Bioprinting Tissue Analogues with Decellularized Extracellular Matrix Bioink for Regeneration and Tissue Models of Cartilage and Intervertebral Discs

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The recent convergence of decellularized extracellular matrix (dECM) methodologies and 3D bioprinting (3DBP) has led to multiple advancements in tissue engineering scaffold design by enabling researchers to recreate a tissue-specific 3D environment in a custom geometry. The application of 3DBP of dECM for articular cartilage and intervertebral disc (IVD) repair, however, is still burgeoning. While cartilage and IVD tissue each possess unique architectures, they are composed of similar macromolecules and therefore pose similar challenges for the successful application of their matrix bioinks. Herein, the state-of-the-art in cartilage and IVD dECM bioink preparation, material properties, and applications are highlighted. The current major obstacles regarding optimal decellularization and printing methods are discussed, which need to be overcome to enable recapitulation of the hierarchical organization, zone-specific matrix composition, and anisotropic biomechanical behavior of the native tissues. Finally, a vision is presented for how this field may continue to evolve in the future to empower the fabrication of scaffolds that serve as effective templates for guiding cellular assembly and organization toward the formation of functional cartilage and IVD tissues.

1. Introduction

Articular cartilage and intervertebral disc (IVD) degeneration are clinically relevant problems which are major sources of disability and socioeconomic burden. Despite the serious impact of conditions like low back pain (LBP), osteoarthritis (OA), and rheumatoid arthritis (RA), there are no clinically effective treatments, with palliative care primarily used to delay invasive and suboptimal surgical interventions. Tissue engineering is a major area of research and development with the purpose of supporting the formation of repair tissue that closely mimics native tissue. However, tissue engineering of cartilage and the IVD has little to no clinical application at the present time and has proven to be a significant challenge. Besides both tissues being avascular with limited innate repair capacity, they are characterized by complex spatial arrangements of collagen fibers and glycosaminoglycans (GAG) which impart unique biomechanical functionality. Considering these factors, it is not surprising that attempts to re-establish healthy structure and function to cartilage and the IVD have yet been unsuccessful.

In the current tissue engineering strategies, cells are traditionally seeded into prefabricated biomaterial scaffolds, and with the aid of appropriate biochemical and mechanical factors, they produce targeted extracellular matrix (ECM). Recently, there has been an emerging research focus on using the tissue microenvironment for guiding cell behavior to engineer more biomimetic tissues. In fact, rather than using single-component biomaterial polymers hyaluronic acid,[1] collagen,[2] alginate,[3] and polyethylene glycol (PEGs),[4] many researchers have shifted to using decellularized extracellular matrix (dECM). The case for using dECM matrices is supported by studies in multiple areas, including liver,[5] cardiac,[6] respiratory,[7] nerve tissue,[8] bone,[9] and muscle.[10] The ECM is composed of tissue-specific cytokines, growth factors, and macromolecules, all of which provides microenvironmental cues that regulate cell behavior.[11] Post decellularization, dECM retains a portion of these ECM components, thus enhancing cell function compared to natural and synthetic single-component biomaterials.[12]

Concurrent with the emergence of new dECM-based scaffold materials, 3D bioprinting (3DBP) has received considerable attention and become a vital tool in advancing regenerative medicine.[13] 3DBP is defined by Guillemot in 2010[14] as "the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bioengineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies." As a cost effective and rapid method of fabricating scaffolds, 3DBP allows for the capture of tissue macrostructure. It enables the precise spatial placement of various biomaterial types, encapsulated bioactive molecules, and cells. Consequently, 3DBP has been extensively used for the creation of customized implants for tissue and organ repair,[15] high throughput screening of therapeutics[16] and cancer models.[17] Significantly, the work of Pati et al. in 2014 marked the first development of bioinks
from cartilage, fat, and cardiac dECM into anatomically relevant sizes, shapes, and architectures.[28]

While still in its early stages of application for cartilage and the IVD, the technological integration of 3DBP and dECM holds significant potential for improving scaffold design. This review aims to highlight general methods of dECM bioink preparation and printing methodologies. In addition, it details recent work on the optimization of cartilage and IVD decellularization processes, which entail special challenges compared to other tissues in terms of balancing adequate removal of cellular components while retaining the matrix components. Next, recent applications of these bioinks and their properties are described. Since cartilage and IVD bioink design for 3DBP is still a flourishing field, the path forward is discussed in terms of next generation macro and microscale constructs that will advance tissue regeneration and disease modeling for clinical treatment of cartilage and IVD degenerative conditions.

