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3D Printed Biofunctionalized Scaffolds for Microfracture Repair of Cartilage Defects

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Abstract

While articular cartilage defects affect millions of people worldwide from adolescents to adults, the repair articular cartilage defects still remains challenging due to the limited endogenous regeneration of the tissue and poor integration with implantations. In this study, we developed a 3D-printed scaffold functionalized with aggrecan that supports the cellular fraction of bone marrow released from microfracture, a widely used clinical procedure, and demonstrated tremendous improvement of regenerated cartilage tissue quality and joint function in a lapine model. Optical coherence tomography (OCT) revealed doubled thickness of the regenerated cartilage tissue in the group treated with our aggrecan functionalized scaffold compared to standard microfracture treatment. H&E staining showed 366 ± 95 chondrocytes present in the unit area of cartilage layer with the support of bioactive scaffold, while conventional microfracture group showed only 112 ± 26 chondrocytes. The expression of type II collagen appeared almost 10 times higher with our approach compared to normal microfracture, indicating the potential to overcome the fibro-cartilage formation associated with current microfracture approach. The therapeutic effect was also evaluated at joint function level. The mobility was evaluated using a modified Basso, Beattie and Bresnahan (BBB) scale. While the defect control group showed no movement improvement over the course of study, all experimental groups showed a trend of increasing scores over time. The present work developed an effective method to regenerate critical

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Disclosure Statement

We have filed an invention disclosure for the 3D printed lid used in the centrifugation assay.

articular defects by combining a 3D-printed therapeutic scaffold with the microfracture surgical procedure. This biofunctionalized acellular scaffold has great potential to be applied as a supplement for traditional microfracture to improve the quality of cartilage regeneration in a cost and labor effective way.

Keywords

aggrecan; scaffold; extrusion 3D printing; microfracture; articular cartilage; Poly(L-Lactide-co-ε-Caprolactone); custom fabrication

Introduction

As of 2016, approximately 14 million Americans experienced symptomatic knee osteoarthritis, [1] demonstrating a huge need for the development of translational clinical therapies. However, little success has been gained to achieve a long term success. Microfracture and Autologous Chondrocyte Implantation (ACI) are the most common surgical treatments for cartilage defects. The microfracture surgery entails drilling small holes into the subchondral bone underlying a cartilage defect to trigger the release of mesenchymal stem cells (MSCs) from the bone marrow which differentiate into chondrocytes to regenerate articular cartilage tissue.[2] However, the post-surgical microenvironment fails to orient and guide the MSCs properly, resulting in comparatively weak fibrous tissue relative to native cartilage.[3] ACI is typically recommended for defects larger than 2 cm² and requires extensive recovery time after the surgery. Additionally, it remains difficult to harvest and cultivate a large enough chondrocyte population to transplant due to the low proliferation rate and differentiation potential during *in vitro* culture.[4, 5] The recently FDA approved product Matrix-Induced Autologous Chondrocyte Implantation (MACI) showed improved treatment results.[6] However, MACI displayed the same drawbacks as ACI, including the dual-surgery procedures and less abundant cell source.

Articular cartilage has is composed exclusively of chondrocytes surrounded by dense extracellular matrix (ECM) composed of water, collagen (primarily type II), and proteoglycans.[7]. The banded structure forms 3 zones: superficial, transitional, and deep corresponding to the cartilage depth. As the ECM constitutes more than 90% of the dry weight of articular cartilage, it is essential to understand and restore the ECM function in cartilage tissue engineering. Proteoglycans play an essential role in the ECM as the overall negative charge retains water molecules; the resulting swelling pressure allows the tissue to resist compressive force.[8] The most abundant proteoglycan within the ECM of articular cartilage is aggrecan, structured as a core protein decorated with several types of sulfated glycosaminoglycans (GAGs), including chondroitin sulfate and keratan sulfate. Multiple aggrecan monomers self-assemble into large molecules through covalent bonds with hyaluronic acid, forming large, biologically active aggregated structures. [9, 10]

Tissue engineering strategies actively pursued to regenerate biomimetic cartilage tissue, such as utilizing various concentrations of collagen type II in a 3D construct. [11] Others have attempted to isolate chondrocytes from the different cartilage zones and seed them onto 3D scaffolds in an effort to replicate the banded structure in native articular cartilage.[12]

However, besides the limited biological complexity involved, extensive preparation and characterization are required for these approaches. Biologically active, acellular scaffolds remain an attractive alternative to cell-laden constructs considering the potential manufacturing and preservation as a clinical product. 3D printing technology has used to manufacture patient-specific scaffolds with custom shapes and consistent quality.[13] To enhance the cartilage healing process, an acellular 3D printed scaffold could be combined with microfracture procedures without a second surgery.[14, 15]

The goal of this study is to improve the quality of regenerated cartilage tissue during microfracture by developing a 3D-printed scaffold functionalized with aggrecan. As the main proteoglycan component of native articular cartilage tissue, aggrecan provides binding sites to recruit stem cells and aggregates growth factors to the scaffold surface, further promoting cell attachment. The physiological environment for the MSCs released from microfracture would be enhanced by the water and biological components trapped by the aggrecan, mimicking the cartilage microenvironment. The first aim of the study was to design and verify the scaffold surface modification with aggrecan. The bone marrow and attached cell population's interaction with the scaffold were analyzed using a customized centrifugation assay. After *in vitro* evaluation, we tested the scaffold functionality in a lapine model, comparing the standard microfracture, with non-functionalized and functionalized scaffolds. The success of this treatment, which combines acellular bioactive scaffold and microfracture, provides an effective way to improve the cartilage tissue repair outcome without excessive labor or complicated procedures.

