Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications

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Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications

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Abstract

Bioprinting is an emerging technique used to fabricate viable, 3D tissue constructs through the precise deposition of cells and hydrogels in a layer-by-layer fashion. Despite the ability to mimic the native properties of tissue, printed 3D constructs that are composed of naturally-derived biomaterials still lack structural integrity and adequate mechanical properties for use in vivo, thus limiting their development for use in load-bearing tissue engineering applications, such as cartilage. Fabrication of viable constructs using a novel multi-head deposition system provides the ability to combine synthetic polymers, which have higher mechanical strength than natural materials, with the favorable environment for cell growth provided by traditional naturally-derived hydrogels. However, the complexity and high cost associated with constructing the required robotic system hamper the widespread application of this approach. Moreover, the scaffolds fabricated by these robotic systems often lack flexibility, which further restrict their applications. To address these limitations, advanced fabrication techniques are necessary to generate complex constructs with controlled architectures and adequate mechanical properties.

In this study, we describe the construction of a hybrid inkjet printing/electrospinning system that can be used to fabricate viable tissues for cartilage tissue engineering applications. Electrospinning of polycaprolactone fibers was alternated with inkjet printing of rabbit elastic chondrocytes suspended in a fibrin–collagen hydrogel in order to fabricate a five-layer tissue construct of 1 mm thickness. The chondrocytes survived within the printed hybrid construct with more than 80% viability one week after printing. In addition, the cells proliferated and maintained their basic biological properties within the printed layered constructs. Furthermore, the fabricated constructs formed cartilage-like tissues both in vitro and in vivo as evidenced by the deposition of type II collagen and glycosaminoglycans. Moreover, the printed hybrid scaffolds demonstrated enhanced mechanical properties compared to printed alginate or fibrin–collagen gels alone. This study demonstrates the feasibility of constructing a hybrid inkjet printing system using off-the-shelf components to produce cartilage constructs with improved biological and mechanical properties.

(Some figures may appear in colour only in the online journal)

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1. Introduction

Naturally-derived hydrogels such as collagen, fibrin and elastin have advanced the field of biomaterials in tissue engineering and regenerative medicine because their composition and structure resembles that of native tissue [1, 2]. Organ/tissue printing using inkjet technology is evolving into a practical method to fabricate viable 3D tissue constructs and has found widespread applications in the field of tissue engineering and regenerative medicine [3, 4]. The bioprinting approach often utilizes naturally-derived hydrogels as cell-printing solutions to construct tissues in layer-by-layer fashion due to their superior biocompatibility and low toxicity on the cells [5]. However, the low to moderate mechanical properties of these hydrogels hinder their utility in load-bearing tissue engineering applications, such as cartilage, because they are unable to provide proper 3D structural integrity during surgical procedures and the regenerative process [6].

Fabrication methods such as solid free form technology, which is based on computer-aided design/computer-aided manufacturing, has been employed to fabricate 3D scaffolds [7]. Despite its ability to control the scaffold porosity, the scaffold, which often is made of a synthetic polymer, does not provide the proper affinitive environment to the cells due to the polymer hydrophobicity properties [8]. Multi-head deposition systems (MHDS) offer the ability to fabricate hybrid scaffolds using both synthetic polymers and hydrogel materials. The hybrid scaffold provides a favorable environment within the hydrogel for the cells to grow and can possess adequate mechanical properties that can be altered depending on the synthetic polymer used during fabrication. Recently, MHDS was employed to fabricate hybrid scaffolds using synthetic polymers such as PCL and polyglycolic acid incorporated within hydrogels such as alginate, gelatin, and hyaluronic acid as a cell-printing solution [6, 8, 9]. While the ability to precisely deposit cells and materials has been well-established, most robotic precision deposition machines are often complex, expensive to manufacture and require special skills to operate. By combining synthetic polymers and natural hydrogels, these systems can offer a hybrid scaffold system, but the main frame of the scaffold, composed of bulk synthetic polymers, is very rigid and lack of flexibility, which might not be suitable for cartilage tissue engineering. Deposition of these components can also be achieved by inkjet printing system using off-the-shelf components and provides a platform in which multiple cell types can be deposited into a construct. This technique can provide many of the similar functions as a robotic deposition system but can be effectively utilized with little specialized equipment [10, 11]. Inkjet technology has been used in many applications to produce high density patterns of DNA for microarrays [12], microdepositions of proteins for enzyme assays [13] and high density arrays of bacterial colonies [14]. The technique has also been applied to high-throughput patterning of mammalian cells, including Chinese hamster ovary cells [15] and hippocampal cells [16]. Even more recently, it has been shown that the environmental conditions generated during the printing process, such as heat and shear stress, can be used to perform high-throughput transfection of living cells [17]. These experiments support the use of inkjet bioprinting as a low cost fabrication method and provide a glimpse of its future potential.