2. Background

2.1. Anatomy, Composition, and Pathophysiology of Cartilage and Intervertebral Disc

The composition and organization of cartilage and IVD play an integral role in their function. Cartilage tissue is predominantly composed of 65–80% water depending on the position,[19] collagen (~25% wet weight), and proteoglycans (PGs, ~5% wet weight).[20] The PGs are dispersed along the collagen fibers and oriented in the same direction as the collagen fibers.[20] Importantly, the ultrastructure of cartilage ECM varies depending on the zone (Figure 1A,B). The superficial zone is primarily composed of collagen fibers (type II and IX). The fibers are oriented parallel to the articular surface, imparting tensile and shear properties to cartilage.[21] Deep to the superficial zone is the middle zone, composed of collagen II and aggrecan. Here, the collagen fibers are thicker than in the superficial zone and arranged in a slanted fashion, providing compressive resistance to cartilage. The deep zone contains the highest amount of PG and the collagen fibers have the largest diameter and are arranged in a columnar orientation, perpendicular to the joint line. Consequently, this zone of cartilage has the highest compressive strength. The tidemark marks the transition to the calcified cartilage and is identifiable histologically by a differing staining pattern than the middle and deep zones. The calcified zone is a stiff mineralized tissue that serves as the junction to bone.[20] Here, the collagen fibers, composed of collagen type II, type IX,[22] and type X,[23] are oriented vertically and directly connected to bone collagen fibers.[24] A single cell type, chondrocytes, exists in cartilage. Chondrocytes are present at the highest density in the superficial layer of cartilage, where they have a flat morphology. In the middle and deep zones, they have a round morphology.[25]

The IVD is composed of two distinct regions, the central gelatinous nucleus pulposus (NP) and the peripheral, multilayered annulus fibrosus (AF) (Figure 1C,D). This composite structure lies sandwiched between two hyaline cartilage endplates. The composition and structure of the IVD varies radially across its structure. Regarding collagen content, the IVD contains types I, II, III, V, VI, IX, XI, XII, and XIV.[24] Types I and II comprise 80% of the total collagen in the disc, followed by type VI, which comprises 10 to 20%.[27] Progressing from inner to outer IVD, the proportion of type II declines and that of type I increases such that the outer AF is predominantly type I collagen. PG content is richer in the central NP compared to the peripheral AF.[28] The majority of PGs in the IVD is aggrecan,[29] which plays an important role in the weight bearing properties of the tissue. Structurally, the NP consists of randomly oriented collagen fibers dispersed in an aggrecan gel with elastin fibers at the periphery.[30] The AF is made up of 15–20 concentric rings, called lamellae,[31] formed from directional collagen fibers oriented at angles of ±30° to 45° from outer to inner AF. The inter-lamellar matrix (ILM) lies between the lamellae, with a thickness of less than 30 µm.[32] The ECM of the ILM is an amorphous gel composed with a matrix of elastic fibers, aggrecan, lipids and collagens I and V.[33] Corresponding with this gradient in structure, the NP, inner AF and outer AF possess unique cell phenotypes with the capability of producing and maintaining the zone-specific ECM. The cells of the nucleus and inner annulus are round and chondrocyte-like,[34] while cells of the outer AF are elongated and fibroblast-like, extending along the collagen fibrils.[35] In addition, the stiffness of IVD tissue significantly varies depending on the location, with the outer AF stiffer than the inner AF and NP.[36]
3. Decellularization

3.1. Standard Decellularization Protocols

Decellularization involves the removal of cellular and nuclear remnants that may elicit an immune response in vivo and interfere with tissue regeneration. The standards for residual xenogeneic DNA in dECM were established by Crapo et al.:<50 ng DNA per mg of ECM dry weight, <200 bp DNA fragment length [39] and lack of nuclear material staining by DAPI or Hematoxylin and Eosin. Decellularization is achieved using compounds or methods which promote the degradation or solubilization of cell membranes. Chemical methods, including ionic and nonionic detergents, are the most frequently used ways of achieving decellularization. Sodium dodecyl sulfate (SDS) is a widely used ionic detergent and is known as potent decellularization reagent. However, it will denature proteins, like collagen, causing them to be dissociated and washed away from the matrix during decellularization. This action results in a looser dECM network, which can be favorable for cell maintenance, viability, and migration. On the other hand, the loss of ECM components can lower bioactivity of the dECM. Triton X-100 is another detergent commonly used in dECM preparation. As a nonionic detergent, it achieves decellularization through the action of dissolving the lipid components of cells, while allowing proteins to retain functional conformation. In general, however, it is reported to be less efficient at decellularization than SDS. For this reason, SDS and Triton X-100 are used in succession to reduce exposure time to the SDS. The removal of SDS with washing has been verified with Fourier-transform infrared spectroscopy (FTIR). It was suggested that articular cartilage retains SDS to a higher extent than other tissues due to its density, which had negative consequences for recellularization.
Enzymes can be used to disrupt cell membrane adhesion to the ECM. Decellularization with enzymes includes the use of nucleases, like deoxyribonuclease (DNase) or proteases like trypsin. DNase catalyzes the hydrolysis of DNA chains. However, it is necessary to remove the nucleases from the tissue, since they could invoke an immune response.\(^{[12]}\) Often trypsin solutions are supplemented with a chelating agent like ethylenediaminetetraacetic acid (EDTA), which can also disrupt cell adhesion by binding magnesium and calcium from cell surfaces. Enzymatic methods are known to effectively remove cellular materials\(^{[15c,40c,47]}\), but they drastically disrupt ECM components, like collagen, and the tissue ultrastructure.\(^{[12]}\) Trypsin causes marked structural disorder, detected by scanning electron microscopy (SEM), which was reported to be more pronounced than the structural alterations caused by SDS.\(^{[48]}\) Similarly, in a comparison of three decellularization protocols for the thoracic aorta with SDS, trypsin, or Triton X-100, Trypsin caused the most disruption to tissue ultrastructure, while the other detergents caused no major changes. Moreover, the tissues decellularized by Triton X-100 or SDS retained similar elastic properties to the native tissue.\(^{[49]}\)

