Using Chondrocyte Redifferentiation as a Tool to Develop Insoluble Type II Collagen Microfiber Scaffolds for Articular Cartilage Regeneration

Shuanhu Zhou, Kevin Capariño, Patrick LeMarble, and Shu-Tung Li*
Laboratory for Tissue Engineering Research, The Shu-Tung and Alice Li Foundation, Oakland, NJ 07436, USA. *sli@thelifoundation.org

DISCLOSURES: All authors have no conflicts to disclose related to this research project.

INTRODUCTION: Articular cartilage damage leads to joint dysfunction and significant patient morbidity. Regenerative medicine strategies utilizing biomimetic scaffolds have gained traction as potential solutions for cartilage repair. Type II collagen (Coll II) is the primary structural protein in cartilage, providing biomechanical strength while supporting the maintenance of the chondrocyte phenotype [1,2]. Although the natural Coll II in cartilage exists as insoluble fibers, most Coll II scaffolds or hydrogels used in cartilage tissue engineering are fabricated from soluble Coll II, partly due to the challenges associated with using purified insoluble Coll II from cartilage to fabricate scaffolds [3]. While the advantages of soluble Coll II scaffolds over soluble type I collagen (Coll I) or synthetic biomaterials in maintaining the chondrocyte phenotype and promoting chondrogenic differentiation are supported, conflicting evidence also exists [4]. In this study, we used chondrocyte redifferentiation as a tool to test the hypothesis that insoluble Coll II microfiber scaffolds have greater potential than insoluble Coll I scaffolds for cartilage tissue engineering. Scaffold pore size is a critical factor influencing tissue regeneration efficiency [5]. To accurately compare the biomaterial differences between insoluble Coll I and II microfiber scaffolds, we developed a method to fabricate both scaffold types with a similar pore distribution and size. Our findings provide valuable insights into the design criteria for insoluble Coll II microfiber scaffolds and may offer a novel approach for improving cartilage repair strategies.

METHODS: Insoluble Coll II microfibers were purified from bovine articular cartilage, and insoluble Coll I microfibers were isolated from bovine tendons using methods modified from our previously described protocols [6,7]. The quality of purified collagen II microfibers was assessed by analyzing hydroxyproline content, DNA quantification, uronic acid levels, SDS-PAGE, and amino acid composition. Coll I and original Coll II microfiber (Coll II-O) inks were prepared using microfibers hydrated in sodium acetate buffer and centrifuged to achieve a 10% (w/v) collagen content. To generate Coll II microfiber scaffolds with a comparable pore size to Coll I, insoluble Coll II microfibers were treated with pepsin at 4 °C for one hour (Coll II-E). The collagen microfiber inks were molded into cylinders by injecting them into 96-well plates, followed by freeze-drying, crosslinking with EDC/NHS, and sterilization with ethylene oxide before cell culture. Primary chondrocytes were isolated from the articular cartilage of bovine calves. Dedifferentiated bovine chondrocytes (P1) were obtained through monolayer expansion of primary chondrocytes in DMEM supplemented with 10% FBS. Chondrocytes were seeded into scaffolds at a density of 2×10^6 cells per scaffold. After 3-6 weeks in chondrocyte differentiation medium under either static or dynamic conditions in a hypoxic environment (5% O_2), the cell-laden scaffolds were harvested for molecular, biochemical, and histological analyses.