However, like many other bioprinting approaches, inkjet bioprinting technology utilizes hydrogels as the primary scaffold materials for printed cells [18]. Although a variety of tissue constructs can be fabricated with these gel-based scaffolds, these gels often have limited structural integrity due to their low mechanical strength [19, 20]. This limitation hinders development of printed constructs for use in load-bearing tissue engineering applications, such as those designed to repair or replace cartilage and bone.

We hypothesized that these mechanical limitations associated with printed hydrogel constructs could be overcome by using inkjet printing techniques in conjunction with electrospinning, which is another fabrication technique that can create nanofibrous scaffolds of synthetic polymer. By combining flexible mats of electrospun synthetic polymer with traditional inkjet printing in a layer-by-layer fashion, functional tissue constructs with enhanced mechanical properties could be fabricated. In this study, we first determined whether electrospinning could be employed in conjunction with inkjet-based cell printing to produce constructs with enhanced mechanical properties that would be suitable for cartilage tissue engineering in a proof-of-concept design. We then evaluated the functionality of the resulting constructs in terms of four parameters: cell survival, maintenance of the layered structure, mechanical properties, and formation of cartilage-specific extracellular matrix. Generation of organized layered cartilage constructs with improved biological and mechanical properties would have numerous applications clinically. In particular, to meet the depth-dependent cellular and matrix properties properties of articular cartilage, biomimetic zonal cartilage repair and regeneration using modular hydrogel systems has been envisioned [21]. Our hybrid method allows for control over construct organization and might have potential to these applications involving complex zonal properties to tissue-engineered constructs for cartilage repair.

2. Materials and methods

2.1. Materials

Poly(ε-caprolactone) (PCL) (704105, average $M_n \sim 42,500$), Pluronic F-127 (P2443), fibrinogen from bovine plasma (F8630) and thrombin from bovine plasma (T4648) were all purchased from Sigma-Aldrich (St. Louis, MO). Collagen type I derived from rat tail (354236) was purchased from BD Biosciences (Billerica, MA). All reagents were chemical grade.

2.2. Construction of the hybrid printing system

To build cartilage constructs, we developed a novel hybrid printing system by incorporating an electrospinning apparatus into an inkjet printing platform. Figures 1(A) and (B) show the schematic drawing of this hybrid printing system and the actual hybrid printing system after construction, respectively.
The inkjet printing platform is composed of a customized XYZ plotter driven by step motors. The printhead was equipped with a dc solenoid inkjet valve (Offshore Solutions Inc, Beaverton, OR). Reservoirs for loading the cell suspension to be printed were connected to the inkjet valve. The cell-printing solution was supplied to the inkjet valve from the reservoirs using pressurized air. The printhead was mounted over a XYZ plotter platform to allow deposition of cells onto the scaffold generated by electrospinning. The XYZ plotter was positioned under the printhead via a customized controller. The controller acquires the positioning information from custom software built on the .NET Framework 2.0 (Microsoft Corp, Redmond, WA). The software converts the image of the target to a special four byte protocol, which is used to activate a specific inkjet valve and coordinate X–Y–Z position.

In this hybrid system, the electrospinning apparatus is used to generate a polymeric fiber-based scaffold. An electrospinning head, in tandem with the inkjet head, was also mounted over the XYZ plotter. The power supply, which is used to provide a high voltage field for electrospinning, was modulated by the same customized controller. To prevent the potential adverse effects of static discharge on the logic circuitry of the customized controller, the controller was conductively isolated from the high voltage power supply through fiber optics.

