Scaffold Synergy: Boosting Bone Healing with a Dynamic Duo of siRNA and Roxadustat

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INTRODUCTION

While the field of orthopedic surgery has made great advances in bone regeneration over the last decade, critical size defects, defined as defects exceeding 2-2.5 times the diameter of the affected bone, remain a major clinical challenge due to their inability to heal naturally. Current clinical treatments, including autografts, allografts, and synthetic implants, have notable limitations. Autografts, often harvested from the iliac crest, are considered the "gold standard" due to their osteogenic, osteoconductive, and osteoinductive properties while minimizing immune response. However, this type of graph often requires a secondary surgical site which can increase the risk of infection, nerve damage, and limits graft availability. Allographs. sourced from cadaveric donors, can overcome the quantity issue but carry risks of immune rejection and disease transmission, contributing to long-term failure rates as high as 50%(1). Polymeric synthetic implants have advanced in promoting osseointegration. However, challenges remain in achieving adequate vascularization and matching the mechanical properties of native bone tissue.

This study aims to bridge this gap by creating a multifunctional scaffold that couples osteogenic and angiogenic cues with the mechanical strength needed in load bearing applications to promote robust bone regeneration. To achieve this, a composite scaffold composed of poly(propylene fumarate) (PPF) and poly(caprolactone fumarate) (PCLF) encompassed by a gelatin methacrylate (GelMA) hydrogel developed. This hybrid design leverages the mechanical strength and biocompatibility of the polymeric scaffold together with the cell-friendly microenvironment and delivery potential of the GelMA hydrogel. The hydrogel serves as a carrier for both the small interfering RNA (siRNA) targeting Noggin, a negative regulator of the BMP-2 signaling pathway, and the angiogenic small molecule drug Roxadustat. The inhibition of Noggin is expected to enhance osteogenic signaling and upregulate markers such as alkaline phosphatase (ALP) activity and calcium deposition, while Roxadustat promotes vascularization within the defect site. Together, these mechanisms aim to accelerate bone regeneration within critical sized defects.

METHODS

Scaffold Preparation: PPF/PCLF scaffolds were fabricated by 3D digital light printing (DLP) using a poly(propylene fumarate) (PPF) and poly(caprolactone fumarate (PCLF) resin (10% w/w PCLF: PFF) dissolved in diethyl fumarate. Cylindrical scaffolds (4 mm height x 5 mm diameter) with 70% porosity and 1 mm pore size (as determined in previous work) were printed and mechanically tested under compression at a rate of 3mm/min using a 2,000 lb load cell (N = 5). Scaffolds were then cleaned, sterilized, and encapsulated in a hydrogel composed of 10% (w/w) gelatin methacrylate (GelMA) and 5% (w/w) poly(ethylene glycol) diacrylate (PEGDA) in Dulbecco's modified eagle medium (DMEM) containing 0.05% lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) as the photoinitiator. The hydrogel was crosslinked under UV light for 60 seconds to form the final PPF/PCFL-GelMA composite scaffold.

SiRNA Osteogenic Differentiation: To establish an effective siRNA concentration, a concentration screening was performed using rat bone marrow stem cells (rBMSCs) and assessed using live/dead viability assay (Invitrogen) and RT-qPCR. GelMA hydrogels loaded with siRNA (2,500 ng RNA/mL or 5,000 ng RNA/mL were submerged in phosphate buffered saline (PBS) to determine release kinetic with supernatants collected at 30 min, 1 h, 2 h, 4 h, and days 1, 3, and 7. SiRNA concentration in each sample was quantified using UV spectrophotometry. For *in vitro* functionality testing, composite scaffolds were either statically seeded with rBMSCs or the cells were encapsulated within the hydrogel. Three treatment groups were used: (1) siNoggin RNA, (2) scrambled siRNA, (3) unloaded control. Samples were collected on days 1, 3, 7, 14, 21 for biochemical assays (N = 5), RT-qPCR (N = 4), live/dead viability assay (N = 2), and histological staining (N = 2). To obtain a baseline, a 2D system was established to differentiate the siRNA effects on rBMSCs from scaffold structure. The 2D *in vitro* experimentation was performed by using rBMSCs seeded on standard tissue culture plates and were treated with the same three siRNA groups as stated above. Cells were collected on days 1, 3, 7, 14, 21 for cellular assays (N = 5), RT-qPCR (N = 4), live/dead viability assay (N = 2), and histological staining (N = 2)

