Infiltration and In-Tissue Polymerization of Photocross-Linked Hydrogel for Effective Fixation of Implants into Cartilage—An In Vitro Study

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Supporting Information

ABSTRACT: Effective and biocompatible fixation of implants into cartilage defects has yet to be successfully achieved. [Poly-D,L-lactic acid/polyethyleneglycol/poly-D,L-lactic acid] (PDLLA-PEG) is a chondrosupportive scaffold that is photocross-linked using the visible-light photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). Interestingly, LAP and its monomer DLLA-EG are able to infiltrate the cartilage and form hydrogels upon the detection of light. After the infiltration of LAP and DLLA-EG into the implant and host cartilage, an interconnected and continuous hydrogel structure is formed which fixes the implant within the host cartilage. A mechanical test shows that the infiltrated group displays a significantly higher push-out force than the group that has not been infiltrated (the traditional fibrin fixation group). Surprisingly, the in-cartilage hydrogel also reduces the release of sulfated glycosaminoglycan from cartilage explants. However, infiltration does not affect the cell viability or the expression of cartilage marker genes. This new strategy thus represents a biocompatible and efficient method to fix implants into host tissues.

1. INTRODUCTION

Articular cartilage injury affects approximately 900,000 people in the United States alone every year and represents a significant challenge for healthcare professionals.1–3 If left untreated, it can cause pain and swelling and eventually lead to the onset of osteoarthritis.4–6 Currently, there are several surgical treatments available, such as microfracture, autologous chondrocyte implantation, and osteochondral transplantation. However, none of the previous ones are able to fully restore the structure and function of the injured cartilage.7,8 One reason for the unsatisfactory results of implantation surgeries can be the insufficiency of fixing the implants.9

Currently, there are various fixation strategies being used in the field, such as press-fitting, suturing, subchondral anchoring,8 fibrin gluing, or a combination of a few. Among these methods, suturing and subchondral anchoring can cause extra damage to the cartilage, and press-fitting can cause cell death because of the high pressure between the implant and the host tissue.10 Fibrin glue has been widely used to temporarily fix grafts, but the fixation is weak and does not last long because of its rapid degradation.11–13

Recently, we have developed [poly-D,L-lactic acid/polyethyleneglycol/poly-D,L-lactic acid] (PDLLA-PEG)-based scaffolds for cartilage repair, which have been polymerized by the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP).14 Interestingly, we found that both monomers, [D,L-lactic acid/ethylene glycol/D,L-lactic acid] (DLLA-EG) and LAP, before polymerization, could infiltrate the cartilage and become photocross-linked. This unique in-cartilage gelation capacity of PDLLA-PEG inspired the potential
application in tissue fixation (Figure 1a). In this new method, the implant and the internal side of the host cartilage that contacts the implant were precultured with a DLLA-EG/LAP solution. This allows for the infiltration of monomers and photoinitiators. Afterward, the implant was transferred into the defect area of host tissue with an additional DLLA-EG/LAP solution to fill the gap in between. We hypothesized that upon illumination, an interconnected and continuous hydrogel network would form from the host cartilage to the implant through the gap zone, resulting in a secure fixation (Figure 1a).

To test our hypothesis, we created a traditional in vitro ring-disk cartilage repair model, in which a bovine cartilage ring [6 mm outer diameter (OD) and 4 mm inner diameter (ID)] is used as the host tissue, and a 3 mm diameter cartilage disk served as the implant (Figure 1b). The gap between the host tissue and the graft ranged from 0 to 1 mm.

2. RESULTS AND DISCUSSION

First, we would like to measure the infiltration rate of monomers, which will help determine the location of polymers within the cartilage. It is difficult to directly monitor the movement of the monomers, so we used the molecular tracer Biocytin Alexa Fluor 546 to help track them; Biocytin Alexa Fluor 546 has a molecular weight of 1209 Da, similar to DLLA-EG (1144 Da). The cartilage disks were soaked into a dye solution for different periods time and then imaged with a fluorescence microscope. As shown in Figure S1 (in the Supporting Information), the fastest travel occurred in the first 5 min. During this period, monomers may infiltrate roughly 0.6 mm. Then, both the cartilage disk and the internal side of the ring were incubated with DLLA-EG/LAP for 5 min. Afterward, an additional monomer solution was used to fill the gap. The entire construct was subject to illumination—the group was named “PreI-5min.” An additional control group “PreI-0min” underwent the same procedure but without the infiltration process.