The last method of decellularization is promoting cell lysis through physical disruptions like freeze-thawing, or pressure.\(^{[2]}\) Freeze-thawing causes the formation of ice crystals, which disrupts cell membranes. The method has been widely used prior to application of detergent or enzymes.\(^{[49]}\) After the application of physical methods, washes with enzymatic or chemical agents, possibly supplemented with sonication or mechanical agitation, are needed to effectively eliminate the residual cellular components inside the matrices.\(^{[50]}\) The extent and duration of these physical treatments, as well as required reagent volumes and concentrations, depend on the density and surface area of a tissue.\(^{[38]}\) Nie et al. reported that the application of three freeze thaw cycles to articular cartilage between ultralow (\(-80 ^\circ\)C) and room temperature improved efficiency of chemical and enzymatic decellularization methods.\(^{[44d]}\)

### 3.2. Specific Protocols on Cartilage and Intervertebral Disc Decellularization

#### 3.2.1. Collagenous Cartilage and IVD Component Retention

Post-decellularization, the collagen from the native tissue matrix is well-retained. Quantitative spectrophotometric measurements of hydroxyproline content generally reveal that the proportion of collagen content is increased compared to the native tissue due to the reduction of the cellular and other ECM content.\(^{[40a,e,51]}\) Immunohistochemistry\(^{[42a,46,52]}\) and enzyme-linked immunosorbent assays (ELISA),\(^{[53]}\) and electron microscopy\(^{[46]}\) have been used to confirm no detectable deterioration of the collagen II epitope or the collagen II ultrastructure after decellularization of cartilage and IVD tissue.

#### 3.2.2. Noncollagenous Cartilage and IVD Component Retention

The high density and low surface area of cartilage and IVD tissues pose unique challenges for decellularization in terms of adequate removal of cellular debris while maintaining noncollagenous components.\(^{[32]}\) GAG loss during decellularization is well documented.\(^{[18,40a,e,41,44a,50b–d,54]}\) GAG loss is known to occur with exposure to SDS\(^{[49b,53]}\) and Triton.\(^{[41,46d,53,55]}\) Trypsin,\(^{[54a]}\) pepsin, hydrochloric acid,\(^{[46]}\) and guanidine hydrochloride.\(^{[56]}\) In some cases of cartilage dECM preparation, GAG is intentionally depleted during the decellularization process\(^{[46,53,56]}\) to open the network porosity, facilitating more efficient decellularization, perfusion and recellularization. This approach is justified by reports that type II collagen alone can support the deposition of cartilage ECM and rounded chondrocyte morphology.\(^{[57]}\) which could be further promoted with addition of transforming growth factor \(\beta\) (TGF-\(\beta\)).\(^{[58]}\)

On the other hand, retaining the GAG tissue content during decellularization could potentially improve dECM properties. GAG retention increases the elasticity, compressive and tensile properties of dECM.\(^{[49b,53,58]}\) GAG and whole aggrecan are known to have chondroinductive effects in vitro and in vivo.\(^{[59]}\) The macromolecules are also implicated in differentiation toward IVD phenotypes.\(^{[60]}\) Therefore, the development of gentler, more efficient decellularization processes that preserve the GAG content of cartilage and IVD tissues, while maximizing depletion of the cellular components, has been the goal of recent research.

Cell removal and ECM preservation in cartilage tissue was compared with SDS and Triton X-100. GAG loss increased with exposure time, with SDS preserving more GAG than Triton X-100.\(^{[58]}\) In another study, decellularization of bovine NP tissue was compared using sodium deoxycholate, sodium deoxycholate and SDS, or sodium deoxycholate, SDS, and Triton X-100, combined with freeze-thaw cycles and DNase treatment.\(^{[41]}\) The decellularization conditions showed no differences in collagen II retention, but there was significant GAG loss in all the schemes, with sodium deoxycholate having the mildest impact. Still, GAG loss was 80% compared to the native tissue. The detergents Triton X-100, sulfobetaine-10 and sulfobetaine-16 were used in short cyclic intervals to preserve GAG content of porcine NP during decellularization.\(^{[54c]}\) The method resulted in 49–55% GAG retention, while effectively removing cellular components.