Methods

Scaffold Fabrication

Poly(L-lactide-co-caprolactone) (PLCL, PolySciTech, West Lafayette, IN) with a lactic acid:caprolactone ratio of 70:30 and molecular weight of 45kD – 55kD was combined with 15% (w/w) amine end capped poly(lactic-co-glycolic acid) (amine-PLGA, PolySciTech) to make a PLCL-amine scaffold material by grinding the polymers together to form a homogeneous mixture. Scaffolds were fabricated using the 3D Bioplotter (EnvisionTEC, Gladbeck, Germany) using direct melt extrusion technique.[16] The scaffold inner pattern was programmed using the provided EnvisionTEC software. Material was loaded in the printing cartridge and melted at 155°C for extrusion at 6.5 bar with an average speed of 4 mm/s using a 0.2 mm inner diameter stainless steel needle. Scaffolds had dimensions of 3 mm (length) x 3 mm (width) x 0.5 mm (height) (Figure 1C). Particularly, in the bone marrow seeding study, scaffolds were fabricated with a disk shape and a diameter of 7 mm to maximize the covered area in the well plate.

Aggrecan Functionalization of Scaffolds through Covalent Bonding

Aggrecan was covalently bound to the printed scaffolds. 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC, Thermo Fisher Scientific Waltham, MA) and N-hydroxysulfosuccinimide (sulfo-NHS, Thermo Fisher Scientific) were used to link the carboxyl group on aggrecan molecules to the amine group on the printed PLCL scaffolds. PLCL-amine scaffolds were soaked in 0.1M 2-[morpholino]ethanesulfonic acid buffer

(MES, Sigma-Aldrich, St. Louis, MO) buffer for 15 minutes prior to aggrecan functionalization. For each scaffold, a functionalization solution was composed of 0.5 mg lyophilized bovine aggrecan (Sigma-Aldrich), 1 mL MES buffer, 1.6 mg EDC, and 4.4 mg sulfo-NHS and allowed to react for 15 minutes based on the EDC-NHS reaction manufacturer protocol. Scaffolds were placed in the functionalization solution and placed on the shaker at 50 rpm to react for 2 hours. Scaffolds were washed with phosphate-buffered saline (PBS, Sigma-Aldrich).

Nuclear Magnetic Resonance Spectroscopy

Bruker Advance III 600 NMR spectrometer with BBFO gradient probe was used for the ^1H NMR experiments. Polymer samples were dissolved in Dimethyl Sulfoxide- d_6 (CD_3SOCD_3). The internal solvent signal δ (CD_3SOCD_3) = 2.5 ppm was used as reference. Aggrecan was dissolved in D_2O at 1mg/ml. Printed scaffold functionalized with aggrecan (PLCL-aggrecan) was dissolved in CD_3SOCD_3 / deuterium oxide (D_2O) at 2:1 ratio. Solvent suppression pulse sequence was used to suppress solvent ^1H signal for Aggrecan and PLCL-aggrecan samples to achieve the optimum sample signals. A total of 64, and 128 scans were used for ^1H , solvent suppression experiments, respectively.

Compressive Mechanical Testing

We performed mechanical testing on the printed PLCL-amine and aggrecan functionalized PLCL-amine scaffolds (N=6 for each group) using an Instron mechanical testing system (33R/4465) (Norwood, MA). The scaffolds were wet in PBS for 24 hours before the compressive mechanical properties were evaluated. All samples were compressed by a 50N load cell at a displacement rate of 0.5 mm/min with a pre-load of approximately 0.05N until the machine protection distance was reached. For every 10 ms, compressive stress and strain were recorded and calculated based on the original cross-sectional area and height of the wet scaffold. The compressive modulus was then determined by definition, which is the slope of the linear region of the stress-strain curve before failure.

Cell Culture and Seeding

Human MSCs (hMSCs, RoosterBio, Frederick, MD) were expanded in RoosterNourish high performance MSC media (RoosterBio) according to the manufacturer's recommendations. After reaching the recommended density, cells were lifted with trypsin-EDTA (Gibco-LifeSciences, Gaithersburg, MD) and centrifuged at 200xg for 10 minutes to form a cell pellet. Cells were re-suspended in RoosterNourish media at a concentration of 10 million cells/mL. Approximately 1 million cells were seeded onto each scaffold in 100 μL of to cover the scaffold. Scaffolds were kept at 37 $^\circ\text{C}$ for 4 hours to allow for attachment to the scaffold surface with culture media changed every other day.

DNA Quantification

hMSCs were lifted from scaffolds using trypsin-EDTA (Gibco) and centrifuged into a cell pellet. DNA was isolated from hMSCs using a DNeasy Plus MiniKit (Qiagen, Frederick, MD). DNA was quantified using a PicoGreen dsDNA Assay Kit (Quanti-iT, ThermoFisher

Scientific, Waltham, MA). DNA isolates from the DNeasy kit were compared to standard curve to quantify DNA.

Centrifugation Device Fabrication

A lid was designed in SolidWorks (Dassault Systèmes, Vélizy-Villacoublay, France) for a slight press fit into the inner wells of a standard 24 well plate, allowing the assembly to be flipped upside down and centrifuged. The designed device was intended to capture the scaffolds and cell solution during centrifugation. The lid was fabricated using an EnvisionTEC Perfactory 4 Mini Multilens (Gladbeck, Germany) using E-Shell 300 (EnvisionTEC, Inc., Dearborn, MI). Excess resin was removed by submerging printed objects in 99% isopropanol (Pharmco-Aaper, Shelbyville, KY) for 5 minutes and blown dry with air. Complete resin curing was achieved with 1000 flashes of broad spectrum light (Otoflash, envisionTEC, Inc., Dearborn, MI). Scaffolds were cleaned in 100% ethanol (Pharmco-Aaper, Shelbyville, KY) for >30 minutes to leach any remaining soluble contaminants. Scaffolds were sterilized under 100% ethanol and UV light for 20 minutes. Scaffolds were rehydrated in a serial rehydration to pH 7.4 sterile PBS in 4 steps (75:25, 50:50, 25:75, and 0:100 ethanol:PBS), with each intermediary step allowed to soak for 5 minutes.