### 2.3. Preparation of polymer solution

PCL (10% wt/vol) and Pluronic F-127 (5% wt/vol) were dissolved in acetone under gentle stirring in a warm water bath (50 °C). Acetone was used as a solvent because it is a natural byproduct of ketone body synthesis in the liver. Pluronic F-127 was added to the polymer solution to reduce the viscosity of molten PCL and also improve the hydrophilicity properties of the resulting electrospun PCL scaffold [22]. The final polymer solution was loaded into the polymer reservoirs of the printing system.

### 2.4. Isolation and culture of rabbit articular chondrocytes

Chondrocytes were obtained from rabbit ear cartilage as described previously [23]. Briefly, the rabbit was sedated and the ear was shaved, cleaned with povidone-iodine, and draped. A pocket was resected under sterile conditions at the subperichondrial level and an approximately 2 × 2 cm piece of cartilage was removed. Biopsy specimens were washed in phosphate-buffered saline (PBS) and finely minced under sterile conditions. To dissociate chondrocytes from the cartilage tissue, the minced cartilage was incubated in Ham’s F12 medium with glutamine/bicarbonate supplemented with 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 2.5 μg ml⁻¹ amphotericin B (Gibco-BRL), and 1 mg ml⁻¹ collagenase type B (Boehringer Mannheim GmbH, Germany) at 37 °C on an orbital shaker. After 24 h, the undigested pieces were discarded and the supernatant was collected and distributed onto fresh tissue culture dishes. Every dish was filled with 20 ml of fresh Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1% antibiotic-antimycotic (ABAM) and 1% L-glutamine. Cell cultures were incubated in a humidified incubator in 5% CO₂ at 37 °C. Culture media was changed every three to four days. To prepare the cell printing solution, cell pellets at passage 6 were re-suspended in a mixture of fibrinogen (10 mg ml⁻¹) and rat tail collagen type I (1.5 mg ml⁻¹) in PBS at a concentration of 3–4 × 10⁶ cells ml⁻¹ and loaded into cell reservoirs.

### 2.5. Fabrication of cartilage constructs

A layer of PCL was electrospun for 30 min at 3 ml h⁻¹ at 20 kilovolts (KV) through an 18.5 gauge blunt needle syringe. The syringe was placed 8 cm above a sterile 10 cm petri dish containing sterile PBS. A sheet of aluminum foil was placed directly under the petri dish and to act as the ground for the system and force the PCL to spin directly into the PBS. Wet electrospinning was necessary because dry PCL/F-127 rapidly absorbs water from printed constructs and leads to desiccation of the cells. After spinning a
layer of PCL, 3 ml of the cell-printing solution containing chondrocytes/fibrinogen/collagen was printed through the inkjet valve onto the electrospun PCL fibrous scaffold layer to form a cell/matrix layer. The cell-printing solution was allowed to gel through subsequent printing of 1.5 ml thrombin (20 U ml$^{-1}$ dissolved in PBS) on the cell/matrix layer to create a transient clot for structural integrity while spinning. The thrombin layer was allowed to react with the fibrinogen for 15 min. Subsequently, PCL was spun again for another 10 min at 3 ml h$^{-1}$ followed by inkjet printing of the cell-printing solution and thrombin. A final layer of PCL was spun for 30 min at 3 ml h$^{-1}$. The final construct consisted of five alternating layers of PCL/F-127 (three electrospun layers) and chondrocytes (two gel layers) with an approximate thickness of 1 mm. Each of the two gel layers was approximately 150 μm, and the central electrospun layer was about 100 μm, and the top and bottom electrospun layers were approximately 300 μm thick. The design that central layer of PCL/F-127 was thinner than the outer electrospun layers was to facilitate more communication of the cells in the two gel layers and subsequent integration of the cells upon the degradation of the electrospun layers. The top and bottom electrospun layers were designed to provide the mechanical property of the hybrid construct. It was observed that the thickness of the constructs was reduced to approximately 0.4 mm as the fibrin degraded and left the collagen matrix behind. Each construct was square with a 10 cm diagonal. Cartilage constructs were then incubated in DMEM medium supplemented with 10% FBS, 1% ABAM and 1% L-glutamine in 5% CO$_2$ at 37 °C.