To validate the in-scaffold formation of hydrogel, the cured constructs were treated by papain, which was able to completely digest the cartilage (both ring and disk), without breaking down the PDLLA-PEG hydrogel. As shown in Figure 1b, the remaining polymer in the PreI-5min group was intact and significantly larger than that of the PreI-0min group. The weight of the polymers in the PreI-5min group (31.37 ± 2.173, n = 3) was more than 2 times higher than the PreI-0min group (14.2 mg ± 1.044, n = 3) (Figure 1c), indicating that a PDLLA-PEG hydrogel network was formed within the cartilage. It should be noted that there is some uncertainty on how the photoinitiator responds. As the wavelength of the light source is 395 nm, it should not pass through the opaque cartilage to activate the photoinitiator. Given the fact that free radicals can penetrate a tissue very quickly, we assume that the free radicals generated in the gap zone might travel into the cartilage and initiate polymerization.

Whether all the monomers in the cartilage had been fully polymerized is unknown. To test whether the infiltration process and the monomer residues, if any, would adversely affect the phenotype of the cartilage, the constructs with and without infiltration were cultured up to 28 days. From the results in Figure 1d, it is observed that samples from the PreI-
5 min group displayed a higher level of aggrecan (ACAN) and collagen type II (COL2), which are the chondrogenic markers. These results could actually be beneficial in associating PDLLA-PEG with the ability to support chondrogenesis. 14

After the implants were fixed in the host tissue, a standard push-out test was conducted to evaluate the binding strength between the two. The traditional fibrin glue-based fixation method was used as the control. We also assessed how different infiltration times would affect the fixation results. As shown in Figure 2a, 5 min of infiltration resulted in a maximum force (MF) of 1504.75 kPa, which was 20 times higher than that of the fibrin group (73.86 kPa). The MF did not increase after 5 min of infiltration; thus, 5 min was used in all future studies. To better demonstrate the result of infiltration, we recorded the force change in a separate pull-out test (Figure S2, Movie S1 in the Supporting Information). As demonstrated in the video, the force that was needed to pull the implant out from the host tissue was significantly higher in the PreI-5 min group (Movie S1).

One disadvantage of the suture and press-fitting technologies is the risk of adversely impacting tissue health.17 For example, press-fitting may cause chondrocyte necrosis and cartilage matrix degradation in the interface area.10 We also examined that the continuous force generated by suturing would induce chondrocyte apoptosis.15 To determine whether the new fixation method developed in this study would have a similar issue, we examined the chondrocyte viability and phenotypes. As shown in Figure 2b, there was no difference in the live cell ratio of all tested groups, suggesting this fixation process is biocompatible.

After 4 weeks of culture in chondrogenic medium, we re-examined the connection between the implant and the host tissue. As shown in Figure 3a, the control group, without the application of any type of fixation, showed no physical connection between the implant and the host tissue. In the fibrin group, some of the implants disconnected from the host tissue during the change in the medium, suggesting poor integration. In the PreI-5 min group, all grafts stayed in position. The safranin O/fast green staining revealed a physical structure between the implant and the host tissue (Figure 3a). As this area was acellular, we assumed it to be a PDLLA-PEG hydrogel scaffold.

Agreeing with the results above, the push-out test showed that measurable data could only be collected from the PreI-5 min group (Figure 3b). After 28 days of culture, the MF in the PreI-5 min group was lower than that at day 0 but was still higher than that of the fibrin group on day 0. The reduction of MF in the PreI-5 min group may be because of the degradation of PDLLA-PEG.19 The reason that we chose the biodegradable PDLLA-PEG was to eventually replace the exogenous scaffolds with the newly formed cartilage, leading to a functional and biological integration. If a stable fixation is necessary, we can simply replace PDLLA-PEG with a PEG scaffold. The PEG scaffold lacks a hydrolysis group, resulting in a very minimal degradation under physiological conditions.