Fernandez et al.\(^{[42b]}\) looked at 13 different decellularization procedures for bovine NP tissue, with the following aspects reported to be consequential for GAG preservation: 1) The inclusion of an ethanol prewash to fully dehydrate the tissue resulting in better uptake of detergents, 2) The use of high concentrations of Triton X-100 and EDTA to decrease required detergent exposure time, 3) Abatement of the ionic detergent deoxycholic acid to improve maintenance of tissue architecture and composition. The best protocol resulted in GAG loss of \(-70\%\). Despite this loss, the mechanical properties of the dECM approached that of the native human NP.

Recently, Zhou et al.\(^{[61]}\) took a unique approach where the GAG lost during decellularization was quantified and an equivalent amount of chondroitin sulfate was codissolved with the digested dECM in the pregel. The resulting pregel was applied as an injectable scaffold and demonstrated capability to support NP-like differentiation of adipose derived MSCs in vitro. The cell seeded constructs were also implanted in an in vivo rabbit model of disc degeneration, resulting in partial restoration of ECM content and structure.
Although collagen and GAG are the most often quantified ECM components in dECM, other types of collagens, laminin, fibronectin, growth factors and cytokines can influence the cellular and tissue response to dECM. Luo et al. compared growth factor retention in cartilage tissue decellularized with Triton X-100 or SDS, and found it similar for both groups, with exposure time being the most critical factor. Ghosh and co-workers quantified basic fibroblast growth factor (bFGF) and TGF-β in extracts of cartilage decellularized with Trypsin EDTA and Triton X-100. The analysis revealed that 68.8% of the bFGF and 42% of the TGF-β was retained after decellularization. Multiplex assay analysis showed no significant decrease in the concentration of cytokines before and after decellularization.

Published studies on optimization of decellularization procedures for cartilage and IVD tissues are summarized in Table 1. Overall, the ideal procedure depends on multiple variables, especially the density and surface area of the tissue during decellularization. The research indicates that minimizing exposure time to the decellularization agents is critical for achieving a balance between DNA loss and preservation of ECM. Applying freeze-thaw cycles to the tissue and grinding or slicing the tissue prior to decellularization, followed by the usage of higher concentrations of decellularization reagents, was shown to be promising across multiple studies.

### 4. Preparation and Application of dECM Bioinks

Multiple studies point toward the utility of dECM from cartilage and IVD tissues, alone or in combination with growth factors, in facilitating targeted cell differentiation and ECM synthesis. Despite the important control over scaffold architecture and cell placement that 3DBP offers, there have been only a handful of applications of 3DBP reported with cartilage dECM bioinks and none yet with IVD dECM. The bioinks that have been developed from cartilage dECM (Table 2) can be categorized into 1) composites formed from dECM particulates encapsulated within a hydrogel network cross-linked postextrusion, or 2) solubilized dECM which gels with the thermally

### Table 1. Summary of published studies quantitatively comparing cell removal and ECM retention for various decellularization methods.