Bone Marrow Seeding

Whole human unprocessed bone marrow (Lonza, Walkersville, MD) was seeded onto the printed scaffolds placed in ultra-low attachment 24-well plate (ThermoFisher). Equal amount of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, MD) supplemented with 1.0% v/v penicillin/streptomycin (Life Technologies) and 0.1 mM nonessential amino acids (Life Technologies) was added to each well. The plate was incubated at 37°C, 5% CO₂, and 80% humidity for 24 hours to allow attachment.

Centrifugation Assay

The centrifugation assay set up is shown in Figure 2A. The concept and determination of the centrifugal force were based on previously established protocol.[17] The 3D printed cover was applied to seal the wells of the 24-well plate in place of the original lid. The plates were centrifuged upside down at a relative centrifugal force of 50 g for 5 min at 22 °C to separate the adhered and non-adhered cells. After first centrifugation, the supernatant containing the non-adhered fraction was collected and enumerated. Trypsin-EDTA (Gibco) was added, centrifuged again to harvest, and scaffolds and wells washed once with PBS to lift the adhered fraction.

Evaluation of hMSCs Isolated from Whole Bone Marrow

Flow cytometry was conducted to analyze the cell population isolated from the bone marrow. The cells were re-suspended at a concentration of approximately 5 million/mL. Cells and controls were stained using Human MSC Analysis Kit (BD Stemflow; BD, NJ) following the manufacturer's instruction. The data was analyzed using FlowJo (FlowJo LLC, Ashland, Oregon) to gate the populations. Briefly, 100 µL of each sample was mixed with 20 µL of the hMSC Positive Cocktail (CD90 FITC, CD73 APC, and CD105 PerCP-

Cy5.5) and 20 μ L of the hMSC Negative Cocktail (CD34 PE, CD45 PE, CD11b PE, CD19 PE, and HLA-DR PE) and incubated at 4 °C in the dark for 30 min. The mixed cell suspensions were washed twice with 500 μ L of PBS containing 1% bovine serum albumin (BSA). The cells were suspended in 500 μ L of PBS containing 1% BSA and analyzed using a BD FACSCanto II. The positive control using hMSCs (Lonza) and the negative isotype controls was used to set up the gate for all samples. Cells of each group were counted during 90-second run at medium rate, as controlled volume.

Animal Surgery and Tissue Harvest

14 female New Zealand White Rabbits (NZW, 7–9 lb) (Covance Inc., NJ) were anesthetized with Ketamine (35–40 mg/kg)/Xylazine (2–5 mg/kg). Total of four groups were evaluated: defect control, microfracture only, microfracture with unmodified scaffold, and microfracture with aggrecan functionalized scaffold. Three rabbits were included in each group, with one extra in the groups treated with scaffold as a preparation for potential unknown side effect. Post sedation, isoflurane was delivered from a precision vaporizer (1.5–3%) in 100% O₂ via face mask during the surgery. The right hind leg served as the defect limb and the left hind leg was not treated in this unilateral defect model. We made a midline knee incision and performed a medial parapatellar approach to the knee. The patella was dislocated laterally to expose the articular surface of the trochlea. A full thickness cartilage defect in the center of the trochlear groove was then created that measured approximately 3mm in length and width. In the microfracture groups, five holes were drilled to a ~8mm depth with a 0.75 mm K-wire. In the two experimental scaffold groups, modified or unmodified polymer constructs placed superficial to the cartilage defect site and secured with fibrin glue (TISSEEL; Baxter, Deerfield, IL). The knee was taken through several cycles of range of motion and the fibrin glue / construct complex was found to be intact. The patella was then reduced and the incision closed with absorbable sutures (Figure 3A).

The joints from both knees were harvested after 8 weeks. The left knee tissue was used as healthy control in the evaluation assays. Euthanasia was performed with Beuthasol (Pentobarbital IV, 100 mg/kg) in the auricular vein after a ketamine 20–40 mg/kg and 2–5 mg/kg xylazine subcutaneous injection. After euthanasia, the bilateral knee joints (femur end and chondyle) were harvested for further analysis. The animal study, including the following locomotion test (protocol #0717009), was approved by Institutional Animal Care and Use Committee (IACUC) at University of Maryland, School of Medicine, Baltimore, MD. All animal studies including the follow up evaluation were conducted as blind tests.

Animal Locomotion Test and Evaluation

After two weeks (starting on week 3), the rabbits taken to an open field restricted by a 1.4 m $\times$ 0.5 m fence to allow for locomotion evaluation. Videos were recorded of test subjects from the top and side view on weeks 3, 4, 5, 6, and 8 post-surgery. Videos were analyzed and test subjects were evaluated by a modified Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale to quantify the subjects' movement, coordination, and weight bearing abilities (Table 1). The score was averaged from three evaluators with a blind study.

Optical Coherence Tomography (OCT)

The frequency-domain OCT imaging system was equipped with a wavelength-swept laser light source centered at 1310 nm with 100 nm bandwidth. [18] The wavelength-swept frequency was 16 kHz and the output power was 17 mW. 3% of the laser output power went into a Mach-Zehnder interferometer and generated a frequency-clock signal with uniformly spaced optical frequency to trigger sampling the OCT signal. The system applied a fiber-based Michelson interferometer and about 97% of the laser power was split evenly to the sample and reference arms of the interferometer. The signals reflected from the sample and reference arms encountered at the fiber couple and formed interference fringes, which encoded different frequencies, were then received by a balanced detector. Depth-resolved tomography was achieved by performing a fast Fourier transform of the interference fringes [19]. The scanning field of view was set at 5 mm x 5 mm to cover the entire defect area with 800 pixels in each direction. During imaging, the tissue was irrigated with PBS to prevent dehydration. For cartilage thickness analysis on the acquired images, 10 random sections were picked on the X-axis (cross-section from top of trochlea groove) and the thickness of each section was calculated from a random point on the Y-axis. The average thickness of each group was calculated from all biological samples of the group.