Our previous results showed that the inclusion of chondroinduced mesenchymal stem cells could compensate for the loss of the mechanical strength of the PDLLA-PEG scaffold by providing a cartilage extracellular matrix.24 A similar strategy can be used in the future to maintain the fixation strength and create biological integration.

Finally, we measured sGAG in the cartilage. We hypothesized that the hydrogel formed within the scaffold would reduce the release of sGAG, a common problem observed in explants.20–22 As expected, the cartilage in the infiltration

Figure 2. (a) Maximal force in the push-out test. (b) Cell viability analysis. After being cultured in chondrogenic medium for 7 days, constructs from different groups were analyzed by live/dead staining; green = live cells, red = dead cells. The live cell ratio was measured based on the imaging (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).

Figure 3. After 28 days of culture in chondrogenic medium, constructs with different fixation methods were analyzed. (a) Macro-appearance (top) and histology (safranin O staining, bottom). Black arrow indicates the fibrin residual. White arrow indicates the PDLLA-PEG hydrogel in the gap between the implant and the host tissue. (b) Maximal force in the push-out test. (c) Measurement of sulfated glycosaminoglycan (sGAG) and dsDNA content in the constructs (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).
group maintained a significantly greater amount of sGAG than the control group and fibrin group did (Figure 3c). Therefore, the hydrogel network not only significantly increased the fixation strength but also enhanced the health of the cartilage.

3. CONCLUSIONS

To summarize, in this in vitro study, we developed a new strategy to fix cartilage implants into host tissue. With this method, we achieved a high-strength bond between the implant and host cartilage without affecting the cell viability and tissue phenotype. To infiltrate the in vivo cartilage with DLLA-EG/LAP, a gauze that is completely soaked with the DLLA-EG/LAP solution should be filled into the defect area. Because the gauze is able to hold the aqueous solution, DLLA-EG/LAP will be able to infiltrate into the cartilage. The utilization of this fixation technology will be further examined in a study on animals in the future.

4. EXPERIMENTAL MATERIALS AND METHODS

4.1. Cartilage, Explants, Isolation, and Culture. Articular cartilage explants were harvested from the knee joint of a newborn bovine within 24 h after being sacrificed (JW Treuth and Sons, Baltimore, MD). After being washed with HEPES buffered sterile saline (HBSS), the cartilage disks with 6 mm diameter and 3–4 mm thickness were balanced in a basic medium [Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Gaithersburg, MD), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and 1% antibiotic–antimycotic (Gibco)] for 24 h. Then, an inner plug with a 3 mm diameter was punched out from the center of the cartilage disk, which would be used as the implant. After the 4 mm diameter biopsy punch was used to make the void space bigger, the cartilage ring, which had an OD of 6 mm and ID of 4 mm, was treated as the host tissue (Figure 1b).

4.2. Infiltration of DLLA-EG/LAP into Cartilage Explants. DLLA-EG and LAP were synthesized using a protocol developed in our lab.13 For infiltration, grafts and host tissues were cultured in a 25% DLLA-EG HBSS solution with 0.2% LAP for 5 min. Afterward, the implant was inserted into the host tissue with an open space ranging from 0 to 1 mm in diameter. The open space was filled with the DLLA-EG/LAP solution, and then the whole construct was subject to illumination for 2 min. We first used the DLLA-EG/LAP solution to fill the open space but without the presoaking (infiltration) process. Then, we used fibrin glue (Tisselle, Baxter Healthcare Corp, Deerfield, IL) to fill the open space (without using PDLA-PEG); these were the two controls. All cartilage explants were cultured in chondrogenic medium (DMEM with 1% l-alanyl-l-glutamine (GlutaMAX), 55 mg/L sodium pyruvate, 1% antibiotic–antimycotic, 1% insulin transferrin-selenium (Invitrogen, Carlsbad, CA, USA), 10 ng/mL transforming growth factor-β3 (TGF-β3; PeproTech, Rocky Hill, NJ, USA), 100 nM dexamethasone, 50 μM l-ascorbic acid 2-phosphate, and 23 μM l-proline).