Roxadustat Angiogenic Studies: Roxadustat working concentrations (0 µm, 20 µm, 50 µm, 100 µm) were screened using monolayer human umbilical vein endothelial cells (HUVECs) viability and CD31 immunofluorescent staining. Drug release kinetics were determined by immersing Roxadustat loaded GelMA hydrogels in PBS and collecting supernatants at 30 min, 1 h, 2 h, 4 h, and days 1, 3, and 7, and quantifying release via Nanodrop spectrophotometry. For *in vitro* angiogenic evaluation, HUVECs were cultured on Matrigel (Corning) and treated with conditioned media from Roxadustat loaded GelMA hydrogels. Cells were analyzed on days 1, 3, 7, 14, and 21 for RT-qPCR (N = 4), live/dead viability assay (N = 4), or immunofluorescent staining (N = 2).

Statistical Analysis: Statistical analyses were performed using R-Studio. For comparison between multiple groups, the Kruskal-Wallis test was used, followed by Dunn's post hoc test with Bonferroni adjusted p-values applied where applicable. A p-value of < 0.05 was considered statistically significant.

RESULTS SECTION

PPF/PCLF scaffolds were successfully fabricated using DLP and yielded uniform cylindrical structures with 70% porosity and 1 mm pore size. Mechanical compression testing confirmed reproducible modulus values within the range of trabecular bone. GelMA hydrogel coatings were uniformly crosslinked around the polymer scaffolds for stable composite constructs.

A working siRNA concentration of 2,500 ng RNA per mL of hydrogel was established from concentration screening studies and used in subsequent experiments. RT-qPCR analysis confirmed Noggin gene silencing by day 3 in both encapsulated composite scaffolds and 2D monolayer rBMSC cultures. The magnitude of Noggin inhibition was greater in the 3D encapsulated scaffold compared to that of monolayer cultures. Early Roxadustat release data suggests a biphasic release pattern with an initial burst followed by sustained release. Live/dead viability assays confirmed high rBMSC viability (>90%) across all rBMSC experimental groups, indicating high cytocompatibility of both the polymeric scaffold and the hydrogel components. Phalloidin staining of the monolayer cultures revealed good cell spreading and attachment. Preliminary Alizarin Red S staining demonstrated increased calcium deposition in the siNoggin transfected rBMSCs compared to both the scrambled siRNA and the control groups.

DISCUSSION

The reproducible fabrication, trabecular range stiffness, and stable GelMA encapsulation confirm the suitability of the PPF/PCLF-GelMA scaffold as a dual-delivery platform for bone regeneration. Enhanced Noggin inhibition in the 3D scaffolds compared to 2D cultures suggests improved siRNA retention and uptake within the hydrogel environment supporting the advantage of a 3D delivery system. Preliminary increases in calcium deposition following siNoggin treatment indicate increased activation of osteogenic pathways and confirms siRNA bioactivity. However, these findings are limited by the early *in vitro* nature of the data, *in vivo* validation will be essential in confirming therapeutic efficacy and vascularized bone formation under physiological conditions.

SIGNIFICANCE/CLINICAL RELEVANCE

This composite scaffold integrates mechanical support with targeted molecular therapy to address the dual challenges of poor osteogenesis and limited vascularization in critical-sized bone defects. Localized siNoggin delivery promotes BMP-2 mediated bone formation, while controlled Roxadustat release enhances angiogenesis. Together, these mechanisms offer a translationally relevant approach for improving bone healing outcomes and reducing complications associated with current grafting techniques.

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REFERENCES

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