Histological Staining and Image Analysis

Tissue samples were fixed in buffered 4% paraformaldehyde solution containing 1% sucrose. Samples were decalcified, dehydrated, and embedded in paraffin. The samples were sectioned to 5 μ m thick slices and placed on positively charged glass slides. For H&E staining, the samples were stained by hematoxylin, followed by counterstaining of eosin. For Alcian Blue staining, samples were stained by Alcian Blue for 30 minutes, then counterstained under nuclear fast red for 5 minutes. The pictures were taken using Nikon Ti2 microscope (Nikon, Tokyo, Japan) mounted with Nikon DS Ri2 camera. The images were analyzed using the automatic measurement tools built in the microscope software. Chondrocytes number was counted in the H&E images by thresholding the RGB color. Circularity (0.33–1.00) and area (> 3.93) were applied as restrictions to determine cell nuclei. For safranin-O staining, after deparaffinization and hydration, the slides were stained with hematoxylin, fast green, and 0.1% safranin-O solution following standard procedures. Immunohistology was conducted using type II collagen antibody (Abcam, Cambridge, UK) following standard protocol. Similar thresholding techniques were applied when quantifying the type II collagen expression. The binary area of Alcian Blue stained GAGs was quantified by adjusting HSI (Hue, Saturation, Intensity) value. Area (0.05–13807 μ m²) was applied as a restrictive factor to eliminate noise and background. The statistics were generated from all three subjects in each group during quantification. In the image analysis, the healthy control image was used to set thresholds and restrictions and applied to all images.

Results

Aggrecan functionalization and in vitro evaluation of cell adhesion

The EDC-NHS reaction to covalently bind the carboxyl groups on the side chains of aggrecan and the amine groups on the printed PLCL-amine scaffold is shown in Figure 1A. After chemical reaction, the modified scaffold sample was compared to PLCL and PLCL-

amine samples using NMR. First, by comparing the spectra of printed plain PLCL sample, raw PLGA-amine, and printed PLCL-amine sample (blended with 85% PLCL and 15% PLGA-amine), we demonstrated that the amine group is preserved during the printing process (Figure 1B). Chemical shift was expressed in parts per million (ppm). Amine shift at 0.93 ppm and 2.61 ppm were observed in the PLCL-amine sample. Further analysis on PLCL-amine sample, pure aggrecan sample, and the functionalized sample revealed matching peaks confirming the presence of aggrecan (Figure 1B). Because aggrecan's complex structure, the acquisition of separate peaks was challenging. Low ^1H signal was due to low aggrecan solubility as a large proteoglycan molecule. After solvent suppression, the matched peaks were primarily observed at 3.94 ppm, sulfate, and 1.5 ppm, carboxylic ester; these two groups are the most abundant side groups in aggrecan. Interestingly, the additional of aggrecan was demonstrated to have an impact on the compressive modulus of the wet scaffold. The Young's modulus of printed PLCL-amine scaffold and aggrecan functionalized scaffold were 2.48 ± 0.29 MPa and 1.93 ± 0.30 MPa (Figure 1E).

After confirming the presence of functional groups on the scaffold, the biological response of surface modifying the scaffolds was evaluated by seeding hMSCs onto PLCL, PLCL-amine, and aggrecan modified scaffolds. The three groups presented DNA concentrations of 231.91 ± 5.73 ng/ml, 386.86 ± 3.73 ng/ml, and 597.24 ± 8.02 ng/ml. From the DNA quantification data, after 7 days the aggrecan modified scaffold showed three times more DNA content than the unmodified scaffold (Figure 1D), indicating that the aggrecan bond to the scaffold played an important role in improving the cell attachment.

With the knowledge of that aggrecan improved cell attachment, we then seeded whole bone marrow onto the scaffold to evaluate the attached cell population. A cell centrifugation assay isolated the adhered cells from whole bone marrow was performed to determine cell phenotype through flow cytometry (Figure 2A). The non-adhered and adhered cell population were separated for further analysis. A hMSC cocktail was used in the flow cytometry to identify the hMSCs population (CD105+, CD90+, CD73+, CD11b-, CD19-, CD45-, HLA-DR-). The aggrecan modified scaffold had approximately 10 times more adhered cells than the control groups (PLCL and PLCL-amine scaffolds). The adhered/attached cell count in controlled 90 second flow for aggrecan modified scaffold, PLCL scaffold, and PLCL-amine scaffold was 286377, 21113, and 32551, respectively (Figure 2B). Among the adhered cells, the positive hMSCs for aggrecan modified scaffold and the two controls are 387, 200 and 214 in a controlled unit volume, which yields a positive hMSCs percentile of 0.1–1% among the adhered cells in the whole bone marrow (Figure 2B).

In vivo assessment of scaffold function

Although the above experiments demonstrated the improved cell recruitment as a result of aggrecan functionalization, the actual healing process associated with microfracture is hard to mimic in the *in vitro* environment. An animal model was used to further test the therapeutic effect of the scaffold. The animal surgery was completed successfully following the approved protocol. All 14 rabbits survived with an overall good health condition until the termination of the study. The implanted scaffolds were mostly degraded after 8 weeks. A

thin layer of regenerated cartilage tissue visibly covered the defect area in the experimental groups. With the additional scaffold, especially when functionalized scaffold, the newly regenerated cartilage tissue showed a healthier appearance (Figure 3B).