Constructs without cells were fabricated using the same method of alternating PCL/F-127 and the fibrinogen/collagen mixture. PCL/F-127 control constructs were fabricated using the same electrospinning method without the fibrinogen/collagen mixture. For the purpose of comparing the printed-electrospun constructs with commonly used hydrogels, alginate and fibrin/collagen hydrogels were used as controls. Alginate controls were prepared by mixing 2% wt/vol sodium alginate (MVG; Pronova Biomedical, Oslo, Norway) with an overall glucuronic acid (G-block) content of 70% (as recommended by the manufacturer) with PBS. Solution was gelled by mixing with 0.25 M CaCl$_2$ and pulled through an even diameter tube before gelling was complete to ensure a uniform thickness through the construct. Collagen/fibrin hydrogels were fabricated by plating the mixture of collagen and fibrin without cells in a petri dish marked to 0.4 mm to ensure an even thickness and measuring the resulting gel with digital calipers.

2.6. Evaluation of cell viability

The viability of chondrocytes within the fabricated cartilage constructs was evaluated at one week after printing by a two-color fluorescence live/dead assay (Molecular Probes, OR) following the manufacturer’s protocol. Briefly, triplicates of printed cartilage constructs were rinsed with PBS to remove residual serum and incubated for 30 min in 5% CO$_2$ at 37 °C with 2 ml of 2 μM calcein AM and 4 μM ethidium homodimer (EdD-1) dissolved in 10 ml PBS solution. Constructs were then rinsed with PBS and viewed using a fluorescent microscope at 200× magnification. Cell viability was assessed by randomly examining five representative fields per sample with a minimum of 50 cells per field, counting the number of live cells stained with calcein AM (green) and comparing this number to the total number of cells.

2.7. Microstructure of cartilage constructs

The microstructure of the cartilage constructs was evaluated using scanning electron microscopy (SEM). Samples were lyophilized and sputter coated with gold. Cross-sectional electron-micrographs were obtained at 200×, 500×, and 4000× magnification at 20.0 kV and 50 Pa using a Hitachi S-2600 scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA).

2.8. Mechanical properties of printed hybrid constructs and other hydrogel constructs

Mechanical properties of hybrid constructs without cells, pure PCL/F-127, alginate, and collagen/fibrin gel were evaluated in fully hydrated conditions using an Instron uniaxial load frame (Instron Corporation, Issaquah, WA). Rectangular samples (n = 3) of 6 mm long and 4 mm wide were prepared from each construct. The thickness of each sample was measured separately using digital caliper and reported as average ± standard deviation (SD). Hybrid samples had a thickness of 0.38 ± 0.055 mm, alginate samples 0.36 ± 0.05 mm, PCL samples 0.29 ± 0.05 mm and collagen/fibrin samples 0.4 mm. The samples were allowed to stretch at 0.2 mm s$^{-1}$ crosshead speed until failure. Ultimate tensile strength (UTS) was calculated at the maximum load immediately before breaking. Young’s modulus was calculated from the slope of the linear segment of the stress–strain curve. Values were presented as average ± SD.

2.9. In vitro evaluation of hybrid cartilage constructs

Hybrid printed cartilage constructs were cultured in vitro to evaluate the cellular organization and proliferation within each construct. In addition, extracellular matrix deposition and organization were observed over time. Hybrid constructs were fabricated as described and cultured in DMEM supplemented with 10% FBS, 1% ABAM and 1% L-glutamine in 5% CO$_2$ at 37 °C for four weeks. Printed hybrid constructed without cells were used as controls.