| Tissue source       | Decellularization procedure                                                                 | Key outcomes                                                                                     | Reference |
|---------------------|----------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|-----------|
| Ovine knee          | Tissue slices freeze-thaw (F/T, 3x), then two groups: 1) 5% or 10% SDS for 8 h, or 2) 10% EDTA for 8 h | 5% SDS was most effective for infusing dense cartilage; minimal GAG loss achieved with adequate decellularization. | [49a]     |
| Bovine costal cartilage | Homogenized tissue treated with $10 \times 10^{-4}$ EDTA for 24 h, lyophilized, and then treated with 0.5% Triton X-100 for 48 h | EDTA treatment reduced GAG by 5%, Triton treatment reduced GAG by an additional 21%.              | [55]     |
| Porcine ears        | Tissue slices treated with SDS (0.1%, 0.5%, or 1%) or Triton X-100 (0.1%, 0.5%, 2%) for 24–72 h, washed with sterile water. | GAG loss increased with exposure time (25–50% for Triton, 60–70% for SDS). GAG loss was higher in the Triton samples than in the SDS samples. | [53]     |
| Porcine femoral condyle | Tissue pieces F/T (2x), treated with 0.1% SDS for 24 h (this was repeated 1, 3, or 6x), then DNase/RNase solution for 3 h. Washed with PBS throughout decellularization process. | Tissue treated with 1, 3, and 6 cycles showed a DNA reduction of 52.3–98.8% and GAG loss of 87–98%. | [49b]     |
| Bovine NP           | Tissue pieces F/T (5x), ground and then three groups: 1) 2% sodium deoxycholate, 2) 2% sodium deoxycholate and 2% sodium dodecyl sulfate, 3) 2% sodium deoxycholate, 2% sodium dodecyl sulfate, and 0.1% Triton X-100. Each step performed for 1 h. Washed with sterile Millipore water after each decellularization step. | Optimal technique used the fewest detergents for the least amount of time (group 1), though GAG loss was 80%. | [41]     |
| Bovine NP           | Tested 13 different protocols. Varied decellularization reagents (Triton X-100, Deoxycholic acid, and SDS) and concentration of DNase/RNase treatment (720 or 1440 mU mL$^{-1}$ each). Washed with distilled water and/or 70% ethanol throughout decellularization process. | Required exposure time was reduced by dehydrating the tissues and increasing the concentration of decellularization reagents. Optimal protocol caused 70% GAG loss. | [44a]     |
| Human femoral condyle | Tested 24 protocols. All decellularization protocols were preceded with F/T cycles (4x), followed by treatments with various decellularization agents (SDS, Triton X-100, sodium deoxycholate, HCl, NaOH) and enzymes (DNase, RNase, trypsin and elastase, pepsin, collagenase II, pronase). Samples washed in PBS for 24 h following decellularization. | The addition of nucleases to SDS treatment was critical for DNA reduction. SDS+nucleases provided the most efficient decellularization, but residual detergent caused cytotoxicity. 0.1 m HCl reduced DNA content as effectively, without interfering with cell attachment. SDS+nucleases caused 35% GAG loss. Use of Trypsin in place of nucleases caused 50% GAG loss. Use of 0.1 m HCl for decellularization caused 50% GAG loss. 0.1 m NaOH was an effective decellularization agent, but it depleted GAG and damaged collagen II matrix. Pronase and pepsin treatment caused 90% and 96% of GAG loss, respectively. | [46]     |
triggered assembly of the collagen network postextrusion. These approaches are summarized in Figure 2.

4.1. dECM Particulate-Based Bioinks

Post-decellularization and lyophilization, fragmented cartilage can be combined with a secondary biomaterial to impart cohesiveness and mechanical robustness. For example, cartilage dECM particulates (BioCartilage) were suspended in alginate/gellan solution (Figure 3A). The composite network was stabilized by coextrusion with a cation-loaded solution, which induced ionic cross-linking of alginate. The presence of BioCartilage stimulated chondrocyte proliferation in the bioink compared to hydrogel alone. However, TGF-β3 was needed to stimulate strong deposition of ECM.[63]

Recently, Chen et al.[64] combined dECM fragments with 7% hyaluronic acid (HA) solution and gelatin/poly(lactic-co-glycolic acid) (PLGA) electrospun fibers to form a bioink. The HA was cross-linked around the fibers and dECM fragments postextrusion using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry. The incorporation of fibers and dECM at a ratio of 1:1 increased the Young’s modulus of the printed construct to 20 kPa, compared with 10 kPa without the fibers. In another study, cartilage dECM fragments were blended with silk fibroin.[65] Postextrusion, the

| Type of bioink          | Bioink composition                                                                 | Advantages                                                                 | Disadvantages                                                                 | Reference |
|-------------------------|-------------------------------------------------------------------------------------|----------------------------------------------------------------------------|------------------------------------------------------------------------------|-----------|
| dECM particulates       | dECM particulates, Alginate/gellan coextruded with cation-loaded solution           | Cell-friendly gelation                                                     | Cells not encapsulated directly within biomimetic dECM environment           | [63]      |
|                         | dECM particulates, hyaluronic acid + gelatin/PLGA electrospun fibers, cross-linked with NHS/EDC chemistry | Multiscale design; fibers yield improved mechanical performance           | Complex design with multiple components                                       | [64]      |
|                         | dECM particulates, Silk fibroin cross-linked with methanol or EDC/NHS chemistry     | Versatile system with covalent and/or physical cross-linking               | Potential cytotoxicity due to residual organic solvent                        | [65]      |
| Solubilized dECM        | Solubilized dECM encapsulated in PLA microspheres and extruded within PCL          | Microspheres prolong release of bioactive molecules from the dECM         | Potential for loss of bioactivity during encapsulation process               | [54a]     |
|                         | Solubilized dECM printed on PCL backing                                             | Cell encapsulated directly within dECM microenvironment                   | Low mechanical properties and slow gelation time of dECM; low biodegradability of PCL | [18]      |
silk was physically cross-linked by methanol only, or covalently with 100 × 10^{-3} m EDC/NHS in water (EDC-W) or methanol (EDC-M). The scaffolds showed tunable mechanical properties ranging from 0.5 to 3 kPa, depending on the cross-linking method, with the EDC-M scaffolds having the highest stiffness.