4.3. Measuring the Weight of Polymers within the Cartilage. After the photocross-linking, the entire construct was digested in papain solution (125 mg/mL papain, 50 mM sodium phosphate buffer, 2 mM N-acetyl cysteine (Sigma), pH 6.5) at 60 °C for 24 h. The weights of polymers from different groups were measured using an analytical balance (MS304S, Mettler Toledo, Switzerland).

4.4. Gene Expression Analysis. Total RNA was extracted with the TRIZOL reagent (Invitrogen) and further purified with an RNAs plus Mini Kit (Qiagen, Germantown, MD, USA). cDNA was synthesized with a SuperScript VILO cDNA Synthesis Kit (Invitrogen) by following the product manual. Real-time polymerase chain reaction (PCR) was performed with SYBR Green (Invitrogen) by the StepOnePlus real-time system (Applied Biosystems). All of the mRNA expression levels were normalized to 18S rRNA. The relative gene expression level was calculated using the 2−ΔΔCt method. All primers used in this study are listed in Table S1.

4.5. Pull-Out Test. The pull-out test was conducted with a mechanical tester (Bose Electroforce model 3230 Series II). The cartilage samples, implants, and host tissues were placed on a custom metal ring with an OD of 6 mm and ID of 4 mm. As the void space of the metal ring is larger than the implant but smaller than the host tissue, the force that was needed to push the implant out of the host tissue comes from the interconnected bond. A 2 mm diameter metal plunger was used to push the implant. During the process, the force was recorded until the implant was completely separated from the host tissue. The displacement rate was 0.1 mm/s. The MP was used to compare how effective the fixation was among the different groups.

4.6. Pull-Out Test. As shown in Figure S2, similar to the push-out test, the whole construct was placed underneath a metal mold whose void space was bigger than the implant but smaller than the host tissue. A suture was inserted into the center of the implant with a spring scale attached to the top. We pulled the spring scale up vertically until the implant was completely disconnected from the host tissue.

4.7. Assessment of Cell Viability. A viability/cytotoxicity kit (Invitrogen) was used to assess cell viability in cartilage, with or without the infiltration procedure. Images were captured with an inverted epifluorescence microscope (Olympus CKX41, Japan). Green fluorescent staining indicated the presence of live cells, and red staining suggested dead cells. The number of live cells (green) was divided by the total cell number (green + red) to calculate the live cell percentage.

4.8. Histology. The cartilage samples were fixed in 4% paraformaldehyde (FD Neurotechnologies, Inc., Columbia, MD) at room temperature for 16 h. All samples were then dehydrated in gradient ethanol and cleared with xylene (Fisher Scientific, Pittsburgh, PA) for 2 h. Finally, all samples were embedded in paraffin. Afterward, 6 μm sections were prepared with a microtome (Leica Biosystems, model RM 2255). The sections were stained with safranin O/fast green to measure the deposition of sGAG.

4.9. Measurement of sGAG Content in Cartilage Explants. The cartilage samples were digested in papain solution at 60 °C for 24 h. After centrifugation at 12,000g for 15 min, a part of the supernatant was used to assess the sGAG content with a Blyscan kit (Biocolor Life Science Assay, UK). Another part of the supernatant was used to measure the dsDNA content, which was conducted using a QuantiT PicoGreen dsDNA Assay Kit (Invitrogen).

4.10. Statistical Analysis. All data in this study were analyzed using unpaired Student’s t test or two-way analysis of variance. All values were expressed as mean ± standard deviation, and p < 0.05 was defined as the statistical significance.
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02270.

Pull-out test in the PreI-0min group and PreI-Smin group (MP4).

Culture of cartilage disks with Biocytin Alexa Fluor 546 for different periods of time, demonstration of the pull-out test, and sequence of used primers (PDF)

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Notes
The authors declare no competing financial interest.

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