2.10. In vivo evaluation of hybrid cartilage constructs

All animal procedures were approved by the Wake Forest University Health Sciences Animal Care and Use Committee. For the in vivo assessment, printed hybrid constructs were prepared, cultured in vitro for two weeks and implanted subcutaneously in immunodeficient mice (nu/nu) (Charles River Laboratories). An unseeded control hybrid scaffold also was implanted in each mouse. The constructs were implanted in the mice for two, four, and eight weeks. Six animals were used to evaluate the performance of the hybrid
cartilage constructs with two at each time point. Each mouse was anesthetized with isoflurane and two small incisions were created on the dorsa. Hybrid cartilage constructs were subcutaneously inserted in the first incision. The control hybrid constructs without cells were similarly inserted in the second incision. After two, four, and eight weeks of implantation, the incisions were closed and mice were examined. At the end of study, constructs with 6 of the 12 experimental groups were explanted from the mice and prepared for histological analysis.

2.11. Histological evaluation

Samples from printed hybrid constructs with cells and without cells from in vitro and in vivo experiments were processed for histological analyses. Samples were fixed overnight with 10% formalin. Fixed samples were then embedded in paraffin and cut into 3–5 μm thick sections. Masson’s Trichrome (MT) staining for collagen and Safranin O (Saf O) staining for glycosaminoglycans were performed to evaluate the deposition and organization of extracellular matrix in the printed hybrid constructs.

To distinguish collagen that had been deposited by chondrocytes in the hybrid constructs from the collagen used to print the constructs, sections were immunostained with anti-type II collagen antibodies (Dako, Denmark). Immunostaining was performed using a DakoAutostained Universal Staining System (Dako, Denmark). Slides were rinsed with Dako Wash Buffer and incubated in 100 μl of 3% hydrogen peroxide for 30 min. After rinsing twice in buffer, sections were incubated with 100 μl of 10% Dako ready-to-use protein block serum-free for 30 min and samples were left to air dry. Sections were then incubated with 100 μl of mouse anti-type II collagen antibody at 1:200 dilution for 90 min, and this was followed by two washes in buffer. Sections were incubated again with blocking solution for 30 min and then incubated with 100 μl of biotin-labeled rabbit anti-mouse antibody at a 1:200 dilution for 30 min. Sections were rinsed twice with buffer and biotin was detected using streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA).

2.12. Statistical analysis

Statistical analysis was performed using the Student t-test. Values were represented as average ± SD where applicable and p <0.05 was considered to be significant.

3. Results and discussion

3.1. Morphology and microstructure of hybrid electrospun/inkjet printed constructs

We have developed a novel hybrid fabrication system that combines both electrospinning and inkjet bioprinting to achieve simultaneous high throughput cell patterning and controlled scaffold production, thus overcoming one of the major limitations of inkjet printing alone. Electrospinning of PCL was incorporated in this hybrid printing system because PCL scaffolds have excellent mechanical properties and because electrospinning allows the composition of the polymers to be easily controlled to produce highly organized porous structures that encourage cellular infiltration and integration into the surrounding tissue [24–26]. Furthermore, manipulation of the electrospinning technique can produce highly aligned fibers [27] that can be used to direct the growth of muscle [28], nerves [29], and cartilage [30]. Like inkjet printing, electrospinning is a low cost fabrication technique with immense potential for tissue engineering. By combining electrospinning with inkjet printing, we were able to obtain a tissue construct consisting of layers of electrospun PCL and printed hydrogels containing cells. This layered structure could have been achieved by alternating electrospinning and inkjet printing in separate machines. However, the combined hybrid system allows production of extremely thin patterns in the electrospun and cellular layers that would not survive repeated movements between machines. In addition, the hybrid system maintains the sterility of the printing field by eliminating transfers between machines. Together, the electrospinning and printing apparatuses create a system with greater capabilities than either apparatus alone.

