Video Article

Human Cartilage Tissue Fabrication Using Three-dimensional Inkjet Printing Technology

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Abstract

Bioprinting, which is based on thermal inkjet printing, is one of the most attractive enabling technologies in the field of tissue engineering and regenerative medicine. With digital control cells, scaffolds, and growth factors can be precisely deposited to the desired two-dimensional (2D) and three-dimensional (3D) locations rapidly. Therefore, this technology is an ideal approach to fabricate tissues mimicking their native anatomic structures. In order to engineer cartilage with native zonal organization, extracellular matrix composition (ECM), and mechanical properties, we developed a bioprinting platform using a commercial inkjet printer with simultaneous photopolymerization capable for 3D cartilage tissue engineering. Human chondrocytes suspended in poly(ethylene glycol) diacrylate (PEGDA) were printed for 3D neocartilage construction via layer-by-layer assembly. The printed cells were fixed at their original deposited positions, supported by the surrounding scaffold in simultaneous photopolymerization. The mechanical properties of the printed tissue were similar to the native cartilage. Compared to conventional tissue fabrication, which requires longer UV exposure, the viability of the printed cells with simultaneous photopolymerization was significantly higher. Printed neocartilage demonstrated excellent glycosaminoglycan (GAG) and collagen type II production, which was consistent with gene expression. Therefore, this platform is ideal for accurate cell distribution and arrangement for anatomic tissue engineering.

Video Link

The video component of this article can be found at http://www.jove.com/video/51294/

Introduction

Bioprinting based on thermal inkjet printing is one of the most promising enabling technologies in the field of tissue engineering and regenerative medicine. With digital control and high throughput printheads cells, scaffolds, and growth factors can be precisely deposited to the desired two-dimensional (2D) and three-dimensional (3D) positions rapidly. Many successful applications have been achieved using this technology in tissue engineering and regenerative medicine[1-9]. In this paper, a bioprinting platform was established with a modified Hewlett-Packard (HP) Deskjet 500 thermal inkjet printer and a simultaneous photopolymerization system. Synthetic hydrogels formulated from poly(ethylene glycol) (PEG) have shown the capacity of maintaining chondrocyte viability and promote chondrogenic ECM production[10,11]. In addition, photocrosslinkable PEG is highly soluble in water with low viscosity, which makes it ideal for simultaneous polymerization during 3D bioprinting. In this paper, human chondrocytes suspended in poly(ethylene) glycol diacrylate (PEGDA; MW 3,400) were precisely printed to construct neocartilage layer-by-layer with 1,400 dpi in 3D resolution. Homogeneous distribution of deposited cells in a 3D scaffold was observed, which generated cartilage tissue with excellent mechanical properties and enhanced ECM production. By contrast, in manual fabrication the cells accumulated at the bottom of the gel instead of their initially deposited positions due to slower scaffold polymerization, which led to inhomogeneous cartilage formation after culture[2,3].

Protocol

1. Bioprinting Platform Establishment

The printer modification was based on a HP Deskjet 500 thermal inkjet printer and HP 51626a black ink cartridge.

1. Remove the top plastic cover of the printer and carefully detach the control panel from the cover.

2. Detach the 3 cable connections between the printer top portion and base. Remove the printer top portion from the base.
3. On the printer top portion, remove the small plastic and rubber accessories (printhead cleaning system) at the right hand side under the ink cartridge.
4. Remove the base of the paper tray with springs.
5. Remove the metallic plate covering the plastic paper feeding bar.
6. Cut off the plastic paper feeding bar at the middle feeding wheel position using a hand saw or other cutting tool.
7. Remove the 2 paper feeding wheels exposed after the previous step. The wheel plastic is very hard and an electronic saw will be helpful.
8. Clean the dust and debris using canned air and ethanol wipes.
9. Attach the printer top portion to the base.
10. UV sterilize the modified printer for at least 2 hr in a laminar flow hood before using.
11. Cut off the cap of a HP 51626a ink cartridge using a hand saw or other cutting tool.
12. Empty the ink and remove the filter that covers the bottom well reservoir of the cartilage.
13. Rinse the cartridge thoroughly using running tap water.
14. Ultrasonicate the cartridge in de-ionized (DI) water for 10 min to remove the residual ink.
15. Examine the cartridge to make sure all the ink has been removed. Rinse or spray the cartridge thoroughly with 70% ethanol for sterilization, followed by sterilized DI water.
16. Set up a long-wavelength ultraviolet lamp over the printing platform to provide simultaneous photopolymerization capacity.
17. Measure UV intensity at the printing platform using a UV light meter. Adjust the distance between the UV lamp and the printer platform so the intensity at the printing subject is between 4-8 mW/cm^2 (approximately 25 cm from lamp to the printer platform).