### 4.2. Solubilized dECM Bioinks

Typical dECM bioinks, like liver, muscle, tendon, and cardiac tissue, are composed of dECM that is extruded in solubilized form, and this has also been applied to cartilage. The solubilization is accomplished after lyophilization and fragmentation via pepsin digestion in the presence of hydrochloric or acetic acid solution. Since the tissues are composed of collagen in majority after decellularization, digestion of the telopeptide bonds of the collagen triple helix to open the collagen fibrils is necessary for solubilization. Subsequently, the pH is neutralized to 7.4 and the solution is maintained below 10 °C. This solution is designated as the “pregel” and it is suitable for combining with cells. When the pregel solutions are incubated at 37 °C for 20 to 60 min, the collagen gradually undergoes spontaneous reformation of its fibrillar structure, forming a hydrogel that encapsulates the cells. The total time required for the assembly process was found to be dependent on the concentration of dECM in the pregel, with lower concentrations showing longer gelation periods. The dECM concentration in the pregel typically ranges from 2% to 3%. Digestion time in acid pepsin, typically ranging from 24 to 72 h, was not found to have an appreciable effect on assembly time.

When dECM is solubilized for the purpose of forming a bioink, extrusion-based 3D printing methods are commonly used. Because the pregel is flowable, it can be dispensed from a 3D printer nozzle at cold temperatures. After deposition, the bioink is maintained at 37 °C and shape retention is imparted by the gradual assembly of the collagen structure. Pati et al. is the only reported bioink composed solely of solubilized cartilage dECM. The decellularization process, comprised of freeze-thawing, treatment with Trypsin, DNase, RNase, and Triton X-100, yielded adequate decellularization, but a 20% loss of total collagen and 80% loss of GAG. The printability of 3% dECM bioink was evaluated with rheological studies using a steady-shear sweep at 15 °C. Shear thinning behavior, or a decrease in viscosity with shear strain, was observed, which has also been seen for particulate-based cartilage bioinks and solubilized dECM bioinks from adipose, cardiac, and tendon. Shear thinning behavior, caused by the alignment of the macromolecular components of the bioink aligning along the direction of applied extrusion force, is indicative that its viscosity will decrease when passed through the nozzle (Figure 3B). Shear thinning is beneficial for maintaining the viability of printed cells. Pati et al. observed greater than 90% viability during the printing process, similar findings with various other printed bioinks.

Oscillatory rheological studies on the solubilized cartilage dECM bioink revealed that complex modulus (G*) increased over time at 37 °C, signifying collagen assembly. Once gelation was complete, the storage modulus (G’) for the cartilage dECM bioink was higher than the loss modulus (G''), indicating that the network behaved more elastic than viscous, a characteristic that is associated with shape fidelity upon...
deposition.\cite{18,40a,c,66b} The complex moduli reported for the cartilage bioink was in the range of $10^3$–$10^4$ Pa between 1 and 1000 rad s$^{-1}$, similar in range to dECM bioinks reported for muscle\cite{40c} and cardiac.\cite{40d} Notably, the mechanical stability of the gelled dECM was low, so it was printed onto a secondary thermoplastic component to provide additional reinforcement (Figure 3C). Despite the weak mechanical properties and low GAG retention, encapsulated human inferior turbinate-tissue-derived mesenchymal stem cells (hTMSCs) exhibited higher upregulation of SOX9 and Type II collagen compared to collagen I controls at day 14 of in vitro culture within the bioink. However, the magnitude of the upregulation in this study was modest, with a 1.5-fold increase for dECM compared to 1.0 for the collagen control.\cite{18}

Though not yet applied to cartilage dECM bioinks, in some studies, the dECM is codissolved with a secondary cross-linkable component, which forms an interpenetrating hydrogel network around the dECM molecules after deposition, usually through photo-cross-linking.\cite{40f,66a} This approach is useful for speeding up gelation, improving shape retention and increasing mechanical properties. Ghosh et al.\cite{54a} took a unique approach that incorporated bioactive, solubilized cartilage dECM into a thermoplastic polymer with high mechanical properties and shape retention characteristics. Solubilized cartilage dECM was first encapsulated in poly(lactic acid) (PLA) microspheres (MPs) and then coextruded with PCL. The encapsulation of digested dECM in the MPs prolonged the release of bioactive molecules retained during decellularization. Chondrogenesis of human MSCs cultured near the scaffolds in transwell plates was demonstrated. Collagen II, aggrecan and SOX9 gene expression were an order of magnitude higher than MSCs cultured in constructs with unloaded microspheres, despite both groups being cultured in the absence of chondrogenic media.

### 4.3. Particulate versus Solubilized Cartilage dECM Bioinks

Using dECM particulates in bioink formulations, without any digestion step, has an advantage that there is less processing, which can help to preserve material and matrix components. The printed cells are not encapsulated directly within the dECM, but they reside approximate to it within the secondary hydrogel network. The dECM particulates, then, serve as reservoirs for bioactive molecule nearby cells.\cite{54a,63} The porosity and stiffness of the printed struts can be controlled via crosslink density and polymer concentration of the hydrogel network. Since the crosslinking occurs around the dECM particulates, there is no need to introduce nonphysiological crosslinks on the dECM molecules, avoiding a potential negative impact on dECM bioactivity.