RESULTS: Scanning electron microscopy and cryosection were used to evaluate the microstructural features of collagen scaffolds and showed obvious differences in the cross-sectional microarchitecture, pore distribution, and average pore size between Coll I and Coll II-O scaffolds (Fig. 1; ***$p < 0.001$; scale bar: 100 μm). The enzyme-treated Coll II-E scaffolds have a similar microstructure, pore distribution, pore size, and porosity to Coll I scaffolds (Fig. 1; NS: no significant). The limited pepsin treatment did not change the mechanical compressive and thermal profiles of collagen II scaffolds (data not shown). Toluidine blue staining for glycosaminoglycans (GAGs) and qRT-PCR analysis of the chondrogenic marker collagen II gene (*Col2a1*) demonstrated greater chondrocyte redifferentiation of bovine chondrocytes in Coll II-E scaffolds under dynamic compared to static culture conditions (data not shown). Under dynamic conditions, there were more intense GAGs staining observed in Coll II-E scaffolds than in Coll I scaffolds at all time points (Fig. 2A; scale bar: 100 μm). Quantification of the GAGs/DNA ratio revealed significantly higher matrix production in Coll II-E scaffolds compared to Coll I scaffolds (Fig. 2B; *$p < 0.05$, **$p < 0.01$). Gene expression analysis showed that after 4 weeks, chondrocytes in Coll II-E scaffolds expressed higher levels of *Col2a1*, resulting in a significantly increased *Col2a1/Col1a1* ratio compared to those cultured in Coll I scaffolds (Fig. 2C; *$p < 0.05$; ***$p < 0.001$).

DISCUSSION: The pore size and mechanical properties of scaffolds are critical factors influencing the efficiency of cartilage regeneration [5, 8]. Despite the technical challenges associated with insoluble Coll II scaffolds, our fabrication method, utilizing limited pepsin treatment, successfully generated insoluble Coll II scaffolds with pore sizes and mechanical properties comparable to those of insoluble Coll I scaffolds. This equivalence allowed for a direct comparison of the intrinsic bioactivity of the two insoluble collagen types. Quantitative GAGs/DNA analysis, histological GAG staining, and gene expression profiling consistently demonstrated superior extracellular matrix production and enhanced chondrocyte redifferentiation in the Coll II scaffolds, which underscores the material's strong capacity to support the reestablishment of cartilage-specific phenotypes of dedifferentiated chondrocytes, an ongoing challenge in cartilage tissue engineering. To our knowledge, this is the first study to demonstrate that insoluble Coll II microfiber scaffolds are more favorable for *in vitro* chondrocyte redifferentiation than insoluble Coll I scaffolds.

SIGNIFICANCE/CLINICAL RELEVANCE: This work highlights the feasibility and therapeutic potential of insoluble type II collagen microfiber scaffolds as a physiologically relevant biomaterial that may serve as a foundation for developing next-generation biomimetic scaffolds to promote cartilage regeneration.

REFERENCES: [1] Pueyo Moliner A, *et al*. Nat Rev Rheumatol. 2025;21:291-308. [2] Bielajew BJ, *et al*. Nat Rev Mater. 2020;5:730-747. [3] Pieper JS, *et al*. Biomaterials. 2002;23:3183-92. [4] Hu X. *et al*. Commun Mater, 2024;5:149. [5] Zhang Q, *et al*. Acta Biomater. 2014;10:2005-13. [6] Li S-T, *et al*. US Patent 6391333B1. [7] Li S-T, *et al*. US Patent 12173048B2. [8] Koh YG, *et al*. J Tissue Eng. 2019;10:2041731419832133.

ACKNOWLEDGEMENTS: This work was supported by the Shu-Tung and Alice Li Foundation.

Fig. 1

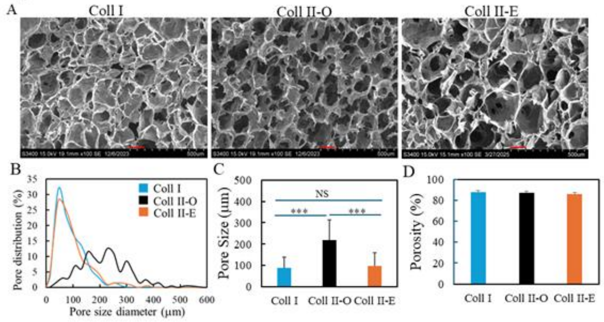

Fig. 2