2. Bioink Preparation

1. Monolayer chondrocyte expansion
    1. Plate 5 million human chondrocytes into each T175 tissue culture flask for cell expansion in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% calf serum and 1x penicillin-streptomycin-glutamine (PSG). Culture cells at 37 °C with humidified air containing 5% CO_2. Change the culture medium every 3 days until the flask is 85% confluence. Use cells from the same passage.
2. Dissolve PEGDA in PBS to a final concentration of 10% w/v. Add photoinitiator I-2959 to a final concentration of 0.05% w/v. Filter sterilize the solution.
3. Suspend cultured human chondrocytes in the prepared PEGDA solution at 5 x 10^6 cells/ml.

3. Cartilage Tissue Printing

1. Turn on the printer and laptop.
2. Create a printing pattern of a solid circle with 4 mm in diameter using Microsoft Word or Adobe Photoshop.
    1. Adjust the position of the pattern and make sure it will print exactly into the plastic mold.
    2. Calculate the number of prints needed to reach the desired thickness of scaffold. For 4 mm in height, 220 prints are required to create the desired scaffold.
3. Load the bioink into the ink cartridge. Cover the cartridge with aluminum foil to protect from the direct UV exposure during printing.
4. Send printing command to the printer. Pull the paper sensor when the printer starts to print. The whole printing process should take less than 4 min for a scaffold with 4 mm in diameter and 4 mm in height.
5. Transfer printed neocartilage to a 24-well plate and add 1.5 ml culture medium to each well.

4. Cell Viability Evaluation in 3D Scaffold

1. Incubate the printed neocartilage in LIVE/DEAD Viability/Cytotoxicity working solution at room temperature for 15 min in dark.
2. Cut the cell-laden hydrogel in half and take fluorescent images of the cutting area.
3. Count live (green) and dead (red) cells by a blinded observer at five randomly taken images. Calculate cell viability by dividing the number of live cells by the total number of live and dead cells.

Representative Results

The modified thermal inkjet printer was capable for cell and scaffold deposition at high throughput and excellent cell viability. Combining with simultaneous photopolymerization and photosensitive biomaterials, this technology is able to fix the cells and other printed substances to the initially deposited locations. According to the properties of the modified thermal inkjet printer, the 2D printing resolution was 300 dpi with a single ink drop volume of 130 pl. There are 50 firing nozzles in each printhead with 3.6 kHz firing frequency[12,13]. Therefore for a representative construct of 4 mm diameter and 4 mm height, the volume and thickness of each printed layer during layer-by-layer construction were 0.23 µl and 18 µm, respectively. The entire printing process took less than 4 min to construct the cartilage tissue (**Figure 1**).

Figure 2A shows an even cell distribution of printed chondrocytes in 3D scaffold due to simultaneous photopolymerization of the surrounding scaffold during cell deposition. By contrast, without simultaneous photopolymerization (scaffold polymerized after cell seeding), the deposited cells sank to the bottom or zonal interface instead of their initially deposited locations due to gravity (**Figure 2B**). This cell accumulation was also observed in previous reports of manual fabrication of cartilage tissue[14,15]. The printed human chondrocytes in 3D PEG hydrogel recovered chondrogenic phenotype and demonstrated gradually increased proteoglycan production during the culture (**Figure 3**)[3].