On the other hand, bioprinting solubilized dECM presents an interesting opportunity to induce spatial arrangement of the macromolecular constituents in the dECM during nozzle extrusion. Shear force during printing has been reported to induce the alignment of collagen fibers\cite{70} and fibrils\cite{71} along the long axis of the printed lines. Using a combination of solubilized collagen I and Matrigel effectively aligned collagen fibers during microextrusion.\cite{72} The presence of Matrigel enabled control over fiber alignment during extrusion by altering the hydrophilic/hydrophobic balance in the bioink and giving rise to molecular crowding. Though this has not been studied yet for cartilage and IVD dECM, controlling collagen alignment via extrusion could potentially serve as a powerful tool for providing desired biophysical and biomechanical cues to printed cells.

### 5. The Next Frontiers in 3DBP of dECM from Cartilage and IVD Tissues

Over the past few decades, tissue engineering strategies have been influenced by principles of developmental biology. The fields parallel each other because they both involve stem cell differentiation, ECM deposition, and tissue assembly. Tissue engineering studies that are inspired by developmental biology are based upon the hypothesis if cell aggregates or tissue fragments are positioned closely together, they can spontaneously self-assemble and self-organize toward the development of a functional tissue.\cite{73} The 3DBP of cartilage and IVD dECM can support these studies by enabling 1) recapitulation of the tissue biochemical composition, 2) layer-by-layer deposition of the ECM to rebuild the zone-specific architecture and biomechanical properties on multiple length scales, and 3) precise placement of appropriate cell type(s) (Figure 4).

For recapitulating the biochemical environment of the ECM after decellularization, it is important to note that the integrity of the aggrecan in cartilage and IVD tissue is important for maintaining healthy biological and biomechanical properties of the tissues. Aggrecan core protein possesses multiple domains that bind other ECM molecules, including hyaluronic acid, as well as cell surface receptors. Since no artificial material has recapitulated the structure and function of aggrecan, aiming to preserve the entire molecule, rather than just the sGAG side chain content, during decellularization could further improve the functionality of dECM from cartilage and the IVD. In addition to collagen and aggrecan, future studies should include proteomic analyses to characterize the presence of matrixome and nonmatrixome proteins after decellularization. This would not only aid in distinguishing between decellularization methods, but also help to elucidate the role of dECM composition in directing cell behavior.\cite{75} Taken further, once decellularization strategies have been improved in terms of ECM retention, region-specific compositions of cartilage and IVD ECM can be mimicked in the 3D printed scaffolds by selecting zone-specific dECM to print. Potentially, a single stem cell population can be spatially cued to a zone-specific phenotype.

Besides the biochemical environment, recapitulation of the architectural features of the ECM is important, as cells sense characteristics like ECM surface topography and fiber organization. Mimicking the native tissue organization on multiple length scales is consequential for both achieving repair and building tissue models for studying cell–cell and cell–biomaterial interactions. However, maximum resolution of extrusion-based printing is only on the order of hundreds of microns,\cite{18,29} which limits its use for replicating physiological structures with microsized features or smaller.\cite{29} For instance, the diameter of collagen fibers ranges from 1 to 300 μm.\cite{76} The randomly
arranged collagen fibrils in the human adult NP have diameters of 100–150 nm, while the aligned fibrils in the AF are 100–200 nm in diameter.\[77\] Recently, other bioprinting technologies have been developed that open new possibilities for achieving high printing resolutions. For example, with digital light processing (DLP) and stereolithography (SLA), the resolution of the printed constructs depends on the focal size of the light beams, which can be on the order of a single micrometer.\[78\] With two-photon polymerization printing, the resolution can be as high as 100 nm.\[79\] We envision using these technologies for 3DBP of dECM will play an integral role in enabling researchers to incorporate appropriate geometric or topographical cues into cartilage and IVD scaffolds for inducing zone-specific cell orientations. Recently, the combination of two or more manufacturing technologies, like 3D printing and electrospinning, has emerged as a trend within biofabrication for manufacturing scaffolds with hierarchical architecture.\[80\]

Notably, light assisted printing techniques require chemical modification of the dECM to achieve light-induced curing after deposition. In any case, chemical modification of cartilage and dECM bioinks is an area that needs further exploration, as a current major limitation is weak mechanical properties. The highest reported Young's modulus for a cartilage or IVD dECM so far is 20 kPa,\[64\] which is not approaching the level of the native tissues, which is 0.1–10 MPa.\[81\] It was reported that using a combination of two crosslinking mechanisms, ionic and covalent, in a bioink composed of sodium alginate and poly(ethylene glycol), increased the stretch-ability and toughness postprinting.\[82\] Also, the inclusion of multiscale constituents into the bioinks, like fibers, is a promising method for increasing mechanical properties.\[64,83\] Shear stress during extrusion could be used to force fiber alignment, thereby simultaneously providing a stimulus for cellular organization.