In the locomotion test, rabbit movement was recorded at each time point from both side view and top view throughout the eight week observations. The locomotion behavior was evaluated using a modified score scale. While the defect control group demonstrated no movement improvement over the course of the study where all experimental groups showed an increasing trend of scores. Rabbits treated with aggrecan functionalized scaffold or plain scaffold had comparable scores with the rabbits treated with traditional microfracture. In this study, with modified BBB score to better represent the rabbit locomotion behavior, all the rabbits received a score higher than 13 (out of 21). Considering the relatively good recovery in regard to the movement ability with treatments, no significant differences among the experimental groups were observed at each time point. All experimental groups with treatments had an average score of around 19 on week eight (Figure 3C). In addition, images were captured to assess the foot pointing angle to their body. If the rabbit's movement was affected by pain or impaired joint function, they would apply unbalanced force on their hind limb or drag the limb when walking. This is reflected by the angle of the surgical site hind limb to their body. From the analysis, all groups with treatment show significantly small angle than the defect control group, indicating a healthier walking pattern (Figure 3D).

The implantation groups showed statistically similar small angles (less than 10°) compared to the microfracture group, demonstrating that the scaffold did not cause discomfort during movement. After 8 weeks, the femur end of the joints was harvested for OCT scanning and histological analysis. The bone and cartilage boundary can be determined by the reflected light contrast due to different tissue structures.[20] The healthy control sample was used as a reference to determine the scan settings and the outer and inner boundary of cartilage tissue. Images reveal aggrecan functionalized scaffolds resulted in a more homogenous thickness distribution (Figure 4 E). This difference, compared to the non-treated scaffold, indicate better integration with the newly regenerated tissue as a result of the biological activity provided by aggrecan. The released cells and growth factors from bone marrow were likely better preserved through the proteoglycan support. Furthermore, the average regenerated cartilage thickness of samples in the aggrecan-PLCL treatment group was $264 \pm 80 \mu\text{m}$, which is significantly higher than all other groups. The samples from the defect control group, microfracture treatment group, and PLCL scaffold group showed regenerated thickness of $47 \pm 41 \mu\text{m}$, $139 \pm 72 \mu\text{m}$, and $155 \pm 80 \mu\text{m}$, respectively (Figure 4 B-E). These results demonstrate the therapeutic effect of functionalizing the scaffold with aggrecan. The combination of microfracture and this acellular bioactive scaffold resulted greatly improved cartilage regeneration in 8 weeks.

The H&E staining shows the nuclei stained dark purple and the eosinophilic structures mainly composed of intracellular and extracellular proteins were counterstained as pink. The regenerated cartilage tissue appeared as lighter pink compared to the subchondral bone tissue (Figure 5B). Chondrocytes number in the cartilage layer was quantified. The defect control group showed limited regenerated tissue with only 55 ± 26 chondrocytes in the imaging area. The microfracture treated group and the PLCL scaffold group showed similar

amount of newly formed tissue with a chondrocytes count of 112 ± 26 and 134 ± 95 , respectively. The slightly improved regeneration of the non-treated scaffold might be caused by the initial support provided the implant; however, the poor integration with the local tissue limited the positive effect. With the addition of aggrecan, the tissue formation was significantly improved with almost three times more (366 ± 95) cell present in the cartilage layer.

The Alcian blue staining further confirmed the therapeutic effect of the functionalized scaffold (Figure 5A). As a major component of native cartilage, the production of GAGs by the chondrocytes has been used as a common way to assess the chondrogenesis or cartilage tissue regeneration. The GAG content stained by Alcian blue was greatly enhanced with the support of scaffold. The GAG expression was quantified as binary area (μm^2) in each image. The calculated average GAG contents of samples from defect group, microfracture group, PLCL scaffold group and aggrecan-PLCL scaffold group were $4412 \pm 940 \mu\text{m}^2$, $21052 \pm 22064 \mu\text{m}^2$, $380120 \pm 32307 \mu\text{m}^2$, and $92603 \pm 30177 \mu\text{m}^2$, respectively. The aggrecan functionalization resulted 20 times more GAG production than the non-treated group and 4.4 times more GAG production compared to the microfracture treatment. The production of proteoglycan is proportional to the safranin-O staining in normal cartilage. The healthy control showed a strong affinity to safranin-O to form a red complex with a top layer of counter stained green color. Compared with regular microfracture treatment, the scaffold treatment showed higher level of proteoglycans, although safranin-O was reported as a not very sensitive indicator when the GAGs were depleted, for example, in the newly formed tissue layer (Figure 6A).[21] From the H&E staining of the subchondral bone area, all samples displayed normal bone morphology indicated by the darker pink stain with normal vascularization (Figure 6B). In general, no visible difference regarding the subchondral structure was observed among all groups compared to the healthy control.

A major concern of microfracture is the variance in success among cases because the released MSCs and biomolecules have limited guidance and support in the defect area. The three rabbits in the microfracture group also showed relatively large standard deviations in multiple assessments. However, such variance seems to be reduced by incorporating the aggrecan-PLCL scaffold, indicating the consistency of the treatment effect. To further evaluate the quality of the regenerated cartilage tissue, immunostaining of type II collagen was performed (Figure 7). The calculated type II expression level as binary area were $59 \pm 12 \mu\text{m}^2$, $3909 \pm 681 \mu\text{m}^2$, $2358 \pm 214 \mu\text{m}^2$, and $32886 \pm 1471 \mu\text{m}^2$ for defect control group, microfracture group, microfracture with unmodified scaffold group, and microfracture with aggrecan scaffold group. All experimental groups displayed a significantly different expression level compared to the defect control from the quantitatively analysis. As a comparison, the type II collagen presence in terms of area in unit area was approximately 10 times more compared to the microfracture treated group.

Discussion

In this study, we developed a bioactive acellular scaffold to combine with the microfracture procedure, promoting cartilage regeneration and demonstrated the therapeutic effect. Our combination technique improved type II cartilage regeneration (10x), tissue thickness (1.9x),

GAG content (4.4x), and number of chondrocytes present (3.2x), as compared to the standard microfracture technique, showing significantly improved cartilage repair. The fabrication of this surface-modified scaffold can be done prior to the surgery with no extensive additional surgical preparation as compared to the normal microfracture procedure. Compared to cell-based treatments and acellular coated-scaffolds, the innovations and advantages associated with this covalently modified therapeutic grant this approach a great potential to bridge the need of clinical solutions and treat large cartilage defects in a cost and labor effective manner.

