml fibronectin solution (F+ group) for 12 hours before seeding. At Day 0, passage 2 bovine bone marrow derived MSCs were seeded at 6,666 cells/mm^2 on test coupons. Specimens were cultured using standard culture conditions (37°C, 5%CO2) on an orbital shaker. To characterize changes in metabolic activity, we performed Alamar Blue Assays (AB) using a 560-590 wavelength setting on a plate reader at day 1,7,14 and 28. One-Way ANOVA with pairwise comparisons were performed to determine significant differences between groups of different surfaces and groups F+/F- of same texture ($p < 0.05$). Two-Way ANOVA with pairwise comparisons were performed to determine significant differences and interactions caused by changes in surface texture and time within F+/- groups ($p < 0.05$).

RESULTS: *Day 1:* Bovine MSCs attached to coupons and were more metabolically active on F- groups overall (p=0.0002; **Fig. 2**). Overall, the Shallow/F- group had significantly increased metabolism in comparison to all groups except Deep/F-. *Day 7:* There was a significant decrease of cell metabolism in all groups, compared with day1 ($p=1x10^{-8}$). F+ groups experienced no changes in cell metabolism as a function of roughness. For F- groups, the Shallow/F- group had significantly increased metabolic activity in comparison to Smooth/F- and Deep/F- (p=0.003). *Day 10:* First visible deposits of biodegradation products were observed during media exchange and continued throughout the experiment. *Day 14:* There was a decrease of cell metabolism in the F+ groups, compared with Day 7 ($p=1x10^{-5}$). Within the F+ cohort, the Shallow/F+ group had significantly increased proliferation in comparison to Smooth/F+ (p=0.006). *Day 28:* There was a decrease of cell metabolism in both groups F+/F-, compared with day 14 ($p=3.2x10^{-8}$, $p=4.8x10^{-17}$). The shallow texture led to significantly increased attachment in comparison to smooth group only in F- group (p=0.04).



a = F- significantly higher than F+
b = F-/Shallow signficantly higher than F-/Smooth
c = F-/Shallow significantly higher than F-/Deep
d = F+/Shallow significantly higher than F+/Smooth

Fig 2: *Results from Alamar Blue testing, which showed high metabolic activity at Day 1, followed by a decline (day 7), plateau (day 14) and another decline (day 28).*

DISCUSSION: Additive manufacturing allows us to 3D print zinc with variable surface textures, which has a high potential for clinical utility in fracture repair. MSCs are known to readily proliferate and differentiate on various nanotextured surfaces [5], and the novel surface textures tested here also fostered cellular adhesion and proliferation. Interestingly, cells attached more readily directly to zinc in comparison to implants coated with fibronectin. At short time points, there existed a "Goldilocks zone" of surface texture, which increased metabolic activity, but it is unclear if this activity leads to improved bone healing efficiency. The biodegradation products observed at Day 10 were also found in a previous study involving zinc-based scaffolds and are likely composed of Zn, C, O, P, and Ca [4]. The influence of surface texture was insignificant by the 28-day timepoint. Since scaffolds were seeded with a relatively high density of cells, it remains unclear how a smaller cell population would proliferate on the different scaffold groups to drive differences by day 28. There was an interesting trend observed when addition of fibronectin not only decreased initial metabolic activity, but also led to no difference between F+ groups at Day 1. Interestingly, this phenomenon was no longer present by Day 7. It remains unclear if the short-term benefits of the Shallow surface texture can be further refined to have longer-lasting effects. Findings of this study are limited because the experiment uses a cell culture model with no mechanical loading. Ongoing and future research within our lab will extend this work into vivo models which would better characterize the responses for the human condition.

SIGNIFICANCE/CLINICAL RELEVANCE: This study represents initial findings that will help us better understand the relationships between 3D printed zinc surface textures and cellular behavior at an early stage of fracture repair. These results will be used to guide the design and development of next-generation 3D printed orthopedic implants created with zinc or zinc coatings.

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