As technology develops in the 3DBP and dECM arenas, identification of suitable cell sources is necessary. MSCs are promising because they are easily accessible from a variety of tissues, have shown potential to differentiate into chondrogenic\[84\] or discogenic phenotypes,\[85\] and have been used efficaciously in clinical studies.\[86\] However, age can reduce MSC therapeutic efficacy\[87\] and extended in vitro culture can reduce their differentiation potential.\[88\] An alternative is the use of induced pluripotent stem cells (iPSCs).\[89\] iPSCs can be derived from patient-derived cells,\[90\] making them a promising therapeutic cell source with potential to differentiate into all types of adult cells. Recently, human induced pluripotent stem cells were differentiated into NP-like cells in vitro.\[91\] Also, peripheral blood cells were transformed into iPSCs, which were further differentiated into human iPSC-MSC-like cells, and subsequently induced toward chondrogenic lineage.\[92\] However, significantly more is left to understand in terms of how to use iPSCs safely and control differentiation to the targeted adult phenotype.

Thermally sensitive bioinks are excellent candidates to be applied for in situ bioprinting. This is where, during surgery, robotic arms reconstruct a tissue defect via 3D printing of a bio-material, so the construct can be tailored to match the defect shape and then it matures in the in vivo niche, rather than in

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Figure 4. dECM-based scaffolds and 3DBP promises to advance tissue engineering of cartilage and the IVD. Upcoming research efforts will focus on optimizing decellularization procedures to retain the native tissue biochemical microenvironment (top right, adapted from Ghatak et al.,\[74\] available for reuse under the Creative Commons Attribution License (CC-BY), Copyright 2015, the Authors. Published by Hindawi), advancing macromolecular designs to improve scaffold stability and mechanical properties (top left), finding suitable sources for repopulating printed scaffolds with cells (bottom right), and developing new biofabrication strategies to build scaffolds mimicking the zone-specific hierarchical architectures of cartilage and the IVD (bottom left). Part of the figure was created with BioRender.com.
vitro. A hand-held 3D printing device (Biopen) was created that could extrude gelatin/hyaluronic acid methacrylate solution, seeded with MSCs, layer by layer into a full thickness cartilage defect. Post extrusion, the macromers were photocured in situ.\[93] Clearly, once new crosslinking mechanisms are developed to improve stability and mechanical properties of cartilage and IVD dECM, there are exciting prospects for application in a clinical setting.

3DBP of cartilage and IVD dECM is most often studied within the context of fabricating constructs for healing tissue voids, yet dECM is also being applied in microfluidic devices. Here, the aim is to recapitulate physiological functions of organs and tissues on one small microsized panel. On these so-called “organ on a chip” platforms, perfusable channels that mimic physiological flow conditions connect chambers representing tissues or organs. The placement of different cell types within the chambers and spatiotemporal control over the chemical microenvironment of the cells in the microfluidic device allow for the study of controlled variables.

IVD degeneration, OA, and RA are multifactorial diseases characterized by pro-inflammatory conditions and interactions between immune cells and resident cells. The latest microfluidic devices commonly utilize multiple cell types, which is meant to simulate in vivo interactions between cell types.\[94] However, the current models are somewhat limited in terms of capturing multiple aspects of the complex in vivo niche. For the next generation of microfluidics studies, the inclusion of dECM components on the microfluidic panels would aid in better mimicking the native cell microenvironment and increasing bioactivity.\[66b] For instance, microfluidics would offer an innovative route for the study of interactions between endothelial cells, nerve cells, and IVD cells to better elucidate the mechanisms of neurovascular ingrowth. Using 3DBP these applications would be of particularly advantageous because it would enable precision placement of the dECM bioinks onto the microfluidic panels with high reproducibility and less labor.

6. Conclusions

New dECM-based biomaterials and 3DBP are a few of the most promising research areas in tissue engineering. Indeed, there is mounting evidence that using dECM-based biomaterials is beneficial in terms of guiding cell proliferation, attachment, and differentiation. The convergence of dECM methodologies and 3DBP has advanced tissue engineering by enabling researchers to recreate tissue-specific 3D environments in custom geometries. For cartilage and IVD repair, new research should be directed toward developing gentler and more efficient decellularization methods that preserve the ECM components of the native tissue, improving printed dECM mechanical properties, and emphasizing the recapitulation of region-specific architectural and biological features of the native tissues. Ultimately, the same principles should be applied on the micrometer scale to engineer microfluidic platforms that enable the high throughput study of disease pathogeneses and treatments. While these studies have tremendous unexplored potential in cartilage and IVD regeneration, we also anticipate that they will be at the center of increasing research efforts in all fields of biofabrication and tissue engineering.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

3D bioprinting, cartilage, decellularized extracellular matrix bioinks, intervertebral discs, organs on chips, tissue regeneration

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