When taking into consideration the type of polymer that should be utilized to fabricate the scaffold, the ability to withstand compressive mechanical stimuli is crucial. In comparison to more commonly used poly(lactic acid)(PLA) and PLGA scaffolds, PLCL scaffold exhibited mechanical properties of toughness relative native cartilage [22] ; further assessments of extracellular matrix accumulation on the cell– inoculated PLCL constructs also demonstrated that the material can significantly enhance chondrogenic differentiation.[23] Interestingly, the addition of aggrecan significantly lowered the compressive modulus of the wet PLCL scaffold, possibly due to the water retaining effect on plasticizing during mechanical testing. Proteoglycans are the most defining factor in force-transmission, growing healthy ECM, and determining the viability of chondrocytes in cartilage. To the best of our knowledge, aggrecan was first used to strengthen the biological function of a 3D-printed scaffold for tissue regeneration. The most prominent indication of aggrecan aiding the chondrocyte microenvironment was the increased cell adhesion as quantified by DNA extraction (10x) and flow cytometry (13.6x) from whole bone marrow. The presence of aggrecan was reported to increase the link protein on the cell surface and thus to improve the cell attachment. [24] Although other biomolecules including other proteoglycans were demonstrated to have the ability to promote cartilage regeneration such as hyaluronic acid, as the main proteoglycan component in native articular cartilage, the unique complex structure of aggrecan provides functions that other molecules cannot replicate. [25, 26]

In the centrifugation assay, a previous established force was applied to separate the adhered and non-adhered cells to achieve a precise quantification of the cell population. [17] A 3D printed lid, which fits the tissue culture well plate, was designed to hold the 3D printed scaffolds in place while collecting the non-adhered bone marrow portion during centrifugation. With the aggrecan functionalization, the cell attachment efficiency was significantly increased. Notably, the presence of aggrecan promoted cell adhesion regardless of the cell types. The percentage of hMSCs among all adhered cells was not affected by aggrecan.

NZW rabbits were used as an animal model to further evaluate the scaffold biofunctionality. NZW rabbits are well-established for use in orthopedic surgery models because the condyle of mature rabbits is large enough to incorporate scaffold treatment with feasible surgical procedures. The thickness of the cartilage layer in rabbit is also enough to prevent the potential intrinsic repair compared to mouse or rat model.[27] The cartilage regeneration process varies by gender as sex hormones play a role in regulating chondrocyte function and collagen production. Particularly, females are associated with higher incidences of OA.[28] Therefore, female rabbits have been used as the standard model in cartilage repair studies in

existing literature, for the purpose of (1) consistent cartilage physiology properties and (2) a model associated with the disease prevalence. Although successful, [29, 30] the random cell differentiation induced by microfracture often leads to the formation of fibrocartilage, which is weaker than articular cartilage and results in the loss of desired tissue function.[31] To overcome the lack of chemical cues in microfracture surgery for the released cellular component, we incorporated an aggrecan functionalized acellular scaffold into the defect area. Cell based treatments require an extra surgery to source autologous cells as well as additional time for *in vitro* maintenance to expand the autologous or allogenic cells. [32] In contrast, this acellular scaffold described in the present study can be combined with microfracture in a single surgery with little additional preparation time, improving this technology's potential for clinical translation over the use of cell-based approaches. However, minor surgical challenges were also discovered in this study using this small animal model. During the implantation procedure into the rabbit model, the scaffolds were fixed in place using fibrin glue. Although most of the scaffolds were degraded after 8 weeks, some debris was found outside the defect area. Due to the limited soft tissue in the trochlear groove after creating the critical defect, suturing the scaffold in place was not feasible. We believe this problem can be remedied in future studies with a larger animal model or by incorporating a more effective biocompatible glue, which would also improve the integration of the local tissue after implantation.[33]

According to previous studies, the tissue regenerated by microfracture matures after 8 weeks,[34] therefore, the knee joints were harvested and evaluated 8 weeks post operation. Clinically, cartilage thickness is an important factor for determining the progression of OA. In this study, cartilage thickness was measured to evaluate the regeneration potential of different treatments. OCT presents advantages over ultrasound including higher resolution, and has been used as a non-invasive method to assess tissue qualitatively and quantitatively, including rabbit cartilage.[20, 35] In this study, OCT provided an effective mechanism to evaluate the 3D structure of the regenerated tissue in addition to histology.

The BBB score was initially developed to evaluate locomotion after spinal cord injury in rats.[36] The application in a rabbit model has also been reported, although a lower range of scores were applied in the study.[37] The rabbits in our study exhibited higher recovery levels so the scale was adjusted to accurately assess differences in healthy and unhealthy walking patterns. The tail balance criterion specific to rats was modified to standing abilities in this application to better differentiate recovery rates among the rabbits. In general, the score each group received matched the histological performance, although more distinct differences among treatment groups could be reflected with a more tailored scale system focusing on post-orthopedic operation. Moreover, considering the challenges of functional level of assessment, it is reasonable that such differences were not shown during the early stages of the tissue regeneration. Even though further refinement and evaluation may establish a locomotion assessment post orthopedic surgery for rabbits, to our knowledge, this was the first time that tissue function was investigated through movement. This trial has a meaningful impact in calling for a system to evaluate the tissue regeneration at a higher level, as well as providing insights on developing the locomotion scale.