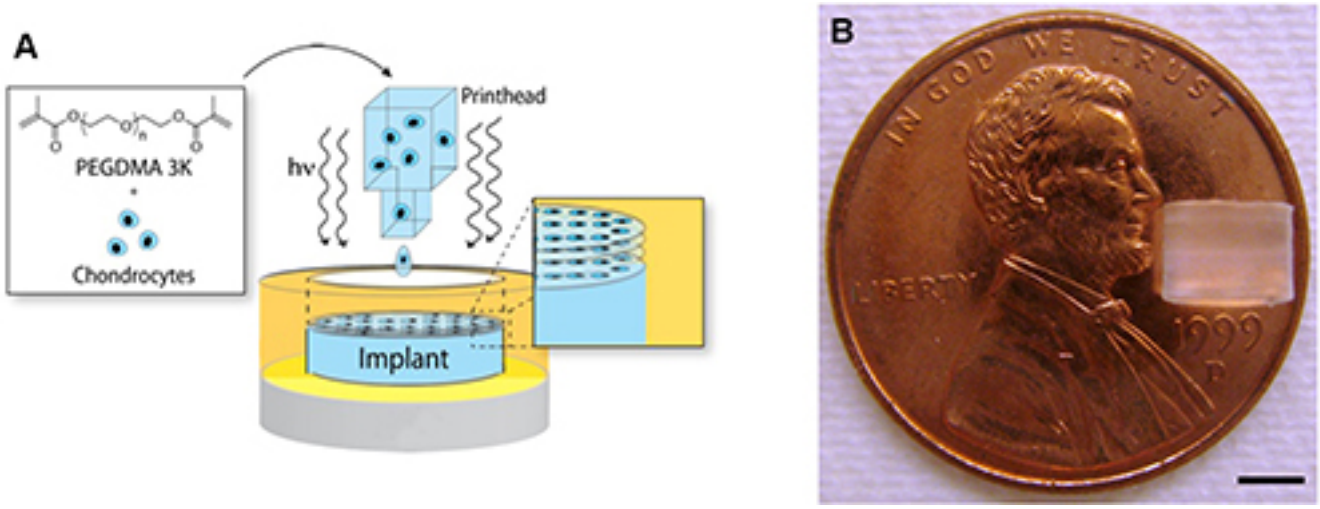

Figure 1. Printed neocartilage tissue. A) Schematic of cartilage bioprinting with simultaneous photopolymerization and layer-by-layer assembly. **B)** A printed neocartilage tissue with 4 mm in diameter and 4 mm in height. Scale Bar = 2 mm. Please click here to view a larger version of this figure.