The printed hybrid constructs produced using our system had adequate mechanical integrity to maintain the structure of the five printed layers as shown in figures 2(A) and (B). Microstructural analysis of the printed hybrid constructs using SEM demonstrated the presence of intact layers of cell/collagen matrix between the sheets of PCL as shown in figure 2(C). Measurements of the electrospun fibers showed an average fiber diameter of 422 ± 62 nm and a pore size smaller than 5 μm. The microstructure of the PCL at high magnification appeared fibrous, and occasional beads were present (figure 2(D)). Formation of beads occurs when the extruded polymer fails to form fibers and instead clusters on the collection screen. This phenomenon is directly related to the viscosity and surface tension of the polymer solution and is inversely related to the charge density of the solution. However, this can be avoided by increasing the concentration of polymer in the solution and slowing the flow rate of the solution through the needle tip. Beading can be controlled to generate structures with porous membranes and cups [31]. Thus, this technique may be used to finely tune the scaffold architecture for different tissue types.

To visualize the attachment of chondrocytes to the collagen matrix, the top layer of PCL was removed from the construct and the cells were visualized under SEM. Chondrocytes appeared to be firmly attached and spread out on the collagen matrix between the sheets of PCL as shown in figure 2(E). SEM demonstrated that the layered structure of the construct could be maintained in vitro. However, we experienced layer separation in two constructs immediately after printing because the electrospun and printed layers were not well anchored to each other.

Our approach is very similar to recent work performed by Yang et al [32], in which skin constructs were produced by manually seeding fibroblasts and keratinocytes on wet electrospun layers. Other similar studies have been performed using electrospayed cells and fibers, which allow a better infiltration of cells into the scaffold [33, 34]. In our technique, direct encapsulation of cells between fiber mats ensures that cells are evenly distributed through the gel layers within the
scaffold, but the cell growth, tissue formation, mechanical properties and functional expression are difficult to control due to the limitations of this technology. These constructs could be adequate for use in engineering some tissues, but the cell placement is not specific enough to build tissues and organs with complex structural and functional requirements. The major advantage of our technique over previous work is that we are using inkjet printing to precisely control deposition of cells in complex patterns within the layers. This is an ability that no other system has been able to demonstrate and illustrates the power of this novel system. The architecture of electrospun scaffolds could already be controlled by manipulating the polymer flow rate, needle size, voltage, and electric field to organize layered tissue constructs in the Z direction. The current prototype we used for this study needed lengthy time to fabricate the constructs. This is mostly due to the time required to electrospin the PCL scaffolds. Further development is necessary to build a high-throughput system that could fabricate constructs in a reasonably acceptable time.

3.2. Cell viability

Printed hybrid constructs with chondrocytes were analyzed using the Molecular Probes™ viability/cytotoxicity assay. The live/dead assay indicated that after one week in culture, $81.58 \pm 3.46\%$ of the chondrocytes in the printed hybrid constructs were viable. This suggests that the combined electrospinning/printing process did not affect the viability of the cells, which showed similar viabilities at similar time points in previous experiments [15]. The electrospinning process did not significantly affect the printed cells, likely because the collection plate acted as the ground and prevented the cells from being directly exposed to the voltage. Furthermore, other studies have shown that cells can be directly deposited at high voltage with no apparent detrimental effects [30, 31].

3.3. Mechanical testing

To evaluate the structural and mechanical properties of the constructs, we examined the Young’s modulus and UTS of each construct and compared these to the values for constructs fabricated with PCL alone by electrospinning and for constructs produced with alginate, the scaffolding material typically used in bioprinting. Mechanical testing demonstrated that the printed hybrid constructs were stiffer and were able to withstand greater tensile stress than alginate, collagen/fibrin or electrospun PCL/F-127-only constructs (table 1). The Young’s modulus of the printed hybrid constructs (1.76 MPa) was four
times higher than that of alginate constructs (0.41 MPa) \((n = 4, p = 0.008)\). Similarly, the UTS of the printed hybrid constructs (1.11 MPa) was five times higher than the UTS of the alginate constructs (0.26 MPa) \((n = 4, p = 0.003)\). Neither the strength nor the stiffness of the printed hybrid constructs was significantly different from those measured for pure PCL/F-127 electrospun fibers \((n = 4, p = 0.067)\). This observation was expected, as the collagen/fibrin gel has very low mechanical strength and generally does not contribute to the combined mechanical properties of the construct. When tested, collagen/fibrin constructs indeed displayed such low tensile strength that the stress-strain profile was indistinguishable from the noise signal generated by the testing machine. These mechanical testing results confirmed our hypothesis that the hybrid constructs would demonstrate increased structural stability over the alginate or fibrin/collagen hydrogels. Both Young’s modulus and the UTS of the constructs were significantly greater than alginate because the constructs took on the structural properties of the PCL. While the observed beading in the PCL reduced the tensile strength of woven electrospun fibers by forming stress defects [35], the hybrid structures still showed better mechanical properties over the alginate controls. Boland et al evaluated the Young’s modulus and UTS of printed alginate constructs to be 89 ± 47 KPa and 56 ± 12 KPa, respectively and were comparable to our alginate mechanical values [36]. The mechanical properties of the hybrid cartilage constructs were lower than those reported for native human cartilage (Young’s modulus 4.1 MPa) [37]. These properties are expected to improve during the tissue regeneration process as cells deposit and align their own ECM. Moreover, the initial mechanical properties of the printed hybrid constructs can be easily improved by changing the diameter, porosity and/or orientation of electrospun PCL fibers.