Previous studies have suggested that covering the microfracture area could result in a more robust cartilage regeneration.[14] It is hypothesized that the mechanism of microfracture to treat cartilage defects is based on the released MSCs from the bone marrow and cellular differentiation with the support of growth factors and cytokines from the platelets.[38] The presence of a scaffold covering the defect area following the microfracture can structurally stabilize the blood clot, which results in better integrated tissue repair.[39] In this study, the subjects treated with an unmodified PLCL scaffold showed an overall improvement compared to microfracture treatment alone. From the OCT 3D scanning, the addition of the PLCL scaffold led to a smoother and more homogeneous regenerated layer of cartilage. Both Alcian blue and safranin-O staining demonstrated the improved proteoglycan formation with the treatment of the bioactive scaffold compared to traditional microfracture procedure. The evaluation of cartilage-bone interface is important to assess the overall success of the surgical procedure. [40–42] In this cartilage defect model, other than the signs from the microfracture drill, no visible difference in the subchondral bone area was observed among all the groups. One of the important functions of aggrecan, as a major proteoglycan in native cartilage, is to mediate the chondrocyte-chondrocyte and chondrocyte-matrix interactions by stabilizing the cell- substratum interactions and sustaining the bioactive molecules in serum and fluids.[43] The G1 domain of aggrecan also plays a role in chondrocyte apoptosis.[44, 45] As such, it is possible that this phenomenon resulted in the subjects treated with aggrecan-PLCL exhibiting a significantly enhanced healing process, which was reflected in the increased thickness of regenerated cartilage and increased chondrocytes and GAGs presence as evidenced in the histology. More deposition of type II collagen in the regenerated area further confirmed the quality of the newly formed tissue and the importance of incorporating aggrecan to improve the biological function of the scaffold.

The native zonal structure of cartilage tissue needs to be taken into consideration in future studies to restore the desired tissue function. [11] Although in this study, the thickness of cartilage tissue in rabbit limited the possibility of implanting a zonal scaffold, it would be of interest to evaluate the ability of a 3D-printed scaffold with a biomimetic zonal structure to induce cell alignment in different regions in a larger animal model with a longer recovery time.[16, 46]

Conclusions

This study developed an effective method to regenerate critical articular defects by combining a 3D-printed bioactive scaffold with the microfracture surgical procedure. The surface of the 3D-printed polymer scaffold was covalently modified with aggrecan to improve cell adherence and maintain cell function at the scaffold surface. With the functionalized scaffold, the microfracture outcome was significantly enhanced by providing extra support to the stem cells and growth factors released from bone marrow during the healing process. The additional aggrecan scaffold treatment resulted in a thicker and healthier regenerated cartilage layer. This biofunctionalized acellular scaffold combined with microfracture shows promise for clinical translation. Such a cellular technologies stand to greatly impact future clinical solutions to improve the quality of repaired cartilage tissue as demonstrated in this study without additional surgeries and with a relatively low cost compared to cell-based therapies.

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Figure 1. Design and characterization of aggrecan functionalization on PLCL scaffold.

A. Schematic diagram of chemical reaction between aggrecan and PLCL-amine scaffold using EDC and sulfo-NHS. **B.** NMR spectrum confirming the presence of amine group after printing the blended PLCL and PLGA-amine material and the aggrecan modification. X-axis shows chemical shift (ppm). **C.** 3D-printed amine-PLCL scaffold with a dimension of 3 mm (L) × 3 mm (W) × 1 mm (H). **D.** DNA quantification of hMSCs seeded on PLCL, PLCL-amine, and PLCL-aggrecan scaffold. After 7 days, significantly higher DNA centration was observed with the aggrecan functionalization. N = 3, data is shown as mean ± standard

deviation. * Shows statistical difference. **E.** Comparison of compressive modulus of wet PLCL scaffold and PLCL-aggrecan scaffold. The modulus of the two groups showed statistically significant difference based on student t test ($p < 0.05$). N= 6 in each group.

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Figure 2. Centrifugation assay to evaluate cell adhesion and adhered cell phenotype.

A. Set up of the centrifugation assay for scaffolds seeded with whole bone marrow. A 3D-printed lid was applied to separate the adhered and non-adhered cells. **B.** Flow cytometry analysis of the adhered cell population. The overlapped area based on 3 positive markers was further quantified to count positive hMSCs. With the addition of aggrecan, total attached cell number was improved by approximately 10 times. More positive hMSCs number was present with aggrecan modification due to overall increased cell number.

Figure 3. Animal surgery and locomotion evaluation.

A. Surgical procedure of microfracture and scaffold implantation. A 3 mm×3 mm full thickness defect was made in the center of the trochlear groove (top left); 5 small holes (0.75mm) were drill at the four corner and the center of the defect on the subchondral bone (top right); scaffold with the defect size was covered the defect area (bottom left); the exposed patellar groove was reverted and the wound was closed by buried absorbable suture (bottom right). **B.** Harvested joints after 8 weeks showing regenerated cartilage tissue. Top left: defect without treatment; top right: defect treated by microfracture; bottom left: defect treated by microfracture and PLCL scaffold; bottom right: defect treated by microfracture and PLCL-aggrecan scaffold. **C.** BBB score evaluation over the 8 weeks of post-surgery observation in open field locomotion test. Compared to defect control, all subjects with surgical treatment showed improved score overtime. The mobility of the animals was not negatively affected by the addition of the implantation. **D.** Measurement of foot alignment

during movement. All experimental groups displayed smaller foot pointing angle, indicating a healthier walking pattern during recovery. $N = 3$. Data is shown as mean $\pm$ standard deviation. ANOVA was performed to compare the significance among groups. Means that do not share the same letter are significantly different.

Figure 4. 3D OCT images of cartilages and thickness quantification.

The representative images of groups were listed: (A) Healthy control group, (B) Defect control group, (C) Microfracture group, (D) Microfracture and PLCL scaffold group and (E) Microfracture and Aggrecan-PLCL scaffold group. Cartilage thickness was calculated from 10 randomly selected sections along y-axis. On each section, a random position on x-axis was selected for calculation. The average and standard deviation of the thickness in the plot was calculated from all samples in each group. The aggrecan functionalization resulted thicker regenerated cartilage layer with more homogenous 3D thickness distribution

compared to the normal microfracture procedure and the group treated with unmodified scaffold. N = 3. Data is shown as mean $\pm$ standard deviation. Groups showing significantly statistical differences were indicated by *.