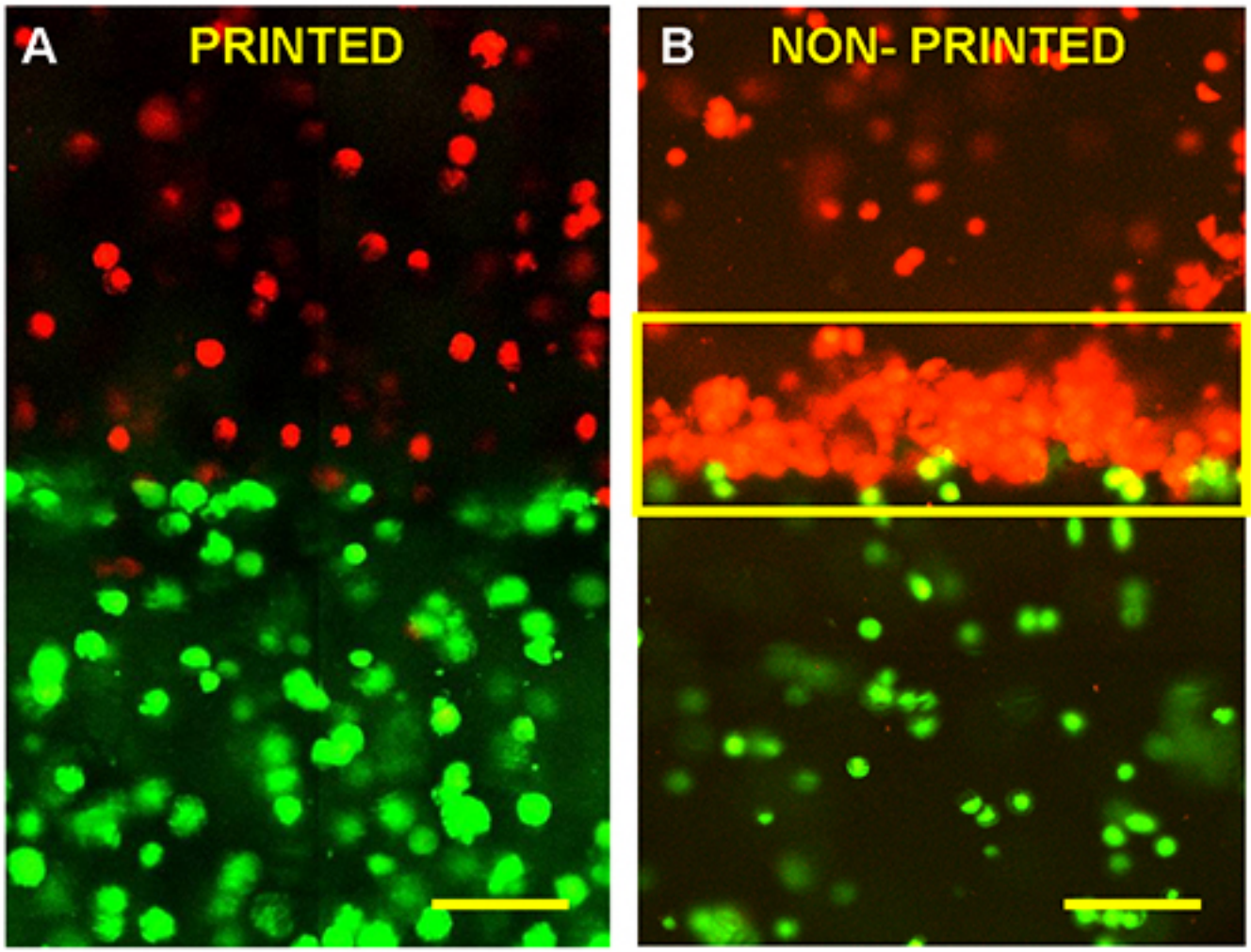

Figure 2. Chondrocytes labeled with green and orange fluorescent dyes demonstrated the zonal cartilage bioprinting feasibility. A) Printed cells maintained their initially deposited positions in the 3D hydrogel. The printing and photopolymerization process completed in 4 min with cell viability of 90% (n = 3). **B)** Cells accumulated to the bottom or interface due to gravity without simultaneous photopolymerization. It took 10 min of UV exposure to gel the construct with the same size of A with a cell viability of 63% (n = 3). Scale bars = 100 μm.