### 3.4. Histological analysis

Having demonstrated that cells were able to survive the printing process and that the printed hybrid constructs were indeed stronger than standard alginate scaffolds, we examined ECM production by the printed chondrocytes in order to determine if the cells retained some or all of their functional characteristics. The main components of elastic cartilage are collagen (types II, VI, IX, X, and XI), glycosaminoglycans (hyaluronan, chondroitin sulfate, and keratin sulfate), and elastin. Of these, type II collagen and the glycosaminoglycans were expected to be most visible in the fabricated constructs. Analysis of sections after two weeks in vitro showed the initiation of cartilage tissue formation, but there was very little cartilage tissue visible. We hypothesize that the lack of ECM deposition at two weeks in vitro was due to lack of nutrient diffusion within the constructs. However, histological analysis confirmed that the chondrocytes were producing the matrix components of cartilage tissue after four weeks of in vitro culture (figure 3). When printed hybrid constructs containing cells were compared to printed hybrid constructs without cells at four weeks, H&E staining showed that a significant population of homogenously organized chondrocytes existed within the matrix of the printed hybrid constructs with cells (figure 3B) compared to the negative control constructs (figure 3A). MT staining demonstrated the clear presence of well-dispersed collagen bands accumulated around the cells (figure 3D). Immunohistochemical analysis revealed formation of type II collagen in the printed hybrid constructs. This was not present in the print mixture and is a major component of cartilage (figures 3G and H). Saf O staining demonstrated the presence of GAGs only in the printed hybrid constructs with cells (figures 3F and E). Interestingly, glycosaminoglycans and type II collagen, the two important components of cartilage, seemed to be concentrated at the edges of the constructs at four weeks in vitro, suggesting that the cells in the center of the construct were less functionally active, possibly due to limited diffusion of nutrients and growth factors from the media to the center of the construct [32, 38]. The parameters of the electrospinning in this experiment generated nanofibers with very low porosity, which may have prevented nutrients in the media from diffusing through these pores. Altering the parameters of the electrospinning would change the porosity of the layers, but a very dense lower layer of PCL should be maintained to prevent the cells from falling through the construct. Alternatively, a mixture of nano- and microfibers may be useful to enhance the diffusion of nutrients and growth factors at the core of the constructs.

**Table 1.** Young’s modulus and UTS of pure alginate gel, electrospun pure PCL, hybrid PCL/collagen/fibrin scaffolds, and collagen/fibrin matrix. Data were presented as average ± SD \((n = 3)\).

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<tr>
<th>Young’s modulus (MPa)</th>
<th>UTS (MPa)</th>
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<tbody>
<tr>
<td>Alginatene</td>
<td>0.41 ± 0.06a</td>
</tr>
<tr>
<td>PCL</td>
<td>0.77 ± 0.22</td>
</tr>
<tr>
<td>Scaffold</td>
<td>1.76 ± 0.71a</td>
</tr>
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<td>Matrixb</td>
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*a p < 0.05 versus hybrid scaffold by two-sample t-test.

