

High-Throughput Fabrication and Analysis of 3D Printed Osteogenic Coral-containing Scaffolds

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INTRODUCTION: In 2021 the bone grafting market was worth €2.72 billion globally, demonstrating the clinical and societal demand for strategies to regenerate bone [1]. As allograft bone, the current grafting gold standard, has a limited supply and risk of disease transmission, the demand for synthetic bone grafting substitutes (BGS) continues to grow, while the use of allograft bone grafts is steadily decreasing [1]. Common synthetic BGS have low mechanical strength and bioactivity, inspiring the development of novel grafting materials. An alternative BGS, coral-derived scaffolds, are a natural, bioactive, high-strength, calcium carbonate bio-ceramic, supports osteogenesis in mesenchymal stem cells, the cells responsible for maintaining bone homeostasis and orchestrating fracture repair [2]. The objective of this study was to develop a novel BGS derived from sustainably grown coral which can be evaluated for its osteogenic properties in a high-throughput (HT) workflow.

METHODS: The HT printing protocol began with a sacrificial boundary (5x5x1 mm) of pluronic bioink in a square bottom 96-well plate (Ibidi) using a CELLINK BIO X printer with a pneumatic printhead. Immortalized bone marrow derived mesenchymal stem cells (iMSC) were incorporated into GelXA or GelXA-coral bioink and dispensed into the centre of the sacrificial boundary by droplet printing for 8 seconds and photocrosslinked for 20 seconds with near-UV LED (405 nm) [3]. The GelXA bioinks utilized here were comprised of gelatin methacryloyl, xanthan gum and alginate. The iMSCs were lentivirally modified to incorporate an osteogenic marker gene promoter-driven fluorescence reporter construct. The resultant cell-bioink constructs were cultured in control or osteogenic differentiation media for 6 weeks. They were non-destructively monitored throughout for cell proliferation and viability with Biotracker NIR694 nuclear dye followed by infrared microscopy on the Operetta High Content Imaging System. The reporter constructs were of a discrete excitation/emission spectrum to the nuclear dye, allowing them to be simultaneously imaged. To further quantify osteogenic differentiation in a non-destructive manner, ALP presence in the conditioned media was monitored weekly by combination with p-nitrophenyl phosphate and spectrophotometry at 405 nm. Endpoint, destructive assays used included quantitative real time polymerase chain reaction for markers of osteogenesis (qRT-PCR), scanning electron microscopy (SEM) imaging and histological staining (Hematoxylin and Eosin (H&E), Von Kossa, Mallory's Trichrome). All quantitative results had at least n = 3 samples, imaging had n = 1. Statistical significance was determined by a t-test or, for more than 2 conditions, a one-way analysis of variance. Significance was considered as p-value of ≤0.05, and statistical analysis of data was performed using Prism 8.0 software.

RESULTS: The described printing methodology allows for one sample to be printed in 60 seconds including print time of the sacrificial boarder, cell-containing bioink and photo-crosslinking. This process resulted in porous (Fig. 1), reproducible printed scaffolds with a weight of 21.52 ± 0.38 mg (n = 5) for GelXA ink and 21.36 ± 0.65 mg (n = 5) for GelXA-coral. The time to image each scaffold in one channel is less than 60 seconds, therefore, a full 96-well plate can be imaged in approximately 1.5 hours fulfilling both a non-destructive and HT workflow criteria. Despite the inclusion of coral particles, the individual cell/cell nuclei can still be clearly imaged and quantified without interference with the coral particles and with homogenous cell distribution achieved (Fig. 2). The reporter iMSCs were validated to fluoresce in conjunction with gene transcription. By all measures, iMSCs in the undifferentiated control media did not show evidence of osteogenic differentiation. In contrast, osteogenic differentiation of iMSCs in both GelXA and GelXA-coral bioinks resulted in increased ALP secretion, increased transcription of marker genes and reporter fluorescence as compared to the undifferentiated control in both bioink formulations. Histologic analysis of osteogenically differentiated constructs similarly indicated an increase in collagen content, as indicated by H&E and Mallory's Trichrome, and calcium deposition, as visualized by Von Kossa staining, in the extracellular matrix. When comparing the osteogenic differentiation of iMSCs in GelXA vs. GelXA-coral bioinks, all markers of osteogenesis were enhanced through the inclusion of coral particles. Furthermore, SEM imaging showed the iMSC's ability to bind and flatten on the coral particles, surrounded by a rich extracellular matrix.

DISCUSSION: Sustainably grown coral was successfully incorporated into bioinks, reproducibly 3D printed, non-destructively monitored throughout cell culture and induced osteogenic differentiation in iMSCs. Despite the use of HT and non-destructive techniques, one limitation is the long-time frame (6 weeks) required to see a substantial differentiation effect. To increase the efficient nature of the technique we aim to incorporate and validate earlier markers of osteogenic differentiation, correlating their reliability with osteogenesis at the 6 week time point. Despite the lengthy assay, the HT fabrication and monitoring workflow offers a more efficient, lower cost and less labor-intensive screening system for the translation of novel BGS to clinic.

SIGNIFICANCE: Demonstrated osteogenic potential of sustainably grown coral offers significant time- and cost-saving for orthopaedic product developers. Besides, the methods developed allow for the rapid screening of new scaffold formulations, enhancing *in vitro* screening methods which can circumvent expensive, elongated and ethically challenging animal testing, through predictive screening. This will revolutionise orthopaedic material development and increase BGS range and availability in the coming decade.

REFERENCES: [1]: Grand View Research, "Bone Grafts and Substitutes Market Size". Available: <https://www.grandviewresearch.com/industry-analysis/bone-grafts-substitutes-market>. Accessed: 20-03-2023. [2]: Sheehy, Eamon J., et al. "The Incorporation of Marine Coral Microparticles into Collagen-based Scaffolds Promotes Osteogenesis of Human Mesenchymal Stromal Cells via Calcium Ion Signalling." *Marine Drugs* 18.2 (2020). [3]: Skårn, Magne et al. "Generation and characterization of an immortalized human mesenchymal stromal cell line." *Stem cells and development* vol. 23,19 (2014): 2377-89.

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IMAGES:

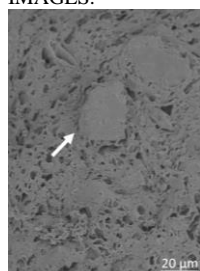


Fig 1. Acellular GelXA-coral hydrogel scaffold. Arrow shows a coral granule.

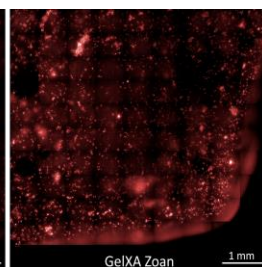
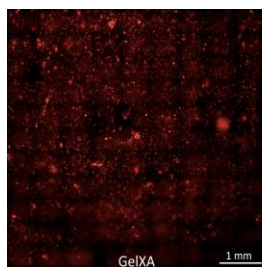


Fig 2. Cell nuclei stained with Biotracker NIR694. Image composed of 99 regions of interest and 8 planes projected into one. Z height is 112 μm.

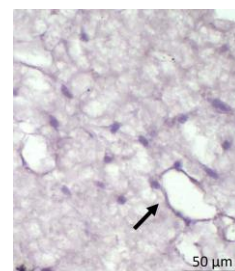


Fig 3. H & E staining of GelXA-coral. Arrow shows cells wrapping around a coral granule.