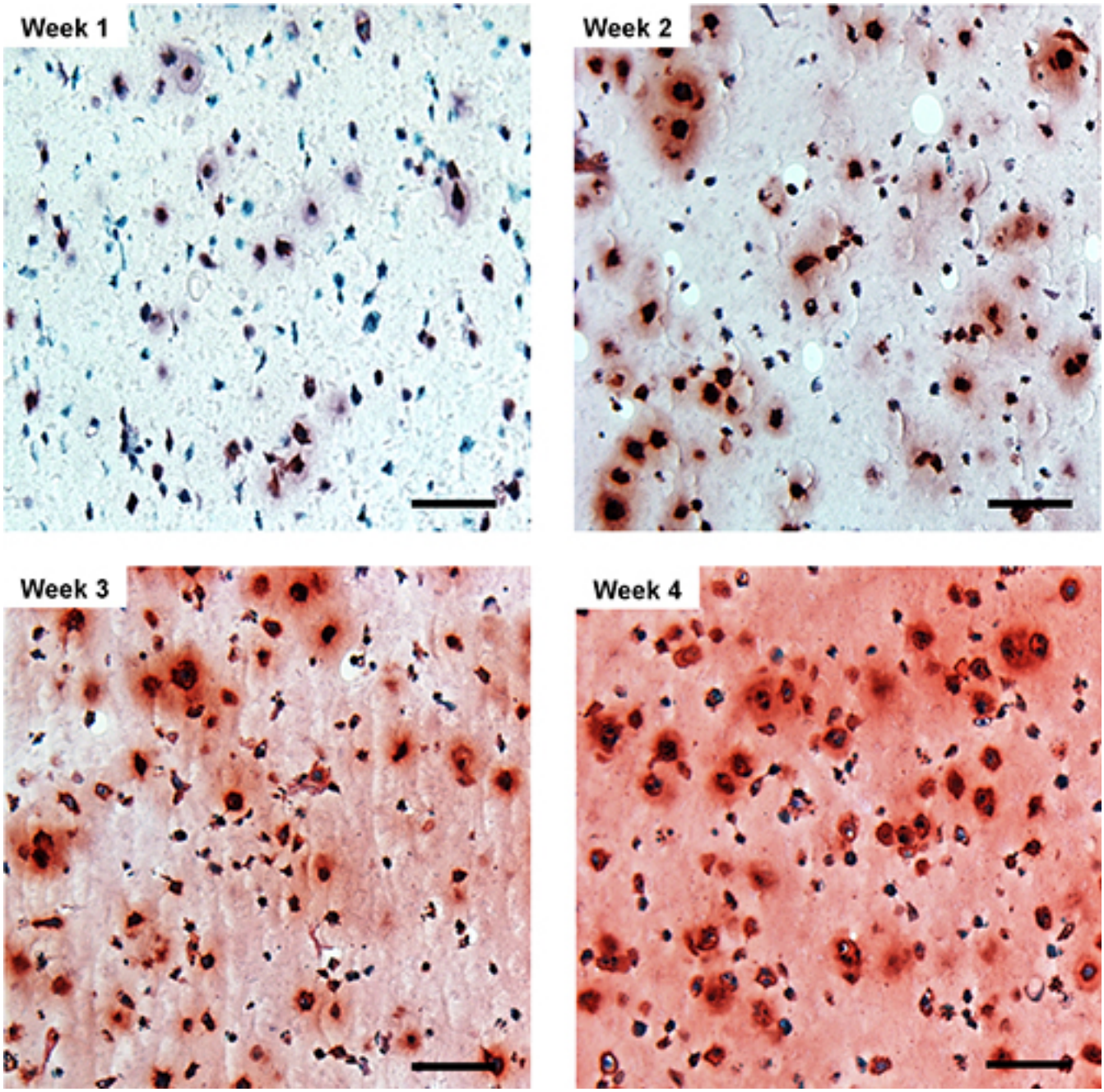

Figure 3. Safranin-O staining of printed chondrocytes in PEG hydrogel shows increased proteoglycan production during the culture. Scale bars = 100 μm.

Discussion

This 3D bioprinting system with simultaneous photopolymerization capacity provides a significantly greater printing resolution than the best previously reported method of *in situ* printing of osteochondral defects using syringe extruded a cellular alginate hydrogel[16]. Higher printing resolution is particularly critical for cartilage tissue engineering to restore the anatomic cartilage zonal organization. Simultaneous photopolymerization during layer-by-layer assembly is crucial to maintain precise deposition of cells and biomaterial scaffolds for 3D construction. Microfabrication with each printed layer also resulted in smooth transitions between zonal layers, minimizing the potential for degradation due to delamination. By precisely adjusting the bioprinting parameters and components of the bioink, we will be able to fabricate the complex 3D structures required to heal a wide variety of cartilage lesions.

With synergistic growth factor stimulation, the bioprinted neocartilage had the best chondrogenic phenotype and most cell proliferation[3]. Therefore, the cell seeding density used in this study, which is feasible for bioprinting, is also ideal for cartilage regeneration when treated with appropriate growth factors. Cartilage repair using autologous chondrocytes is greatly restricted in clinical applications due to the limited number of chondrocytes harvested in biopsy. Implanting the directly harvested autologous chondrocytes or mesenchymal stem cells (MSCs) along with

biomaterials for cartilage repair without monolayer expansion is exceedingly attractive. Therefore, it is critical to expand the printed cell numbers to the optimal cell density required for cartilage formation without compromising the quality of the cartilage matrix given the limited cell numbers. Furthermore, limiting initial cell density will greatly optimize and maximize the bioprinting resolution. Thus, the bioprinting method described here is fully compatible with the low cell numbers in clinical setting and has the potential to be used for cartilage tissue engineering.

In conclusion, our work demonstrates the feasibility of fabricating anatomic cartilage structures by delivering chondrocytes and biomaterial scaffold materials to precise targeted positions. A PEG hydrogel with human chondrocytes was continuously bioprinted via layer-by-layer assembly. Simultaneous photopolymerization maintained the printed cells at their initial deposited positions and reduced phototoxicity. Cells in the printed neocartilage maintained chondrogenic phenotype with consistent gene expression and biochemical analysis[2]. Therefore, this technology is a promising advance for anatomic cartilage tissue engineering.

Disclosures

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