Figure 5. Histological examination of regenerated cartilage tissue using H&E and Alcian blue staining.

A. Alcian Blue staining showing the GAGs production in different groups. Cell nuclei were stained purple and surrounding GAGs were stained blue. The GAGs expression level was quantified by binary area. Compared to defect control, microfracture and PLCL showed increased production of GAGs from the histology staining. The addition of aggrecan further improved the quality of regenerated tissue, with visible lined chondrocytes surrounded by GAGs. **B.** H&E staining showing newly regenerated cartilage tissue. Cell nuclei were

stained purple and the background tissue was stained pink. The cartilage layer presents a lighter pink than the bone tissue. The number of chondrocyte was calculated and compared among groups. The chondrocytes number in the aggrecan functionalized group was significantly higher than other experimental groups. $N = 3$. Data is shown as mean $\pm$ standard deviation. ANOVA was performed to compare the significance among groups. Means that do not share the same letter are significantly different.

Figure 6. Histological evaluation of the cartilage-bone interface.

A. Safranin-O staining showing GAGs production in the regenerated cartilage. The red safranin-O stain is proportional to the proteoglycan content, while Fast Green counterstains the non-collagen sites and provides a clear contrast to the safranin-O staining. **B.** H&E staining at higher magnification (20X) showing the subchondral bone region underneath the defect area. Compared to the healthy control, all groups displayed normal subchondral bone tissue with high density. From top to bottom, representative pictures showing healthy

control, defect control, microfracture only, microfracture + PLCL scaffold, and microfracture + PLCL-aggrecan scaffold for both staining.

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Figure 7. Immunohistochemistry staining of Type II collagen and quantification.

The type II collagen was stained in brown. All experiment groups showed significant higher type II collagen expression compared to the defect control. The addition of aggrecan functionalized scaffold further increased the expression of type II collagen in the regenerated cartilage tissue. The level of expression in each group was quantified. $N = 3$. Data is shown as mean $\pm$ standard deviation. ANOVA was performed to compare the significance among groups. Means that do not share the same letter are significantly different.

Table 1.**Modified BBB Locomotor Rating Scale for Rabbits after Orthopedic Surgery**

0	No observable movement of the hindlimbs.
1	Slight (limited) movement of one or two joints, usually hip and/or knee.
2	Extensive movement of one joint or extensive movement of one joint and slight movement of the other.
3	Extensive movement of two joints.
4	Slight movement of all three joints of the hindlimb (HL).
5	Slight movement of two joints and extensive movement of the third joint.
6	Extensive movement of two joints and slight movement of the third joint.
7	Extensive movement of the three joints in the hindlimbs.
8	Sweeping without weight bearing or plantar support of the paw without weight bearing.
9	Plantar support of the paw with weight bearing only in the support stage (i.e., when static) or occasional, frequent or inconsistent dorsal stepping with weight bearing and no plantar stepping.
10	Plantar stepping with occasional weight bearing and no forelimb (FL)-HL coordination.
11	Plantar stepping with frequent to consistent weight bearing and no FL-HL coordination.
12	Plantar stepping with frequent to consistent weight bearing and occasional FL-HL coordination.
13	Plantar stepping with frequent to consistent weight bearing and frequent FL-HL coordination.
14	Plantar stepping with consistent weight support, consistent FL-HL coordination and predominantly rotated paw position (internally or externally) during locomotion both at the instant of initial contact with the surface or frequent plantar stepping, consistent FL-HL coordination and occasional dorsal stepping.
15	Consistent plantar stepping, consistent FL-HL coordination; predominant paw position is parallel to the body at the time of initial contact.
16	Consistent plantar stepping and FL-HL coordination during gait; the predominant paw position is parallel to the body at the time of initial contact and curved at the instant of movement.
17	Consistent plantar stepping and FL-HL coordination during gait; the predominant paw position is parallel to the body at the time of initial contact and at the instant of movement.
18	Consistent plantar stepping and FL-HL coordination during gait; the predominant paw position is parallel to the body at the time of initial contact and curved during movement. The animal presents partial standing
19	Consistent plantar stepping and FL-HL coordination during gait; the predominant paw position is parallel to the body at the instant of contact and at the time of movement, and the animal presents occasionally full standing.
20	Consistent plantar stepping and FL-HL coordination during gait; the predominant paw position is parallel to the body at the instant of contact and at the time of movement, and the animal presents frequent full standing.
21	Consistent plantar stepping and coordinated gait, consistent movement; paw position is predominantly parallel to the body during the whole support stage; consistent full standing.
DEFINITIONS	
Slight: partial joint movement through less than half the range of joint motion.	
Extensive: movement through more than half of the range of joint motion.	
Sweeping: rhythmic movement of HL in which all three joints are extended, then fully flex and extend again; animal is usually side lying, the plantar surface of paw may or may not contact the ground; no weight support across the HL is evident.	
No Weight Support: no contraction of the extensor muscles of the HL during plantar placement of the paw; or no elevation of the hindquarter.	
Weight Support: contraction of the extensor muscles of the HL during plantar placement of the paw, or elevation of the hindquarter.	
Plantar Stepping: The paw is in plantar contact with weight support then the HL is advanced forward and plantar contact with weight support is reestablished.	

Dorsal Stepping: weight is supported through the dorsal surface of the paw at some point in the step cycle. FL-HL Coordination: for every FL step an HL step is taken and the HLs alternate.
Occasional: less than or equal to half.
Frequent: more than half but not always; 51–94%.
Consistent: nearly always or always; 95–100%. Partial standing: lifting the forelimb off the ground but with trouble holding the body straight for more than 1 second.
Full standing: lifting the forelimb off the ground with a full support provided by the hindlimb.