**b** Mechanical strength of collagen/fibrin matrix was too low to be distinguished from random noise.

**In vivo** evaluation of explanted tissues after 8 weeks showed dense and well organized collagen formation in the printed hybrid constructs compared to very minimal formation of collagen in the controls (figures 4A and B). The structure of the GAGs appears to resemble typical elastic cartilage with areas of dense GAGs interspersed with areas of little deposition (figure 4D). Most importantly, constructs recovered from mice showed rounded chondrocytes with lacunae that are typical of normal elastic cartilage. Type II collagen was also visible in the printed constructs (figure 4F). Control constructs showed no presence of collagen type II or GAGs after eight weeks of implantation (figures 4C and E). Constructs retrieved after eight weeks in vivo had large amounts of collagen and GAGs deposited throughout the entire construct and appeared histologically similar to normal elastic cartilage. This demonstrates that the cells were able to maintain their normal phenotype and produce ECM in vivo despite the in vitro limitations.
Figure 3. Histological analysis of in vitro printed constructs with cells (printed) and without cells (control) at four weeks. Printed samples are five layer constructs alternating PCL and cells in a collagen/fibrin gel. Control samples are five layer constructs alternating PCL and collagen/fibrin gel. (A) Control sample stained with H&E shows no presence of cells. (B) H&E staining of the printed constructs after four weeks in culture. Dark blue staining indicates cell nuclei. (C) Control sample stained with MT shows no blue collagen. (D) MT staining of the printed constructs after four weeks in culture. Dark staining shows cell nuclei. Blue staining demonstrates presence of collagen. (E) Control sample stained with Saf O shows only green counterstain and no red GAGs. (F) Saf O staining of the printed constructs after four weeks in culture. Dark staining indicates cell nuclei and red shows production of GAGs. (G) Control sample labeled with anti-type II collagen antibody demonstrating only counterstain. (H) Anti-type II collagen antibody labeling a section after four weeks in culture. Brown staining indicates production of type II collagen. Magnification: 200×. Scale bars: 100 μm.

Figure 4. Histological analysis of in vivo printed constructs with cells (printed) and without cells (controls) at eight weeks after implantation. Printed samples are five layer constructs alternating PCL and cells in a collagen/fibrin gel. Control samples are five layer constructs alternating PCL and collagen/fibrin gel. All samples were cultured for two weeks prior to implantation. (A) Control sample stained with MT shows no blue collagen. (B) MT staining of constructs implanted in vivo for eight weeks demonstrating large bands of collagen. (C) Control sample stained with Saf O shows no red GAGs. (D) Saf O staining of in vivo constructs shows significant GAG production. (E) Control sample labeled with anti-type II collagen antibody demonstrating only counterstain. (F) Anti-type II collagen antibody labeling a section after eight weeks in vivo. Brown staining indicates production of type II collagen. Magnification: 200×. Scale bars: 100 μm.

3.5. Future direction

Cartilage constructs similar to those created in this experiment could be applied clinically by using MRI images of a defect in the knee as a ‘blueprint’ to bioprint a matching construct. Careful selection of scaffold material for each patient’s construct would allow the implant to withstand mechanical forces while encouraging new cartilage to organize and fill the defect. This technique can also be extended to other tissue types. Although electrospun scaffolds can direct chondrocytes
growth [30], they can also direct the formation of myotubes to enhance strategies for muscle tissue engineering [28]. The use of hybrid system allows creation of highly aligned electrospun fibers and targeted application of myotubes. This technique can then be used to layer patterns of endothelial cells and nerve cells on the aligned myotubes to create functional muscle. By overcoming one of the major obstacles associated with conventional bioprinting, we have expanded the utility of biofabrication technology.

Conclusions

This study demonstrates the feasibility of generating layered cartilage constructs using a combination of electrospinning and inkjet printing. The hybrid scaffold demonstrated enhanced mechanical properties compared to conventional hydrogel constructs generated using inkjet printing alone. Printed cells maintained their viability and produced cartilage-hydrogel constructs generated using inkjet printing alone. This work indicates that the hybrid electrospinning/inkjet printing technique is a promising new technology that could simplify production of complex tissues.

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